Oligomerization and Membrane-binding Properties of Covalent Adducts Formed by the Interaction of α-Synuclein with the Toxic Dopamine Metabolite 3,4-Dihydroxyphenylacetaldehyde (DOPAL)*

Cristian Follmer1, Eduardo Coelho-Cerqueira1, Danilo Y. Yatabe-Franco3, Gabriel D. T. Araujo1, Anderson S. Pinheiro1, Gilberto B. Domont1, and David Eliezer2

From the Departments of 1Physical Chemistry and 2Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro 21941-909, Brazil and the 3Department of Biochemistry, Weill Cornell Medical College, New York, New York 10065

Background: The molecular details of the effects of DOPAL on α-synuclein misfolding and oligomerization are unknown.

Results: DOPAL forms Schiff-base and Michael-addition adducts with α-synuclein Lys residues.

Conclusion: DOPAL modification inhibits αS fibrillation and reduces binding of α-synuclein to synaptic-like vesicles.

Significance: DOPAL modification may interfere with the normal functions of α-synuclein and favor the buildup of potentially toxic oligomers.

Oxidative deamination of dopamine produces the highly toxic aldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL), enhanced production of which is found in post-mortem brains of Parkinson disease patients. When injected into the substantia nigra of rat brains, DOPAL causes the loss of dopaminergic neurons accompanied by the accumulation of potentially toxic oligomers of the presynaptic protein α-synuclein (αS), potentially explaining the synergistic toxicity described for dopamine metabolism and αS aggregation. In this work, we demonstrate that DOPAL interacts with αS via formation of Schiff-base and Michael-addition adducts with Lys residues, in addition to causing oxidation of Met residues to Met-sulfoxide. DOPAL modification leads to the formation of small αS oligomers that may be cross-linked by DOPAL. Both monomeric and oligomeric DOPAL adducts potently inhibit the formation of mature amyloid fibrils by unmodified αS. The binding of αS to either lipid vesicles or detergent micelles, which results in a gain of α-helix structure in its N-terminal lipid-binding domain, protects the protein against DOPAL adduct formation and, consequently, inhibits DOPAL-induced αS oligomerization. Functionally, αS-DOPAL monomer exhibits a reduced affinity for small unilamellar vesicles with lipid composition similar to synaptic vesicles, in addition to diminished membrane-induced α-helical content in comparison with the unmodified protein. These results suggest that DOPAL could compromise the functionality of αS, even in the absence of protein oligomerization, by affecting the interaction of αS with lipid membranes and hence its role in the regulation of synaptic vesicle traffic in neurons.

Parkinson disease (PD),3 the second most common age-related neurodegenerative disorder after Alzheimer’s disease, is a progressive movement disorder that affects ~1–2% of the population over 65 years of age (1). The clinical symptoms (resting tremors, bradykinesia, rigidity, and postural dysfunction) result predominantly from the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and from dopamine (DA) deficiency in the striatum (1–3). The histopathological hallmark of PD is the presence of intraneuronal deposits containing fibrillar aggregates of the presynaptic protein α-synuclein (αS) (called Lewy bodies and Lewy neurites) (4, 5). Missense mutations and genomic multiplication of the αS gene have been linked to autosomal dominant familial PD (6).

Although αS is expressed throughout the brain, formation of protein deposits specifically in dopaminergic neurons in the SN suggests a connection between αS aggregation and DA metabolism. This hypothesis is corroborated by the fact that the neurotoxicity associated with αS overexpression is remarkably reduced when DA synthesis is inhibited (7), and the toxicity of oxidized catechol metabolites is exacerbated by the expression of WT αS or the PD-linked A53T variant (8). The fact that DA itself, at concentrations found in dopaminergic neurons, is not able to impair neuron functionality and cause cell death as found in PD (9) suggests that another molecule produced via

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1 To whom correspondence may be addressed. Tel.: 55-21-2562-7752; Fax: 55-21-2562-7265; E-mail: follmer@iq.ufrj.br.

2 To whom correspondence may be addressed. Tel.: 212-746-6557; Fax: 212-746-4843; E-mail: dae2005@med.cornell.edu.

3 The abbreviations used are: PD, Parkinson disease; ALDH, aldehyde dehydrogenase; CA, citraconic anhydride; DA, dopamine; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DopC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DopE, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; DopQ, DopAL oxidation products include DopAL-quinone; HNE, 4-hydroxy-2-nonenal; HSCC, heteronuclear single quantum coherence; MA, Michael addition; MTSL, 1-oxyl-2,2,5,5,-tetramethylpyrroline-3-methylmethanethiosulfonate; PRE, paramagnetic relaxation enhancement; SB, Schiff-base; SEC, size exclusion chromatography; SN, substantia nigra; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SOD, superoxide dismutase; SUV, small unilamellar vesicle; ThT, thioflavin-T; αS, α-synuclein; ITC, isothermal titration calorimetry; MAO, monoamine oxidase; ROS, reactive oxygen species; HNCA, proton-nitrogen-carbon correlation; NAC, non-αβ component.
The enzymatic oxidation of DA by monoamine oxidase isoforms (MAO-A and MAO-B) produces the highly cytotoxic aldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL) (11), levels of which are enhanced in post-mortem brains of PD patients (12). Elevated DOPAL levels in PD are suggested to result from a combination of decreased vesicular uptake of cytosolic DA (decreased by ~89%) and decreased DOPAL detoxification by the enzyme aldehyde dehydrogenase (ALDH) (decreased by 70%) (13), which converts DOPAL to its non-toxic metabolite 3,4-dihydroxyphenylacetic acid. Interestingly, the inhibition of ALDH is sufficient to cause a parkinsonian phenotype in mice (14). Furthermore, the interaction of aS with DOPAL is reported to lead to an increase of the levels of potentially toxic aS aggregates. For instance, the injection of DOPAL into the intranigral region of mouse brains results in the formation of aS oligomers accompanied by death of dopaminergic neurons (15). This means that the interaction between aS and DOPAL might play a central role in the selective degeneration of dopaminergic neurons and could explain the synergism between the toxicity of DA metabolism and aS toxicity. Unfortunately, the molecular details of the formation of aS oligomers induced by DOPAL remain unclear.

In this work, we demonstrate that Schiff-base (SB) and Michael-addition (MA) adducts formed between DOPAL and Lys residues located in the N-terminal domain of aS are associated with the stabilization of protein oligomers, likely by acting as protein cross-linkers. Interestingly, helical folding of the aS N-terminal domain, which occurs upon binding to lipid membranes, has a protective effect against DOPAL-induced aS oligomerization. However, DOPAL is able to compromise the ability of the aS monomer to interact with lipid vesicles, even in the absence of protein oligomerization, which might represent a potential mechanism for loss-of-function of aS monomers in the regulation of synaptic vesicle trafficking and exocytosis in neurons.

**Experimental Procedures**

**Expression and Purification of aS**—The expression and purification of aS were performed as described previously (16). For production of $^{15}$N-aS or $^{13}$C,$^{15}$N-aS, cells were grown in minimal media containing $^{15}$N-labeled ammonium chloride or $^{15}$N-labeled ammonium chloride plus $^{13}$C-labeled glucose, respectively, and the protein expression was induced with 0.8 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C.

