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Redox Reactions of the α-Synuclein−Cu^{2+} Complex and Their Effects on Neuronal Cell Viability[†]

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ABSTRACT: α-Synuclein (α-syn), a presynaptic protein believed to play an important role in neuropathology in Parkinson's disease (PD), is known to bind Cu^{2+}. Cu^{2+} has been shown to accelerate the aggregation of α-syn to form various toxic aggregates *in vitro*. Copper is also a redox-active metal whose complexes with amyloidogenic proteins/peptides have been linked to oxidative stress in major neurodegenerative diseases. In this work, the formation of the Cu^{2+} complex with α-syn or with an N-terminal peptide, α-syn(1−19), was confirmed with electrospray−mass spectrometry (ES−MS). The redox potentials of the Cu^{2+} complex with α-syn (α-syn−Cu^{2+}) and α-syn(1−19) were determined to be 0.018 and 0.053 V, respectively. Furthermore, the Cu^{2+} center(s) can be readily reduced to Cu^{+}, and possible reactions of α-syn−Cu^{2+} with cellular species (e.g., O_2, ascorbic acid, and dopamine) were investigated. The occurrence of a redox reaction can be rationalized by comparing the redox potential of the α-syn−Cu^{2+} complex to that of the specific cellular species. For example, ascorbic acid can directly reduce α-syn−Cu^{2+} to α-syn−Cu^{+}, setting up a redox cycle in which O_2 is reduced to H_2O_2 and cellular redox species is continuously exhausted. In addition, the H_2O_2 generated was demonstrated to reduce viability of the neuroblastoma SY-HY5Y cells. Although our results ruled out the direct oxidation of dopamine by α-syn−Cu^{2+}, the H_2O_2 generated in the presence of α-syn−Cu^{2+} can oxidize dopamine. Our results suggest that oxidative stress is at least partially responsible for the loss of dopaminergic cells in PD brain and reveal the multifaceted role of the α-syn−Cu^{2+} complex in oxidative stress associated with PD symptoms.

Although many neurodegenerative diseases are manifested by the aggregation of the amyloidogenic proteins (e.g., *β*-amyloid or A*β* in Alzheimer's disease (AD),[1] α-synuclein (α-syn) in Parkinson's disease (PD), and prion protein in Creutzfeldt−Jakob disease) (*1*, *2*), oxidative stress has also been implicated in the pathogenesis of neurodegenerative diseases (*3*, *4*). Consequently, metal-induced oxidative damages have been an area under active pursuit (*5*, *6*). The implication of metal-induced oxidative stress is extremely broad, ranging from acceleration of the formation of reactive oxygen species (ROS) (*7*) in the presence of redox metals such as copper and iron (*8*), mitochondria function impairment (*9*, *10*), and neuronal membrane damages through lipid peroxidation (*11*) to depletion of vital intracellular species (*12*). For example, in the senile plaques of AD patients, large amounts of redox-active metal ions such as Cu^{2+} and Fe^{2+} have been found to coexist with the aggregates of A*β* peptides (*13*). *In vitro* studies have firmly established that these metal ions can strongly bind A*β* peptides (*14*) and the resultant complexes can facilitate the generation of H_2O_2 by reacting with cellular species such as ascorbic acid (AA) and O_2 (*15*, *16*):

$$\text{ascorbic acid} + 2A\beta\text{−}Cu^{2+} + H_2O \rightarrow \text{dehydroascorbic acid} + 2H^{+} + 2A\beta\text{−}Cu^{+} \quad (1)$$

$$2A\beta\text{−}Cu^{+} + O_2 + 2H^{+} \rightarrow 2A\beta\text{−}Cu^{2+} + H_2O_2 \quad (2)$$

Recently, we measured the redox potentials of the Cu^{2+} complexes with several A*β* peptides (*12*). Our results confirmed that the H_2O_2 generation can be catalyzed by the Cu^{2+} complexes of both the aggregation-prone, full-length A*β*(1−42) and the nonaggregating hydrophilic A*β*(1−16) (*12*). H_2O_2 is an important ROS because H_2O_2 itself can react with a variety of cellular species and is a precursor for or product from reactions of other ROS. For example, reaction 2 has been suggested to proceed by reduction of O_2 to oxygen radical anion ($O_2{\cdot}^{-}$, another ROS) (*17*). On the other hand, uncomplexed or free redox metal ions (e.g., redox-active Cu^{2+} and Fe^{2+}) can generate hydroxyl radicals ($OH^{\bullet}$) by reacting with H_2O_2 through the Harber−Weiss and Fenton reactions (*18*, *19*). Since the brain utilizes O_2 for a variety of important functions, continuous reduction of O_2 to generate ROS by trace Cu^{2+} complex with A*β* that is reduced by a cellular reductant (e.g., AA) could lead to pronounced deleterious effects (*5*, *12*). Post-mortem chemical (*20*) and spectroscopic (*21*) analyses have also provided evidence for the possible involvement of these metals or their complexes with A*β* in producing

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[1]Abbreviations: α-syn, α-synuclein; PD, Parkinson's disease; ES−MS, electrospray−mass spectroscopy; α-syn−Cu^{2+}, Cu^{2+} complex of α-syn; α-syn(1−19)−Cu^{2+}, Cu^{2+} complex of α-syn(1−19); AD, Alzheimer's disease; ROS, reactive oxygen species; A*β*, *β*-amyloid; AA, ascorbic acid; DA, dopamine; CV, cyclic voltammogram; DPV, differential pulse voltammogram; LBs, Lewy bodies; NAC, nonamyloid components; GSH, glutathione; IPTG, isopropyl *β*-D-thiogalactopyranoside; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; RP, reverse phase; AFM, atomic force microscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Scheme 1: Sequence of α-Synuclein with the N-Terminus Underlined and the C-Terminus Expressed in Italics

MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFV*KKDQL GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA*

neurotoxins, which eventually result in neuronal cell loss. Interestingly, Aβ has also been suggested to possibly serve as a protective agent through metal ion complexation, which ameliorates the free metal ion-induced hydroxyl radical generation (*22*−*24*).

