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Short Communication

Investigate of the interaction of Cu^{2+} with peptide of α-Synuclein(20-50)

Bijun Feng and You-Nian Liu[*]

College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P. R. China
[*]E-mail: liuyounian@csu.edu.cn

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In this work, the complexes between Cu^{2+} and α-synuclein N-terminus peptides (α -syn(20–50)) was investigated according electrospray-mass spectrometry. The result revealed α -syn(20–50) can bind with Cu^{2+} and the amino acid residues of α -syn(20–50) remained intact and the oxidation of the copper center(s) was 2+. We utlized the electrochemical methods to study the electrochemical behaviors of these complex, and the redox potential of α-syn(20–50)-Cu^{2+} was 0.04 V，0.072 V and 0.025 V, respectively. Then the complex of α -syn(20–50) with Cu^{2+} was studid in the purged with N_2 and O_2, the complex can be readily reduced. According to determ the H_2O_2 concentration, the result proved the complex can catalyze O_2 to reduce to H_2O_2 when bubbled with O_2 to solution.

Keywords: Interaction; α -synuclein; copper; electrochemical behaviors

1. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease[1,2], and it is characterized by a progressive loss of the dopaminergic cells in the substantial nigra which is a small brain region producing dopamine and high concentrations of the metal ions(Cu and Fe) [3,4], but the pathogenic mechanism of PD is unclear at present. α-Synuclein (α-syn), concluded 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)， a presynaptic protein believed to play an important role in neuropathology in Parkinson's disease (PD), is known to bind Cu(II) [5,6], Zn(II)[7] and Fe(II)[8]. Recently, research on the binding of redox active metals (e.g., Cu^{2+} and Fe^{2+}) to α -syn has received increasing attention[9,10]. Part of the attention stems from the observation that Cu^{2+} concentration in the cerebrospinal fluid of PD patients is elevated and aggregation of α -syn is

accelerated by Cu^{2+}[11]. The possible toxicity of the metal-containing α -syn complex or aggregates has also been investigated，the metal ions can accelerate the aggregation of α-syn to form various toxic aggregates in vitro[12,13]. However, it is not clear from these studies whether the neurotoxicity is simply caused by the metal-enhanced α -syn aggregation or a higher toxicity of the metal-containing aggregates.

It is recently shown that the strong anchoring sites for Cu^{2+} are main Met−1 in α -syn (dissociation constant in the sub-micromolar) and His−50 with a dissociation constant determined to be about 40−80 μM [14,15].The residues in the C-terminus bind to Cu^{2+} nonspecifically and weakly[16]. It is necessary to measure the redox potential of the copper complex of α -syn. With the redox potential accurately determined, the feasibility of a given reaction involving Cu^{2+} and α -syn can be addressed from the thermodynamic aspect by comparing the redox potentials between the complex and the species under investigation. Voltammetry is a simple and accurate technique to measure the redox potential of a biomolecule and/or its metal complexes. Determination of redox potentials voltammetrically also obviates the use of redox couples to "bracket" the potential range, as commonly used in estimating the relative redox power of a biomolecule. The avoidance of using various redox couples is particularly attractive, since additives tend to promote the misfolding/aggregation of amyloidogenic proteins/peptides.

The present work employed the electrochemical method to investigation of the copper complex of α -syn(20-50). The N-terminus peptide segment of α -syn(20−50) that contain the anchoring sites were synthesized. Mass spectrometric and voltammetric studies were performed on their complex with Cu^{2+}. To probe the role of these complexes in O_2 reduction, reactions carried out under deaerated condition were contrasted to those proceeded in the presence of O_2. We also determined the extent of H_2O_2 production among the complex.

2. EXPERIMENTAL

2.1 Materials

Wang resin, Fmoc-protected amino acids, diisopropylcarbodiimide, 1-hydroxylbenzotriazole, and piperidine were purchased from Anaspect Inc. (San Jose, CA). Potassium hydrogen phosphate, potassium hydroxide, sodium sulfate, copper sulfate, and organic solvents were purchased from Thermo-Fisher Scientific Inc. (Pittsburgh, PA).