**Preparation of aS-DOPAL Species**—DOPAL was synthesized via the pinacol-pinacolone rearrangement of epinephrine (17). A solution of 50 µM recombinant aS monomer in 20 mM sodium phosphate, pH 7.5, was incubated at 37 °C under agitation (350 rpm) in a Thermomixer (Eppendorf, Hamburg, Germany) in the presence of varying concentrations of DOPAL. Aliquots were withdrawn at different times and analyzed by 12% SDS-PAGE or size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column (void volume, 7.8 ml) (GE Healthcare, Little Chalfont, UK). The percentage of the different oligomeric states of the protein, in the presence or absence of DOPAL, was determined by quantitative densitometry of proteins, in SDS-PAGE stained with Coomassie Blue, using ImageJ software (18). For the production of aS-DOPAL monomer, 10 µM aS monomer in 20 mM sodium phosphate, pH 7.5, was incubated in the presence of DOPAL (DOPAL/protein ratio = 5, 10, or 20) at 37 °C for 24 h (no agitation). aS-DOPAL monomers obtained under these conditions were denoted as aS-DOPAL(1:5), aS-DOPAL(1:10), and aS-DOPAL(1:20), respectively. After removal of unbound DOPAL by dialysis (cutoff, 3.5 kDa), aS-DOPAL monomer was lyophilized and stored at −20 °C for further use. The oligomeric state of aS was evaluated by SDS-PAGE and SEC. Using this protocol, we were able to produce aS-DOPAL essentially in the monomeric state (>95%).

**Preparation of SUV**—1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Phospholipid mixtures containing 60% DOPC, 25% DOPE, and 15% DOPS (molar concentrations) were prepared by drying a mixture of the different lipids dissolved in chloroform under nitrogen gas and resuspending the lipid film in 20 mM sodium phosphate, pH 7.5, 100 mM NaCl at 25 °C. Small unilamellar vesicles (SUVs) were prepared by pulse-sonicating the phospholipid suspensions in a bath sonicator for 10 min in 2-min increments. The size of the resulting SUVs (hydrodynamic radii of 30–50 nm) was determined by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcsershire, UK).

**NMR Spectroscopy**—NMR experiments were performed using 500 MHz (National Nuclear Magnetic Center at Federal University of Rio de Janeiro, Rio de Janeiro, Brazil) or 600 MHz (Weill Cornell Medical College, New York) Bruker Avance spectrometers equipped with cryogenic probes. Phase-sensitive two-dimensional $^1$H–$^{15}$N heteronuclear single quantum coherence ($^1$H–$^{15}$N HSQC) spectra were recorded using Echo-anti-Echo gradient selection. TopSpin 3.2 was used for data acquisition. All spectra were processed with NMRPipe (19) and analyzed with CCPN software. Amide resonance assignments were performed according to previously reported chemical shift assignments for intrinsically unfolded aS (20–22). HNCA experiments of SDS-bound $^{13}$C,$^{15}$N-aS provided α-carbon chemical shifts for secondary shift analysis. For this experiment, 40 mM deuterated SDS was added to 200 µM double-labeled aS-unmodified (aSunmod) or aS-DOPAL(1:10) monomer. Binding to SUV for both aSunmod and aS-DOPAL was monitored via $^1$H–$^{15}$N HSQC experiments with varying lipid concentrations. All NMR experiments were conducted at 10 °C, except those including SDS, which were carried out at 25 °C. Protein concentration was 200 µM in the presence of 20 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 10% D$_2$O. Kinetic experiments were performed at 15 °C instead of 10 °C to increase the rate of DOPAL oxidation.

**Paramagnetic Relaxation Enhancement (PRE)**—Considering that aS has no Cys residues, protein variants G31C, A85C, and P120C were generated by using site-directed mutagenesis. The purified mutant proteins were labeled with 1-oxyl-2,2,5,5,7-tetramethylpyrroline-3-methyl-methanethiosulfonate (MTSL) (Toronto Research Chemicals, Canada) as described (23).
Briefly, 8 mM MTSL (prepared in 100% DMSO) was added to protein samples (300 μM in 50 mM sodium phosphate, pH 7.5) and then incubated for 4 h at 25 °C (300 rpm). The mixture was then dialyzed against MilliQ water and lyophilized for further use. For aS-DOPAL samples, free MTSL was removed by dialysis against buffer and then the protein was diluted to 10 μM and incubated with 100 μM DOPAL (aS-DOPAL(1:10)) for 24 h at 37 °C (no agitation). Removal of unbound DOPAL was done by dialysis against MilliQ water. Pre was measured by collecting $^1$H, $^15$N HSQC spectra using 200 μM aS or aS-DOPAL(1:10) monomer at 10 °C. For control diamagnetic samples, ascorbic acid (5-fold molar excess in relation to the protein) was added to the MTSL spin-labeled sample to reduce the nitroxide spin label. The intensities of cross-peaks in the $^1$H-$^15$N HSQC spectra of both the spin-labeled and reduced samples were measured, and their ratio was calculated.

**Mass Spectrometry (MS)**—Samples of aS-DOPAL-M were prepared by incubation of 100 μM aS with 1 mM DOPAL at 37 °C and 350 rpm for 24 h in 150 mM ammonium bicarbonate, pH 7.8, followed by isolation of modified monomer by SEC and lyophilization. After that, 100 μg of protein were dissolved in 7 M urea and 2 M thiourea, and the samples were then diluted to 1 M urea with 100 mM Tris-HCl buffer, pH 8.5. In sequence, MS-grade trypsin (Promega) was added (1:50 protease/substrate (w/w)) for overnight digestion at 37 °C. Proteolysis was stopped by adding 1% formic acid. Digested samples were desalted in a C$_{18}$ spin column (Harvard Apparatus) following the manufacturer’s instructions. The digested and desalted samples were dissolved in 0.1% formic acid to a final protein concentration of 0.5 mg/ml. Two micrograms of each peptide sample were injected by a nanoHPLC system nanoLC Ultra 2d (Eksigent Technologies) on a self-packed 75-μm inner diameter, 10-cm-long column, packed with Reprosil-Pur C18-AQ, 3 μm, 120 Å, coupled to an LTQ XL Orbitrap ETD mass spectrometer (Thermo Scientific). Chromatography was carried out at 300 nl/min of flow, and mobile phase buffer A was 95% water, 5% acetonitrile, and 0.1% formic acid, and mobile phase buffer B was 95% acetonitrile, 5% water, and 0.1% formic acid. The analytical column was equilibrated with buffer A for 10 min prior to sample injection. The gradient duration was 60 min (5–45% B over 45 min, followed by 45–90% B over 5 min, and 90% B for another 10 min). The mass spectrometer acquisition strategy consisted of data-dependent acquisition, which automatically switches between full scan MS (Fourier transform MS, 60,000 resolution, 500-ms accumulation time, AGC 1 × 10$^6$ ions, range 300–2000 m/z) followed by fragmentation with higher energy collisional dissociation fragmentation of the six most intense ions. Each fragmentation was recorded in a high resolution spectrum (Fourier transform MS, 15,000 resolution, 100-ms accumulation time, AGC 5 × 10$^4$ ions). The recorded spectra were analyzed by Peptide Spectrum Matching, a method in which an algorithm attempts to find similarity between each experimental spectrum and an in silico generated set of spectra, achieved by a theoretical digestion of a sequence in a database and subsequent creation of a “bar code spectrum.” For direct infusion of MS/MS data acquisition, each sample was diluted using acetonitrile/water/methanol 1:1:2 and 0.1% formic acid to a final concentration of 1.0 μg/μl. Using a syringe pump, 500 μl of the solution was infused via electrospray ionization at a flow of 1.0 μl/min. The mass spectrometer acquisition strategy was the same as LC-MS/MS, with the difference of using five microscans per MS1.