PD is pathologically marked by the progressive loss of neurons in the *substantia nigra*, a small brain region producing dopamine (DA) (*25*). A hallmark of PD is that surviving dopaminergic cells contain cytosolic filamentous inclusions known as the Lewy bodies (LBs) (*26*). A major component in LBs is the α-syn aggregates (*27*, *28*), whose monomeric constituent contains 140 amino acid residues encompassing the positively charged N-terminus (residues 1−60), the aggregation-prone nonamyloid components (NAC, residues 60−95), and the negatively charged C-terminus (residues 96−140) (*29*) (Scheme 1).

α-Syn can also bind Cu^{2+}, and several binding sites for Cu^{2+} in α-syn have been detected (*30*). The binding mode contributed by the first four amino acids of N-terminus (*31*), in which Met-1 serves as an anchor, has a dissociation constant of ∼0.2 μM (*31*). This binding mode is much stronger than the nonspecific binding by the C-terminus of α-syn (dissociation constant around millimolar level (*30*)) and also 2 orders of magnitude stronger than the binding sites centered around His-50 (*30*). The relatively high Cu^{2+} binding affinity at Met-1 explains why α-syn can sequester Cu^{2+} released from aberrant proteins (e.g., Cu/Zn superoxide dismutase) of PD patients (*32*, *33*). Together with the abnormal homeostasis of Cu^{2+} in PD patients (*34*−*36*), the Cu^{2+} complex with α-syn (α-syn−Cu^{2+}) has been suggested to play an important role in PD etiology (*37*, *38*). For example, Cu^{2+} is an effective ion in promoting the formation of α-syn oligomers (*39*), which is cytotoxic due to its putative role in pore formation in the cell membrane (*38*). However, given that α-syn is known to be redox-inactive (*40*), the decreased level of redox-active species (e.g., glutathione (GSH) and DA (*41*)) and the increased level of protein oxidation (*42*, *43*) in PD brain cannot be rationalized by the α-syn aggregation mechanism. Prompted by the thought that a mechanism similar to reactions 1 and 2 might be applicable, we hypothesize that easily oxidizable cellular species could react with α-syn−Cu^{2+}. In fact, we recently discovered that the complex formed between α-syn and Fe^{2+} (*44*) can also take part in the generation of H_2O_2 in an analogous fashion (*45*).

To verify our hypothesis, it is necessary to first measure the redox potential of α-syn−Cu^{2+}, which will provide a better assessment of a reaction between the complex and a species that undergoes abnormal homeostasis (e.g., DA (*46*), GSH (*41*), or NADH (*47*)). Since H_2O_2 can cause neuronal cell death (*16*), it is also intriguing to learn whether the α-syn−Cu^{2+} complex can participate in the generation of H_2O_2 in the same manner as Cu^{2+} complexes with Aβ (cf. reactions 1 and 2) and consequently impose toxicity to neuronal cells. In this work, α-syn−Cu^{2+} was characterized by mass spectrometry and voltammetry. To avoid possible α-syn aggregation during the measurements, we also synthesized an N-terminus α-syn(1−19) peptide, which encompasses the strongest Cu^{2+}-anchoring site at Met-1 but does not contribute to the α-syn aggregation. The redox potentials of α-syn−Cu^{2+} and α-syn(1−19)−Cu^{2+} were determined to be 0.018 and 0.053 V, respectively. These potentials suggest that the complexes can oxidize certain cellular reductants (e.g., AA and GSH) and subsequently generate H_2O_2 through O_2 reduction in a mechanism similar to that depicted by reactions 1 and 2. H_2O_2 was verified to be the product of O_2 reduction and shown to be cytotoxic. The implication of the α-syn−Cu^{2+} complex in PD-related oxidative stress is discussed.

MATERIALS AND METHODS

Materials. Wang resin, Fmoc-protected amino acids, diisopropylcarbodiimide, 1-hydroxybenzotriazole, and piperidine were purchased from Anaspect Inc. (San Jose, CA). Potassium hydrogen phosphate, potassium hydroxide, sodium sulfate, ammonium sulfate, trifluoroacetic acid, copper sulfate, copper chloride, nickel chloride, isopropyl β-D-thiogalactopyranoside (IPTG), L-glutamine, and organic solvents were obtained from Thermo-Fisher Scientific Inc. (Pittsburgh, PA). DA and AA were acquired from Sigma-Aldrich (Milwaukee, WI). SH-SY5Y cells (human neuroblastoma) were from American Type Culture Collection Inc. (Manassas, VA), and fetal bovine serum (FBS) was from Omega (Tarzana, CA). Both Dulbecco's modified Eagle medium (DMEM) and Ham's F12 were acquired from Mediatech Inc. (Manassas, VA). *Escherichia coli* BL21 (DE3) and dithiothreitol (DTT) were purchased from Invitrogen Corp. (Carlsbad, CA), and lysozyme was from EMD Inc. (Gibbstown, NJ). Both Aβ(1−16) and Aβ(1−42) were acquired from American Peptide Co. Inc. (Sunnyvale, CA). Water was purified by a Millipore system (Billerica, MA) to be 18 MΩ·cm and used for the preparation of all aqueous solutions, and the mixture of penicillin and streptomycin for cytotoxicity study was also purchased from Millipore. Phosphate buffer was made by mixing 0.1 M potassium hydrogen phosphate and 0.1 M potassium hydroxide.