2.2 Peptide Synthesis

α -syn(20−50) were 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 proceeded for 30 min. Upon dehydration on a freeze-drier (VirTis Benchtop K, Warminster, PA), the crude product was purified by semi-preparative reversed-phase (RP) HPLC (Shimadzu 6AD, Columbia, MO). 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 2 mL/min, purifications of α -syn(20–50) was accomplished with an elution gradient of 25–65% phase B for 12 min and 20–45% phase B for 12 min, respectively. The purity of the synthesized peptides was verified by HPLC and electrospray-mass spectrometry (ES–MS).

2.3 Mass Spectrometric Measurements

$CuCl_2$ and α -syn(20–50) 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 α -syn(20–50). The sampler capillary was kept at 200 ^{o}C. All the mass spectra were collected in the positive ion mode.

2.4 Electrochemical Measurements

Voltammetric measurements of the α -syn(20-50) and its copper complex were carried out on a CHI660B electrochemical workstation (CH Instruments, Austin, TX). 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 phosphate buffer (pH 7.4). Conducting voltammetric experiments under the oxygen-free condition was achieved by transferring the electrochemical cell and solutions into a glove box (Plas Lab, Lansing, MI) that had been thoroughly purged with and kept under high-purity N_2. The oxygen content in the box was found to be less than 0.05 ppm.

3. RESULTS AND DISCUSSION

3.1 Mass spectra analysis of the interaction of Cu^{2+} and α -syn(20–50).

Figure 1 depicts representative mass spectra collected from solutions containing Cu^{2+} and α -syn(20–50). We chose the specific peptide segments based on the report that His–50 are one of the strong anchoring sites for the Cu^{2+} binding. In Figure 1, the quadruplicity charged peaks corresponding to α -syn(20–50) and its 1:1 complex with Cu^{2+} have *m/z* centered around 815.2 and 823.5, respectively. The peaks at around *m/z* 815.2 can be attributed to the α-syn(20–50)/Na^+ adduct produced by trace Na^+ in the solvent and sample introduction system. The result revealed that segment encompassing His–50 can independently anchor Cu^{2+}. The result was similar with the previous report [14,15].

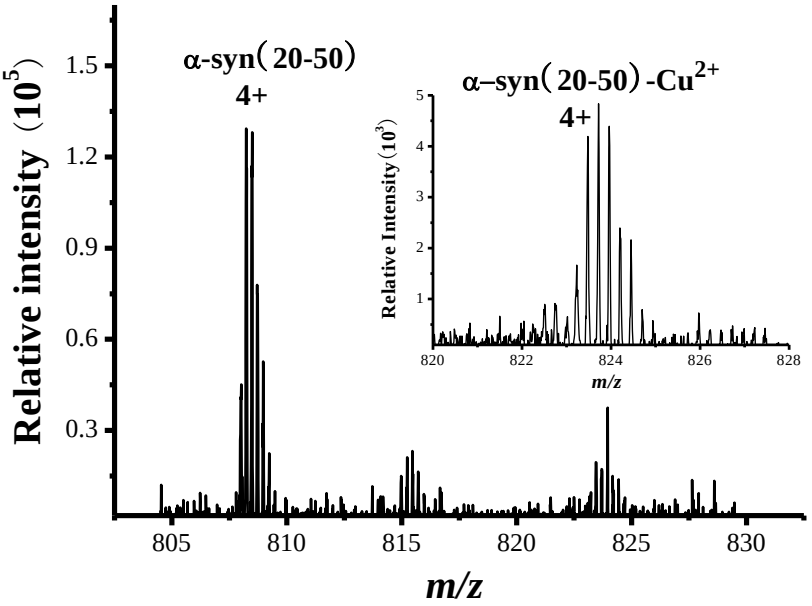


Figure 1. Mass spectra from solutions of α -syn(20-50) and Cu^{2+} showing quadruply charged peaks (inset is the zoomed scan of the complex peak between *m/z* 820–828).