**Isothermal Titration Calorimetry (ITC)**—ITC binding experiments were performed in a nano ITC low volume instrument (TA Instruments, New Castle, PA) with a fixed gold cell by titrating 100 mM SUV (60% DOPC, 25% DOPG, 15% DOPS) into 25 μM aS$_{unmod}$ or aS-DOPAL(1:10) monomer solution. All experiments were performed at 37 °C using 20 mM sodium phosphate, pH 7.5, 100 mM NaCl as buffer. The data were acquired using ITCCure data acquisition software. The incremental ITC experiments consisted of 24 injections of 2-μl at 240-s intervals with stirring speed of 250 rpm.

**Far-UV Circular Dichroism (CD)**—CD measurements were performed using an AVIV Circular Dichroism Spectrometer, Model 410 (Aviv Biomedical Inc., Lakewood, NJ). Solutions of 40 μM aS$_{unmod}$ monomer or aS treated with different concentrations of DOPAL (DOPAL/protein ratio = 5, 10 and 20) in 20 mM sodium phosphate, pH 7.5, 100 mM NaCl in the absence or presence of varying concentration of SUVs were analyzed in a 0.2-mm quartz cuvette at 25 °C. The ellipticity at 222 nm was measured, and the background associated with buffer or SUV solutions was subtracted. The mean residue molar ellipticity at 222 nm ($\theta_{MR, 222}$) was determined using Equation 1,

$$\theta_{MR, 222} = \theta_{222}/10Cn$$  \hspace{1cm} (Eq. 1)

where $\theta_{222}$ is the measured ellipticity at 222 (millidegrees); C is the protein concentration (in molar), n = 140 (number of amino acid residues in aS), and l is the path length of the cuvette in centimeters (0.02 cm). Lipid titration curves generated by plotting $\theta_{MR, 222}$ versus the lipid concentration were analyzed as described previously (24, 25) by fitting to Equation 2,

$$R = R_0 - (R_0 - R_t)K_d + C + L/N \equiv \sqrt{(K_d + C + L/N)^2 - 4CL/N}$$  \hspace{1cm} (Eq. 2)

where R is the measured $\theta_{MR, 222}$ at a given lipid concentration; $R_0$ is the $\theta_{MR, 222}$ in the absence of lipid; $R_t$ is the $\theta_{MR, 222}$ in the presence of saturating lipid; L is the total lipid concentration; C is the total protein concentration; $K_d$ is the apparent macroscopic dissociation equilibrium constant, and N is the binding stoichiometry (lipids/protein). The maximum helical content of aS$_{unmod}$ and aS-DOPAL was determined as described previously (26, 27).

**Determination of H$_2$O$_2$ Content**—The concentration of free H$_2$O$_2$ was evaluated using a fluorometric assay that measured the amount of resorufin produced from Amplex Red (Life Technologies, Inc.) in the presence of horseradish peroxidase (28). These assays were performed in a 96-well microplate at 37 °C and 350 rpm. The resorufin fluorescence was measured by excitation at 571 nm and emission at 585 nm in a Cary eclipse fluorometer (Agilent Technologies, Santa Clara, CA).

**Chemical Modification of Lysines**—To evaluate the role of lysines in DOPAL-induced aS oligomerization, 100 μM aS in 100 mM phosphate buffer at pH 8.0 was incubated for 6 h in the presence or absence of 1.5 mM citraconic anhydride (CA), which causes chemical modification of the lysines of the protein (29). Lys-blocked aS was isolated from free CA by SEC. The
treatment of αS with CA did not cause any detectable protein oligomerization (data not shown). Next, Lys-blocked αS and αS-unmod were incubated with DOPAL, and the formation of oligomers was investigated using SEC and SDS-PAGE.

Results

DOPAL Induces the Formation of Fibril-incompetent Soluble Oligomers of αS in Vitro—Fig. 1A shows that DOPAL promotes, in a concentration-dependent manner, the formation of soluble oligomers of αS. No effect of the treatment of αS-DOPAL sample with the protein cross-linker glutaraldehyde was observed on the gel profile, suggesting that these oligomers are not dissociated from larger species under SDS denaturing conditions (data not shown). In addition, these oligomers were stable enough to be isolated by SEC, and further analysis by SDS-PAGE indicates they are predominantly dimers and trimers of αS-DOPAL, denoted as αS-DOPAL-D and αS-DOPAL-T, respectively (Fig. 1B). Fig. 1C shows that the populations of αS-DOPAL oligomers do not evolve efficiently to larger oligomers even after long term incubation, although a small population of larger species is present after 1st hour of incubation (denoted as H) (Fig. 1C). This result contrasts with the effect of DA, which induces the formation of large oligomers of αS (Fig. 1D). Although it has been suggested that DA specifically stabilizes αS oligomers, the presence of fibrils can be observed after long term incubation with DA (30). This misleading conclusion might be attributed to the fact that the effect of DA oxidation products on ThT fluorescence has been neglected. Oxidized derivatives of DA strongly quench ThT fluorescence, which can mask the formation of ThT-positive fibrillar aggregates (31). The rate of αS fibrillation can be increased by abolishing the rate-limiting nucleation step through the addition of pre-formed fibrils (seeds) to the aggregating solution, which act as nuclei for monomer accretion and fibril growth (32). Because DOPAL oxidation products (essentially DOPAL-quinone, referred to here as DPQ) also strongly quench ThT fluorescence, αS fibrillation in the presence of DOPAL cannot be properly evaluated by using ThT (31) unless DOPAL oxidation is inhibited (see below). For this reason, we monitored αS aggregation/fibrillation using light scattering. Fig. 1E shows that the αS_unmod displayed a remarkable increase in light scattering after the addition of αS seeds, while no effect of the seeds was observed for αS incubated in the presence of DOPAL. This implies that αS-DOPAL species cannot be effectively incorporated into pre-formed nuclei, preventing the growth of fibrils. The absence of any increase in light scattering intensity also suggests that the formation of non-fibrillar high molecular aggregates of αS-DOPAL does not occur under these conditions.

αS-DOPAL Species Are Potent Inhibitors of αS Fibrillation—Given that αS-DOPAL is not incorporated into αS seeds, we evaluated whether αS-DOPAL species may influence the fibrillation of αS_unmod. Monomeric αS_unmod was incubated in the
Covalent Adducts Formed between α-Synuclein and DOPAL

FIGURE 2. Inhibitory activity of aS-DOPAL species on aS_unmodified fibrillation. A, aS_unmodified was incubated at 37 °C and agitation (350 rpm) in the absence or presence of SEC-purified aS-DOPAL-M or aS-DOPAL-Olig at a molar ratio of 1:8 and 1:16 (aS-DOPAL/aSunmod), and the formation of fibrils measured through ThT fluorescence. B shows the normalized kinetic curves obtained in A (molar ratio of 1:16). The kinetic assays were performed in sextuplicate and the results are expressed as mean ± S.D. C, content of aS monomer at the end of the aggregation assay. The population of the monomer was estimated by using SEC and expressed as the peak area of the monomer, at the final time, divided by peak area at the initial time. In the case of aSunmod incubated in the presence of aS-DOPAL-M, the peak areas were corrected by the values obtained for aS-DOPAL-M alone. Control corresponds to aSunmod incubated in the absence of aS-DOPAL species.