Peptide Synthesis. α-Syn(1−19) was synthesized via solid-phase Fmoc chemistry on a Symphony Quartet peptide synthesizer (Protein Technologies, Tucson, AZ). The Fmoc groups were deprotected with 20% piperidine in dimethylformamide (v/v) after the coupling reaction had proceeded for 30 min. Upon dehydration on a freeze dryer (VirTis Benchtop K, Warminster, PA), the crude product was purified by semipreparative reversed-phase (RP) HPLC (Shimadzu 6AD, Columbia, MO) using a column (Jupiter-10-C18-300, 10 mm i.d. × 250 mm) from Phenomenex (Torrance, CA). The eluents were 0.1% trifluoroacetic acid in water (v/v, mobile phase A) and 0.1% trifluoroacetic acid in acetonitrile (v/v, mobile phase B). At a flow rate of 4.75 mL/min, purification of α-syn(1−19) was accomplished with an elution gradient of 25−65% phase B for 12 min. The purity of the synthesized peptides was verified by HPLC and electrospray−mass spectrometry (ES−MS).

Expression and Purification of α-*Syn*. Expression and purification of α-syn followed the reported procedure (*48*). In brief, *E. coli* BL21 (DE3) were transfected with pRK172/α-synuclein plasmids kindly donated by Prof. P. Lansbury (Harvard University). After the expression was induced by IPTG, cells were harvested, resuspended in 10 mM phosphate buffer solution (pH 7.4), and lysed by adding lysozyme. Following sonication, lysate was separated from the precipitate by centrifuge, and the supernatant was mixed with 1 mM DTT. The crude

product was purified by RP-HPLC, and the elution gradient was 25−75% B for 20 min at a flow rate of 4.75 mL/min.

Mass Spectrometric Measurements. ES−MS was conducted on a Thermal Fisher LTQ linear ion-trap mass spectrometer (San Jose, CA). $CuCl_2$ and α-syn(1−19) peptide were dissolved in water at 1 mM and 100 μM, respectively. Aliquots of the copper solution were then added into the peptide solution to form the complex. The mixture solution were subsequently diluted with a water/methanol (v/v = 1:1) solution to a final concentration of 10 μM for the peptide. The sampler capillary was kept at 200 °C, and all of the mass spectra were collected in the positive ion mode. The ES−MS measurement of the copper complexes with α-syn was performed in the same manner.

Electrochemical Measurements. Voltammetric measurements of the Cu^{2+} complexes with the two α-syn species were carried out on a CHI832 electrochemical workstation (CH Instruments, Austin, TX) using a homemade plastic electrochemical cell with an internal volume of ~400 μL. The three-electrode system is composed of a glassy carbon disk working electrode (3 mm in diameter), a platinum wire auxiliary electrode, and a Ag/AgCl reference electrode. The electrolyte solution was a 5 mM phosphate buffer (pH 7.4) in the presence of 0.1 M Na_2SO_4. Conducting voltammetric experiments under oxygen-free condition was achieved by transferring the electrochemical cell and solutions into a glovebox (Plas Lab, Lansing, MI) that had been thoroughly purged with and kept under high-purity N_2. The oxygen concentration in the glovebox was measured to be less than 0.5 ppm by a portable conductivity meter (Orion 3-Star Plus; Thermo Electro Corp., MA).

Detection of Hydrogen Peroxide. H_2O_2 detection kit was purchased from Bioanalytical System Inc. (West Lafayette, IN) and calibrated as described in Supporting Information (*49*). H_2O_2 was generated by two methods in this work. For the first method, the complexes of Cu^{2+} formed with α-syn and with α-syn(1−19) were electrolyzed at 0.04 V vs Ag/AgCl for predefined times. The final solutions were injected through a six-port rotary valve (Valco, Houston, TX) into a flowing stream of phosphate buffer delivered by a syringe pump (Kd Scientific, Holliston, MA) at a flow rate of 10 mL/h. The amount of H_2O_2 was determined by comparing the measured current to that of a calibration curve constructed with H_2O_2 standard solutions (Figure S1 in Supporting Information). As for the second method, α-syn−Cu^{2+} or α-syn(1−19)−Cu^{2+} was mixed with 1 mM AA in aerated solution for a predetermined time. The concentration of H_2O_2 generated by AA reduction of these complexes was much higher than that produced by electrochemical reduction of these complexes. Therefore, the sample mixtures containing H_2O_2 were diluted by 25 times before the analysis with the above-mentioned H_2O_2 detection kit.

Cell Culture and Cytotoxicity. SH-SY5Y cells were cultured in a medium of 44.5% DMEM containing 4 mM L-glutamine/Ham's F12/FBS/mixture of penicillin and streptomycin (v/v/v/v = 44.5%/44.5%/10%/1%). For toxicity assay, cells collected at 45% confluence and resuspended in the same DMEM/F12 media with a smaller FBS content (5%) were added into a 96-well plate, and the plate was placed in a humidified incubator at 37 °C and under 5% CO_2. SH-SY5Y cells were treated with α-syn-containing solutions (*vide infra*) for 24 h. Viability of cells exposed to each solution was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (EMD Inc., Gibbstown, NJ). Briefly, MTT was first dissolved in water to 5 mg/mL. Media from the wells in the plate were replaced by the mixture of 10 % MTT (5 mg/mL) in media (v/v), and the well contents were subsequently incubated for 4 h. After removal of the MTT-comprising media, 150 μL of dimethyl sulfoxide was added into each well to dissolve the formazan precipitate. UV−vis absorption at 595 nm in each well was recorded by a plate reader (Tecan, San Jose, CA). For each type of solution, MTT assays in five separate wells were conducted, and the final viability is the average from MTT assays conducted on three different days.

Atomic Force Microscopy. We used atomic force microscopy (AFM) to investigate whether aggregations had formed with α-syn or α-syn−Cu^{2+} during the measurements of voltammetry, H_2O_2 detection, and cytotoxicity assays. AFM experiments were carried out on an MFP-3D-SA microscope (Asylum Research, Santa Barbara, CA) equipped with the tapping mode in air. The AFM cantilevers were purchased from MikroMasch (San Jose, CA). Freshly peeled mica was treated with Ni^{2+} by immersing the substrate in 10 mM $NiCl_2$ for 15 min, followed by thoroughly rinsing with deionized water and purging with nitrogen. Aliquots of α-syn, α-syn/Cu^{2+}, or α-syn/Cu^{2+}/AA mixture at a predetermined incubation time were casted onto Ni^{2+}-treated mica sheets for 15 min. The substrate was then rinsed with water to remove any residual salt and dried with nitrogen before AFM imaging.