Table 1. Extraction of the copper oxidation state from measured α-syn(20-50)-Cu *m/z* values

Isotopic species	Measd. *m/z*	Rel. Abundance	Calcd. *m/z* of Cu^{2+}	Calcd. Rel. Abundance	Deviation (ppm)	Calcd.*m/z* of Cu^{+}	Calcd. Rel. Abundance	Deviation (ppm)
A	823.2346	34.07	823.1915	47.07	52	823.4434	47.07	254
A + 1	823.4795	85.80	823.4422	84.20	45	823.6941	84.20	261
A + 2	823.7226	100.00	823.6925	100.00	37	823.9444	100.00	269
A + 3	823.9675	90.06	823.9428	89.07	30	824.1948	89.07	276
A + 4	824.1952	49.05	824.1932	61.38	2	824.4452	61.38	303
A + 5	824.4555	44.56	824.4437	33.95	14	824.6956	33.95	291

Previously, it has been reported that tyrosine residues are more susceptible to oxidation, given that its oxidation potential (0.83 V vs NHE) is lower than methionine(1.86V vs NHE)[17,18]. Since the *m/z* values correlate very well with the α -syn(20–50) and its copper complex, we conclude that all of the amino acid residues must have remained intact and the oxidation of the copper center(s) should be 2+. The 2+ oxidation state is confirmed by the smaller deviation value shown in Tables 1, because the deviation between the experimentally observed and the theoretical *m/z* ratios would be much greater if the oxidation state of the copper center(s) were assumed to be +1.

3.2 The electrochemical behaviors of Cu^{2+} and α -syn(20–50).

Cyclic voltammograms (CVs) of α -syn(20–50) and α -syn(20–50) with Cu^{2+} were overlaid in Figure 2. The redox wave, recorded in ambient atmosphere, exhibited quasi-reversible behaviors,

which is in contrast to that of the irreversible reduction peak of free Cu^{2+} (dashed line curve). The anodic and cathodic peak potentials (E_{pa} and E_{pc}) are 0.081 and –0.034 V for α -syn(20–50)/Cu^{2+} respectively. All of the potential and CV shapes are close to those of the complex formed between Cu^{2+} and a Cu^{2+}-histidine complex. Thus, we conclude that the Cu^{2+} center in the complex of the α -syn(20-50) can be reduced to Cu^{+}. The voltammograms of the α -syn(20–50)-Cu^{2+} in the anodic range also revealed a small irreversible oxidation peak at ca. 0.730 V. This irreversible peak can be attributed to the irreversible oxidation of the Tyr residue(s) at a high oxidation potential.