TABLE 1

| Sample                               | t1/2 (h) | Lag time (h) | Elongation rate (10^{-3} h^{-1}) |
|--------------------------------------|----------|--------------|----------------------------------|
| aS_unmodified (buffer)               | 21.2 ± 2.8 | 9.1 ± 1.1    | 37.4 ± 4.8                       |
| aS_unmodified + aS-DOPAL-M (16:1)    | 55.4 ± 6.5 | 14.1 ± 1.6   | 12.1 ± 1.4                       |
| aS_unmodified + aS-DOPAL-Olig (16:1)| 37.7 ± 3.6 | 12.1 ± 1.2   | 18.9 ± 1.8                       |

presence of SEC-purified aS-DOPAL-M or aS-DOPAL-Olig, and the formation of fibrils monitored by ThT fluorescence. Note that free DOPAL and its DPQ oxidation by-products are removed by SEC, enabling the use of the ThT assay. Fig. 2A shows that both aS-DOPAL-M and aS-DOPAL-Olig act as strong inhibitors of aS fibrillation at concentrations as low as 3 μM (molar ratio of aS_unmodified monomer to aS-DOPAL of 16:1). Analysis of the kinetic parameters extracted from the normalized curves (Fig. 2B) revealed that the lag time duration and t1/2 (time required to reach 50% of maximum fibrillation) both increased in the presence of the aS-DOPAL-M or -Olig, while the elongation rates decreased (Table 1). Determination of the monomer content at the end of the aggregation reactions confirmed the anti-fibrillogenic activity of aS-DOPAL-M and aS-DOPAL-Olig (Fig. 2C). These results indicate that a small population of aS-DOPAL species can interfere with the conversion of unmodified aS monomer into fibrils and that this ability is shared by both monomeric and oligomeric forms of aS-DOPAL.

DOPAL-induced aS Oligomerization Requires DOPAL Oxidation—The spontaneous oxidation of DOPAL is largely inhibited at acidic pH, as demonstrated by the lower absorbance at 340 nm (which reports on the formation of quinone) of DOPAL solutions at pH 3.0 compared with pH 7.5 (Fig. 3A). In this context, we evaluated the effect of pH on DOPAL-induced aS oligomerization. An increase in ThT fluorescence is observed when aS_unmodified is incubated at pH 7.5 or 3.0, indicating the formation of amyloid fibrils. Aggregation occurs more rapidly at pH 3.0, as observed previously (33). aS plus DOPAL at pH 7.5 did not display any increase in ThT fluorescence due to the fluorescence quenching caused by DPQ (Fig. 3B). However, when this incubation was performed at pH 3.0, the oxidation of DOPAL was prevented, and an increase in ThT fluorescence was observed. Notably, DOPAL itself does not cause any quenching of ThT fluorescence when its oxidation is inhibited, e.g. by acid pH (31). SDS-PAGE analysis indicates that the formation of dimers and trimers occurs in aS incubated with DOPAL at pH 7.5 but not at pH 3.0 (Fig. 3C). For DA also, high molecular weight aS oligomers were observed only after incubation at pH 7.5 and not at pH 3.0. For both DOPAL and DA, incubation at pH 3.0 does not alter the fibrillization kinetics observed for aS_unmodified alone. Taken together, these findings clearly indicate that DOPAL, similar to DA, has no effect on aS oligomerization or fibrillation under conditions in which its oxidation is prevented.

Taking into account the importance of DOPAL oxidation in the formation of aS-DOPAL oligomers, the effect of the antioxidants ascorbic acid (1 mM), selegiline (1 mM), melatonin (1 mM), trans-resveratrol (1 mM), reduced glutathione (GSH) (1 mM), mannitol (50 mM), and superoxide dismutase (SOD) (60 units) was evaluated. We found that ascorbic acid and GSH are capable of inhibiting the oxidation of DOPAL and diminish the DOPAL-stimulated formation of aS dimers and trimers (Fig. 3, D and E). The other antioxidants did not exert a significant effect on DOPAL oxidation or aS oligomerization at these concentrations, although SOD, a selective scavenger of superoxide radicals, reduced the reactive oxygen species (ROS) generated in the early stage of DOPAL oxidation, notably in the presence of aS, but it lost effectiveness over time, potentially due to the inactivation of SOD by direct reaction with DOPAL (data not shown).

DOPAL Interacts with N-terminal Domain of aS—Fig. 4A shows that DOPAL causes a concentration-dependent decrease of NMR cross-peak intensity in 1H-15N HSQC spectra of monomeric aS. The effects are localized to residues in the N-terminal domain of the protein, especially to residues 1–60, as well as to the vicinity of Met-116 and Met-127 in the C-terminal tail. To investigate whether the DOPAL-induced spectral changes occur synchronously with DOPAL oxidation, we compared the rates of oxidation of DOPAL with the rates for the appearance of perturbations in aS HSQC spectra caused by DOPAL. 1H-15N HSQC spectra were recorded at intervals of 3 h during a total period of 7 days. In parallel, the oxidation of
DOPAL was monitored using an identical sample under the same conditions of the NMR experiment. Fig. 4B shows the $^1$H-$^{15}$N HSQC spectra for aS after 0, 24, and 135 h of incubation at 15 °C. Resonance intensities were fit using a single exponential to extract intensity decay rate constants for individual residues, which were observed to be higher for residues located in the N-terminal region of the protein (Table 2). For example, the decay rate of the Met-5 signal is significantly higher than that of Met-116 and Met-127 (58.5 ± 10$^{-3}$ compared with 15.7 ± 10$^{-3}$ and 13.9 ± 10$^{-3}$ h$^{-1}$, respectively) (Fig. 4C and Table 2). Similarly, decay rate constants for Lys residues at the N terminus are higher than for those at the C terminus (Fig. 4D). The rate constant obtained for the oxidation of DOPAL (55 ± 10$^{-3}$ h$^{-1}$) (Fig. 4E) is in the same range as the rate constant observed for the intensity decrease of residues in the N-terminal domain of aS, strongly suggesting that these processes occur simultaneously. Together, these data indicate that spectral changes in the N-terminal portion of aS are caused by DOPAL oxidation and that the N-terminal region is more susceptible to DOPAL-induced structural modifications than the C terminus of the protein.

**DPQ and DOPAL Have Different Effects on aS Oligomerization**—DOPAL undergoes spontaneous oxidation in aerated aqueous solution, resulting in the formation of quinone derivatives with concomitant generation of ROS (Fig. 5A) (17). Considering that DOPAL oxidation is a key step for its effect on aS oligomerization, we compared the effect of DOPAL with its oxidized form, DPQ. DPQ was prepared by incubation of 1 mM DOPAL at 37 °C and agitation for 48 h, resulting in a brown-colored solution with enhanced absorbance at 340 nm. Although the incubation of aS with freshly prepared DOPAL lead to the formation of dimers and trimers, the incubation with DPQ produced a small population of large oligomers (Fig. 5, B and C), somewhat similar to those found after incubation with DA. This means that DPQ and DOPAL may have different mechanisms for inducing aS oligomerization. From these data, we hypothesized that the generation of ROS from DOPAL oxidation and its effect on aS might be important for the effects of DOPAL on aS.

The influence of aS on both the kinetics of oxidation of DOPAL and the formation of ROS generated during this process was also investigated. The rate of formation of DPQ, monitored by the absorbance at 340 nm, is significantly enhanced, in a concentration-dependent manner, by the presence of aS (Fig. 6A). Considering that DOPAL oxidation generates not only DPQ but also ROS, which, in turn, might be converted to H$_2$O$_2$, the influence of aS on the H$_2$O$_2$ levels produced from DOPAL oxidation was investigated. Fig. 6B indicates that the level of H$_2$O$_2$ detected decreased with increasing aS concentrations. This suggests that aS might react either with the free radicals generated from DOPAL oxidation and/or with H$_2$O$_2$. In both cases, a reduction in the levels of H$_2$O$_2$ would be observed. Collectively, these results suggest that aS might act as a scavenger of ROS generated from DOPAL oxidation and, consequently, stimulate the oxidation of DOPAL by shifting the equilibrium of the reaction toward to the formation of DPQ.