RESULTS

Cu^{2+} Binds to α-Syn Protein/Peptide, and the Resultant Complexes Are Redox-Active. Figure 1 depicts representative electrospray mass spectra (ES−MS) collected from a solution containing Cu^{2+} and α-syn (panel A) and that comprising Cu^{2+} and the peptide segment α-syn(1−19) (panel B). The peaks in panel A correspond to α-syn and the α-syn complex with one Cu^{2+} center and one K^+ adduct (given rise by K^+ in the phosphate buffer used for the α-syn expression and preparation; peaks labeled with asterisks). No other peaks showing stoichiometric ratio higher than 1:1 between α-syn and Cu^{2+} were detected when more Cu^{2+} was mixed with the α-syn solution. This suggests that Cu^{2+} binds less strongly with and might have dissociated from other sites (e.g., the binding site around His-50 or the nonspecific binding in the C-terminus) (*30*, *50*, *51*).

In Figure 1B, the peaks corresponding to the uncomplexed α-syn(1−19) and its 1:1 complex with Cu^{2+} have m/z centered around 977.0 and 1007.5, respectively. Notice that the number of charges on these peaks is much less than those on the whole α-syn protein, because the α-syn protein has much more ionizable amino acid residues. The binding stoichiometry also correlates well with a previous report showing that residues near the N-terminus can complex Cu^{2+} with Met-1 as the anchoring site (*31*). Previously, it has been reported that residues of methionine and tyrosine can be oxidized in the presence of Cu^{2+} (*16*). Since the m/z values of the peaks in Figure 1A correspond to intact protein bound to Cu^{2+}, we conclude that all of the amino acid residues are not chemically modified or oxidized upon the Cu^{2+} complexation.

Cyclic voltammograms (CVs) of Cu^{2+} complexes formed with α-syn (solid line curve) and the α-syn(1−19) peptide (dashed line curve) are overlaid in Figure 2. To ensure extensive complexation, the concentrations of α-syn and α-syn(1−19) used are twice as high as that of Cu^{2+}. The redox waves of α-syn−Cu^{2+} and α-syn(1−19)−Cu^{2+} both exhibit quasi-reversible behaviors, which are in contrast to that of the irreversible reduction peak of free Cu^{2+} (dotted line curve). Thus, we conclude that Cu^{2+}

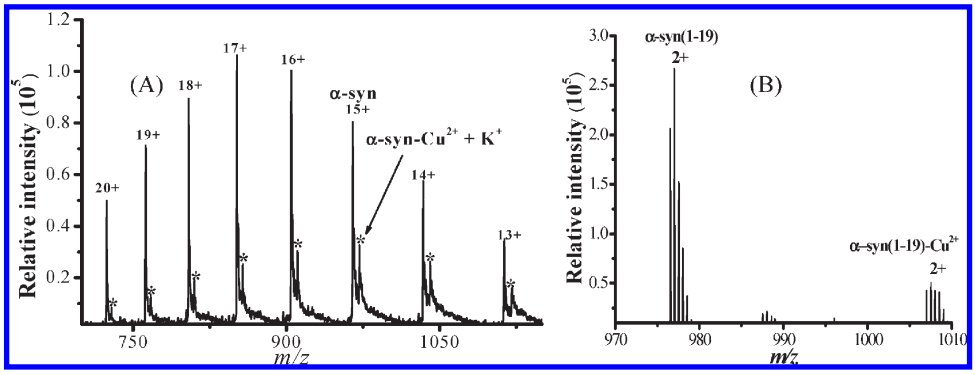

FIGURE 1: Electrospray mass spectra from solutions of (A) α-syn and Cu^{2+}, with peaks bearing 13+ to 20+ charges, and (B) α-syn(1−19) and Cu^{2+}, with peaks corresponding to doubly charged peaks. The asterisks in (A) indicate the α-syn−Cu^{2+} + K^+ adduct ions.

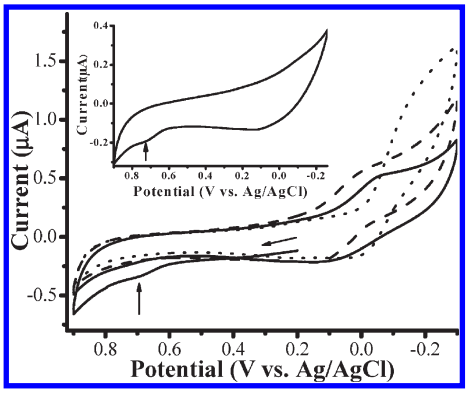

FIGURE 2: Cyclic voltammograms of 100 μM α-syn and 50 μM Cu^{2+} (solid line curve), 100 μM α-syn(1−19) and 50 μM Cu^{2+} (dashed line curve), and 50 μM free Cu^{2+} (dotted line curve) in 5 mM phosphate buffer (pH 7.4) containing 0.1 M Na_2SO_4. The scan rate was 5 mV/s, and the arrow indicates the initial scan direction. A voltammogram of 100 μM α-syn only is shown in the inset with the vertical arrow indicating the irreversible tyrosine oxidation peak.