**FIGURE 3. Role of DOPAL oxidation on DOPAL-induced aS oligomerization.** A, effect of pH (3.0 and 7.5) on the kinetics of oxidation of DOPAL monitored as a function of time through the increase in absorbance at 340 nm (formation of DPQ). B, effect of pH on the fibrillation of 50 μM aS, in the presence or absence of DA or DOPAL (250 μM) was monitored using ThT fluorescence. C, SDS-PAGE of the samples obtained in B. D, effect of 1 mM ascorbic acid, 1 mM selegiline, 1 mM melatonin, 1 mM trans-resveratrol, 1 mM reduced glutathione (GSH), 50 mM mannitol, or 60 units of SOD on DOPAL oxidation measured by the intensity of the absorbance at 340 nm. E, 50 μM aS in 20 mM sodium phosphate, pH 7.5, was incubated in the presence of 250 μM DOPAL plus antioxidants, and the formation of oligomers after 3 days of incubation (37 °C, agitation) was determined by SDS-PAGE. The experiments in B and D were performed in sextuplicate, and the results are represented as mean ± S.D.
DOPAL Forms Covalent Adducts with α-Synuclein and Promotes Oxidation of Met Residues of αS—Chemical modifications induced by DOPAL on αS monomers were investigated using MS. Because of their low oxidation potential, methionines are readily oxidized by many oxidants, producing the corresponding sulfoxide as the major product. Methionine oxidation is also a target for oxidation promoted by DA in which Met-127 seems to play an important role in the formation of oligomers (34). Incubation of αS in the presence of DOPAL leads to the oxidation of Met residues to Met-sulfoxide (Table 3), as indicated by
an increased m/z = 16 in peptide fragments generated from aS-DOPAL-M. A product ion MS/MS spectrum of the peptide 1MDVF MK of an aS-DOPAL-M sample is shown in Fig. 7A. This peptide was detected with double positive charge with an m/z value of 401.7 Da (unmodified control, [M + 2H]²⁺ detected at 385.7 Da, data not shown), which matches with the oxidation of two Met residues. Fragments b₂⁻ and y₂⁻ present in the MS/MS spectrum confirm that both Met-1 and Met-5 are oxidized to sulfoxide in this peptide. NMR experiments indicate that Met-5 is more susceptible to DOPAL-induced oxidation than the C-terminal methionines (Met-116 and Met-127), consistent with previous data that indicate that the C-terminal Met residues (Met-116 and Met-127) are protected by a residual structure of the disordered aS monomer (35, 36). This is corroborated by our MS analysis, which indicates the co-existence of non-oxidized and oxidized forms of Met-116 and Met-127, with variable proportions depending on the sample preparation (data not shown). In contrast, Met-1 and Met-5 were found mostly in their oxidized form.

In addition to the oxidation of Met residues, the mass analysis of peptides generated after trypsin digestion of aSunmod and aS-DOPAL-M indicates the formation of covalent adducts between DOPAL/DPQ and Lys residues of aS (Table 3 and Figs. 7 and 8). Notably, aS-DOPAL-M shows more missed cleavages than the aSunmod (Table 4), which is consistent with the fact that trypsin might have an impaired performance during digestion of aS-DOPAL-M because of the formation of DOPAL adducts with Lys residues.

In interpreting the MS data in detail, we considered previously proposed mechanisms for protein modification by molecules structurally similar to DOPAL. Rauniyar and Prokai (37) detected the formation of SB between protein lysine residues of cytochrome c oxidase and the toxic aldehyde 4-hydroxi-2-nonenal (HNE) through the utilization of neutral loss-triggered MS3 experiments. Li et al. (38) suggest the formation of MA adducts between ortho-quinone, produced from the oxidation of catechol, and lysine residues of aS. Interestingly, such adducts were not detected in the reaction between aS and DA, which also exhibits a catechol scaffold (39). We therefore examined the data for indications that DOPAL could react with aS lysines via these two mechanisms as follows: MA involving the catechol ring or SB involving the aldehyde’s carbonyl (Fig. 8).

The product ion spectrum of the peptide 7GLSKAKEGV-VAAAEKT K23 (Fig. 7B) from aS-DOPAL-M sample illustrates the formation of SB adducts. This peptide was detected with triple charge and m/z value of 657.7 Da. The m/z values of the full peptide along with MS/MS fragments such as b₁₂⁻ and b₇⁻ confirm the presence of two chemical modifications localized...
to the two N-terminal lysines, Lys-10 and Lys-12. The formation of an SB/catechol for Lys-10 is indicated by fragment b4\(-\text{H}_2\text{O}\) for DOPAL-catechol. This result indicates that, despite the oxidation of DOPAL being essential for its interaction with aS, its reduced form is also able to bind to the protein via SB. In all, formation of SB adducts involving the carbonyl group of DOPAL was found in its catechol form, catechol, quinone, and hydrated forms for lysines in which DOPAL's quinone ring were also detected for lysines in which DOPAL oxidation; SB, Schiff-base; MA, Michael-adduct.

The mechanism of modification was proposed based on the results from three independent experiments. Cat, catechol; Qui, quinone; Hyd, hydrated; Mox, methionine oxidation; SB, Schiff-base; MA, Michael-adduct.

The modified residues and mechanisms of modification of aS-DOPAL monomers detected by MS/MS

| DOPAL-modified peptide sequence | Modified residue | Mechanism of modification |
|---------------------------------|------------------|---------------------------|
| MDVEMK5\*                     | Met-1            | Oxidation                 |
| MDVEMKGLSK10\*                | Met-5            | Oxidation                 |
| MDVEMKGLSKAEGVVAEEK23\*       | Met-5            | Oxidation                 |
| GLSKAEGVVAEEK23\*             | Lys-12           | MA/Cat/Hyd                |
| GLSKAEGVVAEEK23\*             | Lys-12           | MA/Qui/Hyd                |
| EGVVAAAEK21\*                 | Lys-21           | MA/Qui                    |
| KTK45\*                       | Lys-32           | SB/Cat                    |
| YEGVLYYGSKTK45\*              | Lys-34           | MA/Cat/Hyd                |
| YEGVLYYGSKTK45\*              | Lys-43           | MA/Qui/Hyd                |
| YEGVLYYGSKTK45\*              | Lys-43           | MA/Qui                   |

FIGURE 6. Effect of aS on DOPAL oxidation. A, rate of oxidation of 250 \(\mu\)M DOPAL in 20 mM sodium phosphate, pH 7.5, 37 °C, monitored by the absorbance at 340 nm, in the presence of varying concentrations of aS (0–150 \(\mu\)M). B shows the effect of aS on the levels of \(\text{H}_2\text{O}_2\) produced from the oxidation of DOPAL. The kinetic assays were performed in triplicate, and the results are represented as mean ± S.D.

TABLE 3

Modified residues and mechanisms of modification of aS-DOPAL monomers detected by MS/MS

The modified residues and mechanisms of modification of aS-DOPAL monomers detected by MS/MS.

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Membrane Binding Protects aS against DOPAL Effects—To investigate whether the effect of aS on DOPAL oxidation, observed in Fig. 6A, depends on the intrinsically disordered VGSKTK45, shown in Fig. 7C, confirms the presence of MA adducts containing hydrated DOPAL. This peptide appears as a \([M + 2H]^{2+}\) with an \(m/z\) value of 863.4 Da. The \(m/z\) values of the full peptide as well as fragments in the MS/MS spectrum such as b4\(^{2+}\) and y4\(^{2+}\) confirm the presence of an MA/catechol/hydrated modification for Lys-34, as well as a MA/quinone adduct for Lys-43. The presence of hydrated DOPAL bound to aS in a detectable amount could explain the predominance of MA adducts over SB, because the latter requires DOPAL in its aldehyde form. In all, MA modifications were detected for lysines Lys-12, Lys-21, Lys-34, and Lys-43 (Table 3). Reported SB and MA modifications were detected in three independent experiments. Modifications in additional lysines were only detected sporadically and were not taken into account.