Table 1: Redox Potentials of the α-Syn−Cu^{2+} and Select Biological Redox Couples

system	E (V vs Ag/AgCl)[a]
norepinephrine	0.188
dopamine (DA)	0.174
O_2/H_2O_2	0.099
cytochrome *a*	0.094
α-syn−Cu^{2+}/α-syn−Cu^+	**0.018**
hemoglobin	−0.044
$CoQ/CoQH_2$	−0.096
ascorbic acid (AA)	−0.145
myoglobin	−0.191
crotonyl-CoA/butyryl-CoA	−0.211
$FMN/FMNH_2$	−0.316
glutathione (GSH)	−0.424
vitamin B_{12}	−0.440
NAD^+/NADH	−0.516
$FAD/FADH_2$	−0.523

[a]The potential values are converted from those listed in ref *12* in the scale of Ag/AgCl.

complexed by both α-syn and α-syn(1−19) can be reduced to Cu^+, and the Cu^+-containing complexes are stable. These characteristics are also similar to those of the complexes formed between Cu^{2+} and various Aβ peptides (*12*) or a Cu^{2+}−histidine complex (*52*). Furthermore, the redox potentials (approximated as the average of the anodic and cathodic peak potentials) are 0.018 V for α-syn−Cu^{2+} and 0.053 V for α-syn(1−19)−Cu^{2+}. This implies that, similar to the Aβ−Cu^{2+} complex (*12*), α-syn−Cu^{2+} should be able to facilitate the ROS generation (*vide infra*). Notice that the α-syn voltammogram in the absence of Cu^{2+} shows a small irreversible oxidation peak at ca. 0.730 V (identified by the vertical arrow in the inset). This peak is due to the irreversible oxidation of the Tyr residue(s) at a high oxidation potential (*12*). The oxidation peak of tyrosine (identified by the vertical arrow in the solid line curve) is still observable in the CV of the mixture of 100 μM α-syn and 50 μM Cu^{2+}. This suggests that the Tyr residues in α-syn are not oxidized upon Cu^{2+} complexation, a point consistent with the aforementioned ES−MS results.

For easy prediction of the likelihood of a redox reaction involving α-syn−Cu^{2+} and a redox molecule, we list the redox potentials of select cellular species (*12*) and α-syn−Cu^{2+} in Table 1. Note that the α-syn−Cu^{2+} complex has a redox potential higher than those of cellular species such as AA, GSH, and NADH. Therefore, thermodynamically α-syn−Cu^{2+} should be able to oxidize these cellular reductants. On the other hand, the redox potential of α-syn−Cu^{2+} is lower than that of DA, indicating that it is not possible for α-syn−Cu^{2+} to directly oxidize DA. To verify these predictions, we mixed α-syn−Cu^{2+} with AA or DA and collected differential pulse voltammograms (DPVs) to determine whether there is a change in the AA or DA concentration over a given period of time.

The black curve in Figure 3A is a DPV of a phosphate buffer containing only 100 μM DA. To avoid possible DA oxidation by O_2 permeated into the solution, the entire electrochemical cell was again placed in a glovebox under N_2. Three hours after the addition of 50 μM Cu^{2+} and 100 μM α-syn, the height of the DA peak remained unchanged (red curve in Figure 3A). Thus, it is apparent that α-syn−Cu^{2+} does not directly oxidize DA. Interestingly, the presence of α-syn and Cu^{2+} significantly decreased the AA oxidation peak (Figure 3B) after 3 h. The red curve in Figure 3B has a peak height of only ~55% of that of the black curve, which corresponds to the DPV of AA in a solution that did not contain α-syn−Cu^{2+}. Also, an oxidation peak emerged at ca. −0.06 V, which can be ascribed to the electrochemical oxidation of the α-syn−Cu^+ complex that had been generated in the presence of AA. The decrease in the peak height thus verifies that α-syn−Cu^{2+} can directly oxidize AA (*53*).

Because the analyte concentration can affect its DPV peak potential for an irreversible redox reaction (*55*), the anodic shift in Figure 3B after the addition of α-syn−Cu^{2+} can be attributed to the decrease in the AA concentration. We should point out that the peak potential corresponding to the DPV oxidation peak

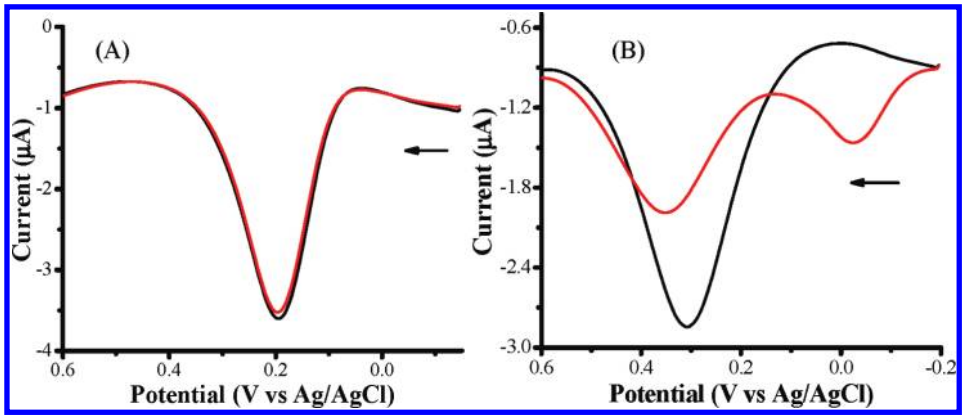

FIGURE 3: Differential pulse voltammograms of 100 μM dopamine (A) and 100 μM ascorbic acid (B) dissolved in deaerated phosphate buffer (pH 7.4) in the absence (black) and presence of 100 μM α-syn and 50 μM Cu^{2+} (red). The arrow indicates the initial scan direction.

of AA (0.281 V in Figure 3B) is much more positive than the standard redox potential of AA (−0.145 V; see also Table 1) (*54*). The reason for this deviation is attributable to the fast irreversible chemical reaction that converts the AA radical cation to dehydroascorbic acid, which shifts the redox peak in the anodic direction (*55*).

Generation of H_2O_2 Facilitated by the Cu^{2+} Complex of α-Syn. Another prediction that can be made from the data in Table 1 is that O_2 in solution should be able to oxidize the reduced form of α-syn−Cu^{2+} (i.e., α-syn−Cu^{+}). As mentioned in the introduction, this reaction is analogous to that occurring between O_2 and Aβ-Cu^{+} (cf. eq 2 given in the introduction) (*12*):

$$2\alpha\text{-syn}-Cu^{+} + O_2 + 2H^{+} \rightarrow 2\alpha\text{-syn}-Cu^{2+} + H_2O_2 \qquad (3)$$

In the presence of a cellular reductant (e.g., AA), α-syn−Cu^{2+} generated from the above reaction can be reduced, regenerating α-syn−Cu^{+}:

$$AA + 2\alpha\text{-syn}-Cu^{2+} + H_2O \rightarrow \text{dehydroascorbic acid} + 2H^{+} + 2\alpha\text{-syn}-Cu^{+} \qquad (4)$$

Reactions 3 and 4 set up a catalytic cycle in which α-syn−Cu^{2+} acts like a "catalyst" to continuously facilitate the O_2 reduction to H_2O_2 and depletion of cellular reductant(s). Indeed, the redox behavior of α-syn−Cu^{2+} was found to be affected by O_2 (cf. Figure S2 in Supporting Information), which indicates the feasibility of reaction 3. To verify the formation of H_2O_2 from the O_2 reduction by α-syn−Cu^{+} (shown in reaction 3), we performed electrolyses of the α-syn−Cu^{2+} in an aerated solution for different times, because H_2O_2 generation in reaction 3 requires the Cu^{2+} center to be reduced to Cu^{+}. H_2O_2 in these solutions was then quantified by the method described in Supporting Information (cf. Figure S1 and the detection scheme). As shown in Figure 4A, the amount of H_2O_2 increases with the electrolysis time. In addition, no H_2O_2 was detected if the α-syn−Cu^{2+} complex were not reduced. We also conducted a control experiment by holding the electrode potential at 0.04 V in a Cu^{2+}-only solution, which generated little H_2O_2 (less than 1 nM). This is conceivable since free Cu^{+} is not stable in an aqueous solution.

As illustrated in Figure 3B, AA can reduce α-syn−Cu^{2+} to α-syn−Cu^{+}. We also determined the H_2O_2 content in an air-saturated solution containing α-syn−Cu^{2+} and AA at different reaction times. The results are shown in Figure 4B. Because free Cu^{2+} can also participate in the AA oxidation reaction (*56*), the concentration of α-syn used was twice as high as that of Cu^{2+} so

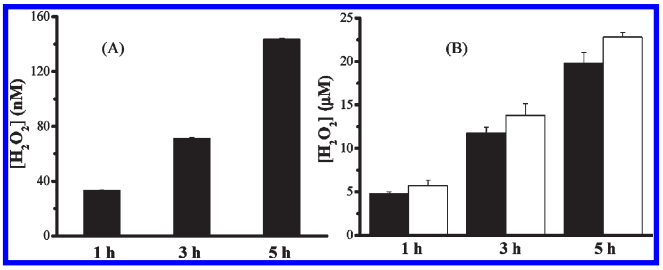

FIGURE 4: Concentrations of H_2O_2 generated from solutions of (A) 100 μM α-syn and 50 μM Cu^{2+} after electrolyses at 0.04 V and (B) 100 μM α-syn and 50 μM Cu^{2+} (black bars) or 100 μM α-syn(1−19) and 50 μM Cu^{2+} (white bars) mixed with 1 mM AA. Both of the measurements were made in ambient atmosphere for different periods (1, 3, and 5 h), and the error bars correspond to relative standard deviation values of three replicates.

that little free Cu^{2+} remained in solution. Again, it can be seen that the longer the reaction time, the greater the amount of H_2O_2 produced. A control experiment was also carried out by mixing 1 mM AA with 0.2 μM free Cu^{2+} (the equilibrium Cu^{2+} concentration predicted from the micromolar binding affinity constant (*30*) for a mixture of 100 μM α-syn and 50 μM Cu^{2+}). Little H_2O_2 (less than 0.1 μM) was detected even after 5 h, suggesting that free Cu^{2+} does not significantly generate H_2O_2 when excess AA is present. We also determined H_2O_2 produced by an air-saturated mixture containing α-syn-(1−19)−Cu^{2+} and 1 mM AA (white bars in Figure 4B) and contrasted the results to those from the α-syn−Cu^{2+} solution. Because both α-syn−Cu^{+} and α-syn(1−19)−Cu^{+} complexes, instead of the α-syn species alone, are capable of producing H_2O_2, the binding of Cu^{2+} must occur within residues 1−19. This point is in line with the discussion of Figure 1. That a slightly higher (~15−19%) H_2O_2 amount was generated in the α-syn-(1−19)−Cu^{2+} solution can be rationalized by the fact that the Cu^{2+} center in the shorter and more hydrophilic α-syn-(1−19)−Cu^{2+} complex is more exposed.

Cytotoxicity of α-Syn−Cu^{2+}. The data presented in Figures 3 and 4 clearly indicate that α-syn−Cu^{2+} can be reduced by AA and its reduced form α-syn−Cu^{+} can be reoxidized by O_2, producing H_2O_2 as a ROS. Because both AA and O_2 are abundant in brain (*57*), it is intriguing to know whether the amount of H_2O_2 formed is significant to cause neuronal cell death. To this end, we conducted cytotoxicity of α-syn−Cu^{2+} under various experimental conditions.