To confirm the role of lysines in the formation of the aS-DOPAL oligomers, we used a protocol for chemical modification of lys residues by CA. Unlike aS

\(\text{Synuclein, which present a higher sequence identity to aS}\), were detected for lysines Lys-10, Lys-12, Lys-21, Lys-34, and Lys-43 (Table 3). Reported SB and MA modifications were detected in three independent experiments. Modifications in additional lysines were only detected sporadically and were not taken into account.

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structure of the protein, we measured the oxidation of DOPAL in the presence of aS plus SDS micelles. Fig. 10A shows that the ability of aS to accelerate DOPAL oxidation was remarkably reduced in the presence of SDS micelles, although SDS alone had no effect on DOPAL oxidation. Next, we investigated whether helically folded aS is protected against the effects of DOPAL by comparing the effect of DOPAL on either membrane-free aS or aS in the presence of SDS micelles or SUVs. SDS-PAGE analysis clearly indicates that the binding of aS to SDS micelles or SUVs blocks DOPAL-induced aS oligomeriza-
Covalent Adducts Formed between α-Synuclein and DOPAL

I) Schiff Base

\[
\text{DOPAL} + \text{Protein} \rightarrow \text{SBcat (+134.13 Da)}
\]

II) Michael Addition

\[
\text{DOPA} + \text{Protein} \rightarrow \text{MACat (+150.13 Da)}
\]

III) Methionine Oxidation

\[
\text{ROS} + \text{Protein} \rightarrow \text{Mox (+15.99 Da)}
\]

Fig. 10B. The intensity ratio of signals of the \(^{1}H-^{15}N\) HSQC spectrum of aS incubated in the presence of DOPAL (DOPAL/protein ratio = 10) in which the SDS micelles were added to the samples before ((aS + SDS) + DOPAL) or after ((aS + DOPAL) + SDS) incubation at 37 °C for 24 h, both normalized by the intensity for aS\(_{\text{unmod}}\) plus SDS. The continuous line (Fig. 10C) indicates a three-residue average. When SDS is added only after the incubation, the intensity decrease of signals at the N terminus of aS plus DOPAL is similar to that observed for the free protein (Fig. 4A), which corresponds to the perturbation caused by the reaction of the protein with DOPAL. However, when SDS micelles are added to the system before the incubation with DOPAL, aS-DOPAL and aS\(_{\text{unmod}}\) exhibited nearly identical intensities in the \(^{1}H-^{15}N\) HSQC spec-

### TABLE 4

| Sample           | \(\Sigma \) # missed cleavages | \(\Sigma \) # PSM | Ratio, missed cleavages per PSM | Mode | Median |
|------------------|--------------------------------|------------------|---------------------------------|------|--------|
| aS\(_{\text{unmod}}\) | 468                            | 16,021           | 0.0292                          | 1    | 1.53   |
| aS-DOPAL-M       | 928                            | 11,282           | 0.0823                          | 1    | 2.34   |

Fig. 9. Role of Lys residues on DOPAL-induced aS oligomerization. SEC (A) and SDS-PAGE (B) analysis of 50 μM Lys-blocked-aS (citraconic anhydride-treated aS (aS\(_{\text{cca}}\)) and aS\(_{\text{unmod}}\) after incubation with 1 mM DOPAL at 37 °C and agitation. C, SDS-PAGE analysis of truncated forms of aS (aS(del2–11) and aS(1–103)) and synuclein family members (β-synuclein and γ-synuclein) (protein concentration = 50 μM) after 24 h of incubation in the presence of 500 μM DOPAL, pH 7.5. Lane 1, aS(del2–11); lane 2, aS(del2–11) + DOPAL; lane 3, aS(1–103); lane 4, aS(1–103) + DOPAL; lane 5, β-synuclein; lane 6, β-synuclein + DOPAL; lane 7, γ-synuclein; lane 8, γ-synuclein + DOPAL; lane 9, aS; lane 10, aS + DOPAL.

Fig. 10. Chemical modifications of aS monomer produced by the incubation with DOPAL as determined by MS/MS analysis. Cat, catechol; Qui, quinone; Hyd, hydrated; Ox, oxidized.
Covalent Adducts Formed between α-Synuclein and DOPAL

tra, suggesting that SDS-bound αS is protected against the effects of DOPAL. Similar results were observed for αS incubated with DOPAL in the presence of 8 mM SUV (Fig. 10D). For these experiments, αS plus DOPAL was incubated at 15 °C for 72 h (no agitation), a condition in which the protein remains essentially monomeric and SUVs are fairly stable.

DOPAL-αS Adducts Strengthen the Interaction of N Terminus with NAC Domain of αS—Intramolecular long range interactions between the C terminus and the N terminus of αS may influence both the aggregation of the protein and its interaction with lipid membranes. Because the formation of DOPAL-Lys adducts decreases the positive charge of the N-terminal domain of αS, we examined long range intramolecular interactions in αS-DOPAL(1:10) using PRE. PRE can establish the physical proximity of different sites within a protein through the degree of broadening observed in the NMR signals caused by the presence of a spin-labeled site (23). Here, PRE was evaluated by measuring the intensity of cross-peaks in the 1H-15N HSQC spectra of MTSL spin-labeled αS, in the absence or presence of ascorbic acid, which was added to the MTSL spin-labeled sample to reduce the nitroxide spin label. PRE measurements for spin-labeled αSunmod mutants G31C, A85C, and P120C showed that spin labels near the N terminus, or in the NAC region (G31C and A85C), result in a broadening of residues in the C-terminal tail in the vicinity of residue 128, whereas the spin label in the C-terminal tail leads to broadening in the NAC region as well as near the N terminus of the protein (Fig. 11), as reported previously (40). However, for αS-DOPAL(1:10) monomer, the incorporation of spin-label at position 31 caused a remarkable broadening in the NAC region, which was not observed for the unmodified protein. Similarly, the presence of MTSL at αS-DOPAL position 85 caused much more extensive
Covalent Adducts Formed between α-Synuclein and DOPAL

broadening near the N terminus of the protein than for aS
unmod. In contrast, the broadening at C terminus domain caused by
MTSL at position 31 was less pronounced for aS-DOPAL in comparison with the unmodified protein. Overall, these results
suggest that the formation of DOPAL-aS adducts increase the
interaction of the N terminus with the NAC domain but
decrease long range interactions involving the N- and C-termi-
nal regions.

DOPAL Decreases the Affinity of aS for Synaptic Vesicle-like Membranes—The formation of DOPAL-aS adducts could affect the propensity of aS to bind to and to form helical struc-
ture upon binding to membranes, either by altering the electro-
static and hydrophobic interactions that promote membrane
binding or by altering the stability of α-helical backbone con-
formation. Fig 12A shows that the far-UV CD spectra for SDS-
bound aSunmod and aS-DOPAL(1:10) samples are nearly iden-
tural, with the negative bands at 208 and 222 nm that are characteristics of \( \alpha \)-helical conformations, suggesting that their interaction with the negatively charged headgroups and hydrophobic interior of SDS micelles is very similar. To investigate the secondary structure of SDS-bound \( \alpha \)-S-DOPAL monomer in greater detail, we measured the \( C_1\) chemical shifts, obtained from HNCA spectra, as indicators of secondary structure preferences in the protein (Fig. 12, B and C). Empirical values expected for a random coil polypeptide (41, 42) were subtracted to generate \( C_1\) secondary shifts, which provide secondary structure information. Positive \( C_1\) secondary chemical shifts indicate a preference for helical conformation, whereas residues that sample more extended conformations show the opposite pattern. Surprisingly, both samples display nearly identical values for \( C_1\) secondary chemical shifts (Fig. 12 D).