Previously, it has been shown that Aβ−Cu^{2+} in the presence of AA and O_2 can result in the loss of PC12 cells (*16*), presumably

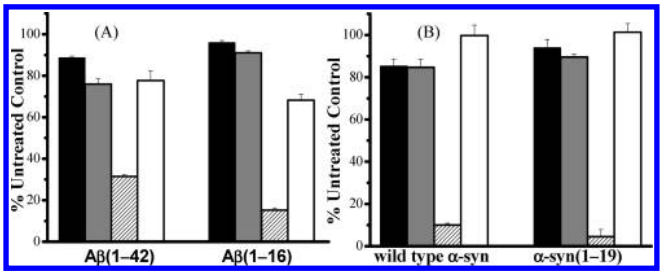

FIGURE 5: Cytotoxicities of (A) Aβ−Cu^{2+} and (B) α-syn−Cu^{2+} in the following solutions: peptide/protein only (black bars), peptide/protein together with Cu^{2+} (gray bars), peptide/protein together with Cu^{2+} and 1 mM AA (patterned bars), and peptide/protein together with Cu^{2+}, 1 mM AA, and 1000 units/mL catalase (white bars). For Aβ(1−42), the concentration of Aβ(1−42) was kept at 10 μM to avoid significant aggregate formation. The concentrations of all other peptides/proteins were 50 μM, and the concentration of Cu^{2+} was half of those of the peptide/protein solutions.

due to the oxidative damages imposed by H_2O_2 generated in the process shown by reactions 1 and 2. Since SH-SY5Y neuroblastoma cells are more commonly used in research concerning the neuropathology in PD, we first investigated whether H_2O_2 produced from the O_2 reduction reaction that is "catalyzed" by Aβ−Cu^{2+} can similarly affect the viability of the SH-SY5Y cells. As shown in Figure 5A, Aβ(1−42) does not oppose apparent toxicity toward the SH-SY5Y cells, but its toxic effect on the SH-SY5Y cells is slightly greater in the presence of Cu^{2+}. Interestingly, further addition of 1 mM AA substantially aggravates the cell viability, which decreased by 2.5 times with respect to a cell medium containing Aβ(1−42) and Cu^{2+}. In a separate control experiment (data not shown), we found that 1 mM AA is not pernicious to the SH-SY5Y cells. Interestingly, when catalase, an enzyme that can decompose H_2O_2 with an extremely high efficiency (*58*), was added, the cell viability dramatically improved (recovered back to ~80%). Bush and co-workers have also used catalase to scavenge H_2O_2 to enhance PC12 cell viability (*16*). Thus, we have shown that H_2O_2 can inflict similar oxidative stress to the SH-SY5Y cells. To exclude the possibility that the toxicity might have originated from the Cu^{2+}-induced Aβ(1−42) aggregates (*59*), we also conducted a parallel experiment with Aβ-(1−16), which encompasses the metal-binding domain but not the aggregation-prone segment of Aβ(1−42). As shown in Figure 5A, a trend in the cell viability similar to that involving Aβ(1−42) was observed.

As for the cytotoxicity of the α-syn−Cu^{2+} and α-syn(1−19)−Cu^{2+} complexes (Figure 5B), results remarkably analogous to those shown in Figure 5A were obtained. Again, the Cu^{2+} complexes with α-syn or α-syn(1−19) are not regarded to be toxic, unless a high concentration of a cellular reductant (AA in this case) is present. The fact that catalase largely abolishes the toxicity effects confirms our observation that H_2O_2 is a major product. Similar to the trend exhibited by Aβ(1−42) and Aβ-(1−16), that α-syn(1−19) affects the cell viability slightly more than α-syn suggests that the former is capable of producing more H_2O_2, a point in line with the data shown in Figure 4B. The trend also suggests that aggregates of α-syn either did not form during the time frame of the cell viability assay (24 h) or did not impose discernible toxic effects. In fact, we used AFM to identify possible aggregates at various times throughout the assay and did not find any forms of α-syn aggregates (cf. Figure S3 in Supporting Information). This is not entirely surprising since it has been reported that α-syn aggregation is not significant in the first 24 h (*29*, *60*).

DISCUSSION

The ES−MS (Figure 1) and electrochemical results (Figure 2) clearly indicate that Cu^{2+} can bind to α-syn and α-syn(1−19). The quasi-reversibility of the redox waves of the α-syn−Cu^{2+} complex suggests that cycling between Cu^{2+} and Cu^{+} in the complex is relatively facile. With the redox potential of the α-syn−Cu^{2+}/α-syn−Cu^{+} couple measured, the likelihood of redox reactions between the α-syn−Cu^{2+}/α-syn−Cu^{+} couple and cellular species (including amino acid residues on proteins) can be assessed. We demonstrated that AA, but not DA, can be directly oxidized by α-syn−Cu^{2+} (see Figure 3). Based on the fact that the oxidation potential of α-syn−Cu^{2+} is also more positive than the redox potential of the GSH/GSSG couple (−0.424 V) (*61*), it is likely that α-syn−Cu^{2+} can oxidize GSH to GSSG. It has been reported that the level of GSH in the *substantia nigra* of PD patients is lower than that in control tissues (*41*). The declined level of GSH is strong evidence for the oxidative stress hypothesis (*3*). Therefore, our data suggest that the α-syn−Cu^{2+} complex might be at least partially responsible for the depletion of GSH.

A physiologically relevant redox reaction closely examined in this work is the α-syn−Cu^{2+}-triggered H_2O_2 formation. The redox potential we measured has accurately projected that, upon the α-syn−Cu^{2+} reduction, O_2 can be reduced to H_2O_2 by α-syn−Cu^{+}. As shown in Figure 4B, after 5 h of reaction, the concentration of H_2O_2 reached as high as ~20 μM. Due to the high concentrations of AA (10 mM) (*57*), GSH (2.5 mM) (*57*), O_2 (20 μM) (*62*) and other cellular species (cf. Table1) in brain, the amount of H_2O_2 produced in vivo can be significant. Since α-syn−Cu^{2+} behaves as an enzyme for the catalytic reduction of O_2 and is not consumed in the catalytic cycle depicted in reactions 3 and 4, the H_2O_2 production would not cease until O_2 and cellular reductants have been completely exhausted. In vivo, in the presence of H_2O_2, many other aberrant processes can take place. As mentioned in the introduction, H_2O_2 can participate in the Fenton reaction with free Fe^{2+} in the labile iron pool (*63*) within the neuronal cytosol to generate hydroxyl radical, a more reactive and potent ROS:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (5)$$