Therefore, the loss of positive charge due to the formation of the DOPAL-Lys adduct does not affect the helicity of the protein in the presence of SDS and does not introduce breaks in the helical structure of the protein beyond those already found in SDS-bound unmodified \( \alpha \).

To investigate the effects of DOPAL modification on the binding of \( \alpha \) to lipid vesicles, we compared the HSQC spectra of \( \alpha_{\text{unmod}}\) and \( \alpha\)-DOPAL (1:10) and (1:20) in the absence or presence of SUVs with a lipid composition similar to that found in synaptic vesicles (60% DOPC, 25% DOPC, 15% DOPS). The interaction of \( \alpha \) with lipid membrane occurs in the slow-exchange limit on the NMR frequency scale, and the slow rotational motion of the membrane-bound protein results in a resonance linewidth too broad for detection. Thus, the use of this approach allows us to relate the normalized signal attenuation of resonances from specific residues in the presence of SUVs to the binding of those residues to the membrane. Fig. 13, A–C, shows the intensity of cross-peaks in the \( ^1\)H-\( ^{15}\)N HSQC spectra for \( \alpha_{\text{unmod}}\), \( \alpha\)-DOPAL (1:10), and \( \alpha\)-DOPAL (1:20), respectively, in the presence of varying concentrations of SUVs (2, 5, and 8 mM) normalized by the intensity in the absence of SUV. The attenuation caused by the presence of SUVs is observed primarily in the N-terminal and NAC domains of the protein. Importantly, this attenuation is higher for \( \alpha_{\text{unmod}}\) in comparison with \( \alpha\) incubated in the presence of DOPAL, especially for \( \alpha\)-DOPAL(1:20), indicating that the modifications induced by DOPAL on \( \alpha \) lead to a decrease in the affinity of the protein for synaptic vesicle-like SUVs.

The interaction of the protein with SUVs may be expected to be highly dependent on temperature, particularly due to the increase of the protein hydrophobicity caused by the formation of DOPAL-Lys adducts. Thus, the parameters of binding derived from NMR experiments at 10 °C may change considerably when the temperature rises to 37 °C. For this reason, we measured the dissociation constant \( (K_d)\) at 37 °C using ITC. Fig. 13, D and E, shows the heat release curves for SUVs titrated into a calorimeter cell containing solutions of \( \alpha_{\text{unmod}}\) or \( \alpha\)-DOPAL (1:10), respectively. The negative heat exchange upon SUV injection shows that the lipid-protein interaction is exothermic with saturable lipid-binding sites on both protein samples. After integration of the obtained heat signals, a binding curve can be derived (Fig. 13F), which provides \( K_d\) values of 1.53 ± 0.3 and 2.57 ± 0.2 \( \mu\)M for \( \alpha_{\text{unmod}}\) and \( \alpha\)-DOPAL,
respectively. These $K_d$ values are somewhat higher than those reported previously based on ITC data, which indicated that the aS monomer interacts with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (4:1) vesicles with a $K_d$ of 0.22 M at 25 °C (62). The difference can likely be attributed to different salt concentrations (100 mM NaCl in our samples versus no salt in the previously reported experiments), although the different temperatures of the experiment (25 °C versus 37 °C) and the different compositions (headgroup and acyl chain saturation) of the vesicles used may also contribute. In any case, as observed in the NMR experiments at 10 °C, the ITC data indicate a decreased affinity of aS-DOPAL for SUVs at 37 °C.

Binding to SUVs was also analyzed by measuring of the gain of helical content upon lipid titration. Far-UV CD titration curves for aS and aS-DOPAL(1:10) monomer with SUVs are shown in Fig. 14. Less helicity is induced for aS-DOPAL than for unmodified aS at equivalent SUV concentrations. Plots of ellipticity at 222 nm versus SUV concentration obtained for aS-DOPAL monomer (1:5, 1:10, and 1:20) are shifted significantly to the right compared with unmodified aS, and the degree of the rightward curve shift increases with the increase of the concentration of DOPAL, indicating that the degree of modification by DOPAL is correlated with a reduced affinity to SUVs. Fits of the titration data used Equation 2 yielded $K_d$ values of 2.2, 4.0, 4.8, and 5.1 μM for aSunmod, aS-DOPAL(1:5), aS-DOPAL(1:10), and

FIGURE 13. Binding of aSunmod and aS-DOPAL monomers to SUVs. A–C show the intensity ratios from $^1$H-$^15$N HSQC spectra of 200 μM aSunmod and aS-DOPAL monomers (DOPAL/aS ratio = 10 and 20) in the presence of varying concentrations of SUV to the peak intensities in the absence of lipids. The continuous line indicates a three-residue average. D and E show the ITC-measured heat release curves (baseline corrected raw data) for 25 μM aSunmod and aS-DOPAL (DOPAL/aS ratio = 10) upon titration with 100 mM SUV at 37 °C. F, integrated heat signals, with subtracted background contributions (SUV injected into buffer), for titrations of SUV into protein solutions presented in D and E. Vesicle composition: 60% DOPC, 25% DOPE, 15% DOPS. All experiments were carried out using 20 mM sodium phosphate, pH 7.5, as buffer.

FIGURE 14. Titration of aSunmod and aS-DOPAL monomers with SUVs analyzed by far-UV CD. Protein solutions (40 μM in 20 mM sodium phosphate, pH 7.5, 100 mM NaCl) were incubated at 25 °C with increasing concentrations of SUVs and analyzed by CD to determine $[\theta]_{222}$ (Equation 1), a measure of protein $\alpha$-helicity. The data were fit to Equation 2 to calculate the values for $K_d$, minimum $[\theta]_{222}$, and maximum $\alpha$-helical content. SUV composition: 60% DOPC, 25% DOPE, and 15 DOPS.
aS-DOPAL(1:20), respectively (Table 5), in reasonable agreement with the ITC, given the differences between the two techniques. The maximum \( \alpha \)-helicity of the samples at saturating lipid concentration indicates that, beyond the reduced affinity for SUVs, aS-DOPAL exhibits a reduced \( \alpha \)-helical content. Although aS\(_{\text{unmod}}\) presents a maximum helical content of 52.6\%, aS modified by DOPAL has its helical content reduced with increasing concentrations of DOPAL, yielding 46.8, 41.5, and 31.9\% helicity for aS-DOPAL(1:5), aS-DOPAL(1:10), and aS-DOPAL(1:20), respectively.

### Discussion

The basis for the selective degeneration of dopamine-producing neurons in Parkinson disease remains a critical unresolved issue in Parkinson disease research. Here, we investigate the link between the most toxic by-product of dopamine production, DOPAL, and the Parkinson disease protein \( \alpha \)-synuclein. Levels of DOPAL are found to be augmented in the post-mortem brains of patients with PD (43), potentially associated with different mechanisms as follows: (i) An increase in DA levels in the cytosol due to leakage of vesicles is possibly promoted by aS oligomers (44). DA accumulation would lead to increased MAO activity and hence to elevated DOPAL levels. (ii) An increase in MAO activity is shown with aging. Positron emission tomography experiments indicate a slight increase in MAO-B levels with age in neuronal tissue in normal healthy human subjects, which could result in elevated DOPAL levels due to MAO-catalyzed DA degradation (45). (iii) A reduction of ALDH cytosolic isoform (ALDH-1A1) expression in surviving neurons in SN of PD, as observed in post-mortem studies (12). The inhibition of mitochondrial complex I, which synthesizes NAD, a cofactor for ALDH, also results in elevated DOPAL levels. (49). Similarly, 4-oxo-2-nonenal and HNE, generated from lipid peroxidation, results in the formation of very stable and cytotoxic protein adducts in vitro. HNE has also been found to induce oligomerization of aS and cause toxicity to dopaminergic neurons in vitro (49).