In cellular milieu, any rogue Cu^{2+} that is not readily complexed by copper-binding proteins (including α-syn) will also react with H_2O_2. The following Harber−Weiss reaction involving Cu^{2+} and H_2O_2 can also produce hydroxyl radicals:

$$H_2O_2 + Cu^{2+} \rightarrow Cu^{+} \cdot O_2H^{\bullet} + H^{+} \quad (6)$$

$$Cu^{+} \cdot O_2H^{\bullet} + H_2O_2 \rightarrow Cu^{+} + O_2 + OH^{\bullet} + H_2O \quad (7)$$

$$H^{+} + Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + H_2O \quad (8)$$

The hydroxyl radical generation may explain the DA lesion and extensive protein oxidation in PD brains (*64*). As described in the Results section, DA cannot be directly oxidized by α-syn−Cu^{2+}. However, it has been shown that H_2O_2 is able to oxidize DA (*65*). Similarly, the fact that the oxidation potential of α-syn−Cu^{2+} is lower than those of tyrosine (0.78 V vs AgCl/Ag) (*12*) and methionine (1.30 V vs AgCl/Ag) (*12*) indicates that complexation of Cu^{2+} by α-syn should not result in oxidation of these residues. This is consistent with the fact that these residues in the α-syn−Cu^{2+} complex have remained intact (see the mass

spectra in Figure 1A). More importantly, H_2O_2 or $OH^{\bullet}$ has been reported to result in oxidation of methionine residues in α-syn−Cu^{2+} to sulfoxide and sulfone groups (*66*). Tyrosine residues are more susceptible to oxidation than methionine (*12*), forming the quionone analogues (loss of protons) (*67*). Although not examined here in detail, we do not rule out the possibility that the production of ROS facilitated by the α-syn−Cu^{2+} complex (i.e., H_2O_2 and $OH^{\bullet}$) may ultimately lead to the oxidation of methionine and tyrosine residues of α-syn. Moreover, we did not examine the role of any uncomplexed Cu^{2+} in producing hydroxyl radicals in the presence of AA or other cellular redox species (*22*−*24*).

It has been widely reported that α-syn aggregates, especially in the oligomeric forms, are highly neurotoxic (*68*−*70*). The Cu^{2+}-containing α-syn aggregates further exacerbate the cytotoxicity (*70*). The role of the α-syn−Cu^{2+} complex in eliciting neuronal cell death (by depleting essential cellular species and/or generation of ROS) was implicated (cf. the cytotoxicity results shown in Figure 5B). Since no aggregation of α-syn was detected during our cytotoxcity studies, the oxidative stress/damage associated with α-syn−Cu^{2+} could have occurred long before aggregates of α-syn had been extensively produced. We should caution that our results do not exclude the possible toxic effects concertedly exerted by oxidative stress of the α-syn−Cu^{2+} complex and any damaging processes that are inherent in the α-syn aggregates. For instance, a hypothesis that has received wide acceptance is the pore formation within cell membrane that is induced by α-syn oligomers (*68*−*70*). In such a process, to our knowledge, redox reactions of α-syn have not been invoked for the interpretation of the experimental results.

Finally, as revealed by data shown in Figure 4B, under the same experimental condition, the amount of H_2O_2 generation facilitated by α-syn(1−19)−Cu^{+} is more than that by α-syn−Cu^{+}. Previously, we found that the rate of H_2O_2 generation by the Cu^{+} complexes with different Aβ peptides also decreases with the peptide length (*12*). If the accessibility of the Cu^{2+} center is crucial to the rate of the H_2O_2 generation or other redox reactions, it is plausible to suggest that the rate at which the various Cu^{2+}-containing α-syn aggregates generate H_2O_2 should be slower than the Cu^{2+} complex formed with the monomeric α-syn. On the basis that the hydrophilic and/or metal-binding domains of other amyloidogenic proteins/peptides are emanated from the hydrophobic cores (e.g., fibrils and bundles of fibrils (*71*)) it is possible that the Cu^{2+}-binding N-termini in the α-syn aggregates are also exposed to solution, albeit the amount of H_2O_2 produced might be lower. However, higher ordered α-syn aggregates (e.g., insoluble oligomers, protofibrils, and fibrils) that also contain Cu^{2+} are not diffusible, and consequently, the ROS accumulation over a period of time in a localized region can be substantial, rendering more severe damages to cell membranes and organelles.

CONCLUSION

The Cu^{2+} complexes with α-synuclein or an N-terminus peptide (i.e., α-syn(1−19)) were detected by ES−MS, and the redox potentials of the two copper complexes have been measured electrochemically. The Cu^{2+} center(s) can be reduced to Cu^{+} readily and are well accessible to solution species. The occurrence of a particular redox reaction that might involve α-syn−Cu^{2+} can be predicted by comparing the redox potentials between the complex and other redox reactants. Because the redox potential of α-syn−Cu^{2+}/α-syn−Cu^{+} is higher than that of AA/dehydroascorbic acid but lower than that of O_2/H_2O_2, α-syn−Cu^{2+} was verified to facilitate the H_2O_2 generation in the presence of AA. On the other hand, the redox potential of DA is higher than that of α-syn−Cu^{2+}/α-syn−Cu^{+}, indicating that DA cannot be directly oxidized by α-syn−Cu^{2+}. However, H_2O_2 and other ROS (e.g., hydroxyl radicals produced from Fenton reaction) can oxidize DA. We also demonstrate that the resultant H_2O_2 can cause neuronal cell death and the scavenging of it by catalase can retain the cell viability. These observations might provide new insight into the gradual loss of dopaminergic cells in PD brain. Taken together, our results about the depletion of oxidizable redox molecules (e.g., GSH, DA, and AA), the reduction of O_2 to H_2O_2 by α-syn−Cu^{+}, and the cytotoxicity of the generated H_2O_2 have helped to link the redox properties of the α-syn−Cu^{2+} complex to previous in vivo observations that are symptomatic of oxidative stress/damage in PD etiology.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental details about the H_2O_2 detection and electrochemical and AFM studies of the complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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