To identify specific sites at which aS is modified after incubation with DOPAL, we employed MS analysis of monomeric aS-DOPAL adducts. The data reveal that in addition to methionine oxidation, which has been previously reported to occur for aS under various oxidizing conditions, the presence of DOPAL leads to the following two types of modifications of the amino group of lysine side chains: SB formation via the carbonyl group of DOPAL or DPQ, or MA to the quinone ring of DPQ. The specificity of DOPAL modification to lysine side chains is supported by data showing that pretreatment with a lysine-blocking reagent prevents DOPAL-induced modification and oligomerization of aS. Lysines in the N-terminal domain of aS were more sensitive to adduct formation than those in the NAC or C-terminal regions of the protein. To our knowledge, this is the first report conclusively documenting the selective covalent modification of aS lysine side chains by dopamine metabolism by-products.
Covalent Adducts Formed between α-Synuclein and DOPAL

FIGURE 15. Hypothetic mechanism of DOPAL-induced oligomerization of αS mediated by the formation of Schiff-base (SB) and Michael addition (MA) reactions. αS-DOPAL monomer (generated via SB reaction between αS and DOPAL) (a) might react with αSmonom (c) via MA mechanism, generating cross-linked dimers of αS (d). Alternatively, c can also react with αS-DOPAL monomer generated by MA reaction between αS and DOPAL (b) to form dimers via either SB or MA mechanisms. The former leads to the formation of d, and the second generates e.

DOPAL May Interfere with αS Function by Reducing Its Affinity for Membranes and Decreasing Its Propensity for Helical Structure—Our results demonstrate that the modification of αS monomer by DOPAL significantly reduces both the affinity of the protein for SUVs and the formation of helical conformations by αS upon membrane binding. DOPAL-induced impairment of binding of αS to lipid vesicles might be caused by a combination of several effects. First, we observe a large increase in intramolecular interactions between the N terminus and the NAC domain, likely caused by the reduced charge and increased hydrophobicity that results from the modification of Lys residues by DOPAL/DPQ. These increased intramolecular interactions could hinder both the initial interactions of αS with membranes and the conversion of the protein to the extended highly helical conformation associated with membrane binding (53). Second, the reduction of positive charge in the N-terminal domain of αS upon DOPAL modification would decrease the electrostatic interaction between the normally highly positively charged N-terminal domain and the negatively charged lipid headgroups present both in the SUVs used in our experiments as well as in the synaptic vesicles they are intended to represent. Finally, the formation of helical structure by αS upon membrane binding depends on the amphipathic nature of the resulting helices, including the presence of the positively charged Lys residues at the interface between the polar and apolar faces (22, 54). By converting these positively charged lysines to hydrophobic lysine adducts, the amphipathic character of the helical conformations formed by αS would be altered, potentially decreasing the ability of the protein to adopt helical structure upon membrane binding. How these different factors combine to perturb the affinity of αS for lipid membranes remains to be investigated. It is interesting to note, however, that binding of αS to SDS micelles appears to be unaffected by DOPAL, suggesting that the strong nature of this interaction, or perhaps the different broken-helix conformation adopted by the protein in the micelle-bound state (53, 55), mitigates the effects of DOPAL modification both on binding and on the formation of helical structure.

Potential Role of DOPAL in αS Function and Pathology—The physiological functions of αS remain poorly understood (56), but at presynaptic nerve terminals the protein is thought to have some role in the genesis, trafficking, or fusion of synaptic vesicles. It has been proposed that αS may function as a SNARE chaperone and promote SNARE-complex assembly (57), but αS also reportedly can inhibit SNARE-mediated vesicle fusion. Recently, it has also been proposed that αS may play a role in protecting membrane lipids from oxidative damage by coupling oxidation of its N-terminal membrane-bound methionines to reduction of oxidized lipids, leading to its release from the membrane and subsequent reduction by cytosolic methionine sulfoxide reductases (58). All of these putative functions require the protein to bind membranes (likely in the form of synaptic vesicles) via its N-terminal lipid-binding domain, which contains a series of seven imperfect 11-residue repeats centered on a KTKEGV consensus sequence. This domain adopts an amphipathic helical structure upon binding to detergent micelles or phospholipid vesicles, and Lys residues within the N-terminal repeats play an important role in the interaction of the protein with membranes (54) and may also regulate its pathophysiology (59). Importantly, 11 of 15 lysines found in αS are located in this N-terminal domain, which is the primary target of DOPAL.

Because DOPAL weakens the affinity of αS monomers for membrane vesicles, it is likely that DOPAL modification of αS in vivo would alter or compromise the function of the protein. To the extent that alterations in αS function contribute to the etiology of PD, this aspect of DOPAL’s effects on αS could be important. It is also possible that by releasing αS from membrane surfaces, DOPAL may enhance the concentration of soluble monomeric αS and thereby drive aggregation of the soluble form of the protein. At the same time, release of αS from the membrane surface enhances its susceptibility to DOPAL/DPQ-mediated cross-linking of αS into small oligomers. These oligomers may potentially be toxic themselves, but in addition they, as well as monomeric αS-DOPAL, can retard the formation of mature fibrils by unmodified αS, likely increasing the population of other potentially toxic oligomeric species as well. It is also interesting to note that a number of disease-linked αS mutations, including A30P and G51D, are now known to promote release of αS from membranes (60, 61). Such mutations could thereby facilitate the modification of αS by DOPAL and promote the toxic effects of this interaction.
Conclusions—Our findings lead to the following model for selective dopaminergic neuron degeneration in PD based on the relationship between DA metabolism, oxidative stress, and aS aggregation (Fig. 16). Cytosolic disordered forms of aS can stimulate DOPAL oxidation, whereas binding of aS to lipid membranes may prevent, at least in part, aS-induced DOPAL oxidation. Oxidation of DOPAL leads both to the production of ROS, resulting in aS Met oxidation, as well as to the formation of covalent adducts between DOPAL and aS Lys side chains. DOPAL-modified aS forms potentially toxic small oligomers via a mechanism that remains to be fully elucidated but that likely involves the formation of DOPAL-mediated protein cross-links. These highly stable aS-DOPAL oligomers, as well as DOPAL-modified monomers, act as potent inhibitors of the formation of putatively inert or even neuroprotective mature aS fibrils, increasing the population of other potentially toxic oligomers of unmodified aS. Both Met oxidation and DOPAL modification of aS also act to reduce the affinity of the protein for synaptic-like vesicles and its ability to form helical structure, increasing the population of membrane-free aS and facilitating further DOPAL-mediated oxidation and oligomerization. Ultimately, further studies are necessary to determine the neurotoxic activity of DOPAL-induced aS oligomers and to ascertain whether these contribute directly to dopaminergic cell death and/or whether their additional effects, including potential inhibition of membrane-binding-dependent aS functions and enhancing the buildup of unmodified non-fibrillar oligomers of aS, contribute to the pathogenesis of PD.

Author Contributions—C. F. and E. C. C. contributed equally to this work. C. F. and E. C. C. conducted most of the experiments and analyzed the results. C. F. conceived the idea for the project. C. F. and D. E. supervised the project, analyzed the results, and wrote the paper. D. Y. Y. F. conducted the experiments for determination of hydrogen peroxide. A. S. P. contributed to the NMR experiments. G. D. T. A. and G. B. D. conducted the MS experiments and analyzed the results.

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