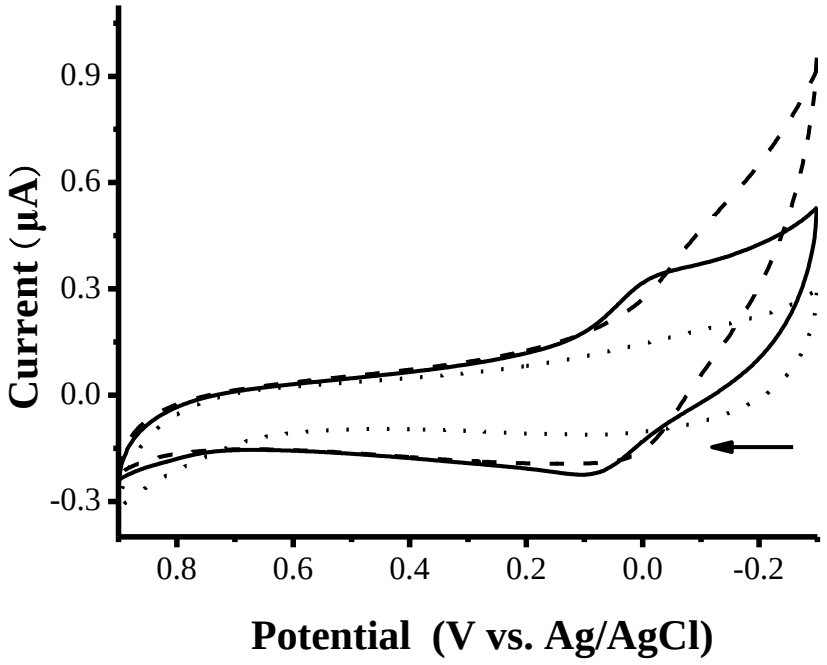


Figure 2. Cyclic voltammograms of 200 μM free Cu^{2+} (dashed line), 200 μM α -syn(20–50) (dot line), and the mixed solution of 200 μM α -syn(20–50) and 200 μM Cu^{2+} (solid line). The scan rate was 5 mV/s and the arrow indicates the initial scan direction.

3.3 The influnce of O_2 to the oxidation of Cu^{2+} and α -syn(20–50)

A major implication of the oxidative stress associated with neurodegenerative diseases is the generation of ROS by redox metal ions (i.e., Cu^{2+}) and/or their complexes with amyloidogenic proteins/peptide[19,20]. We therefore first investigated the possibility of H_2O_2 generation that might be catalyzed by the copper complex of α -syn(20–50). The dashed line in Figure 3 is the CV of the copper complex of α -syn(20–50) in a phosphate buffer that had been thoroughly purged with N_2. Compared to the CVs shown in Figure 2 (solution not degassed with N_2), the redox waves are even better defined, indicating that the reduction reaction of the complex is influenced by O_2 in solution.

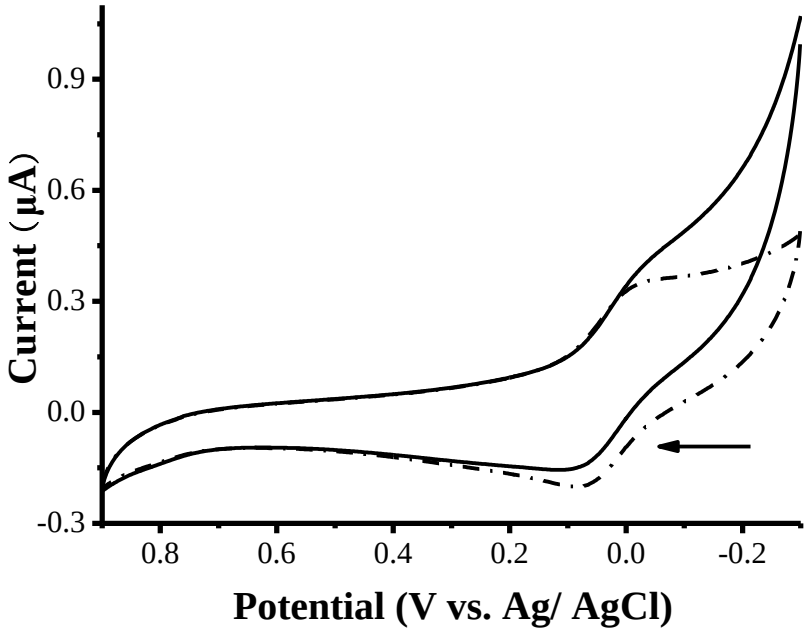


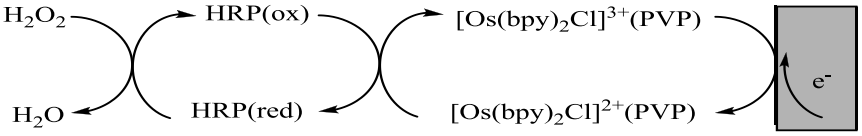
Figure 3. CVs acquired from a N_2-purged phosphate buffer solution (pH 7.4) containing 200 μM α -syn(20–50) and 200 μM Cu^{2+} (dashed line) and an O_2-purged solution (solid line) containing the same amounts of α -syn(20–50) and Cu^{2+}. The scan rate was 5 mV/s and the arrow indicates the initial scan direction.

When the buffer solution was saturated with O_2 (magenta), i_{pc} increased on the expense of i_{pa} and a plateau can be discerned at ca. 0.01 V. The appearance of a plateau is indicative of an electrocatalytic reduction of O_2 as followed:

$$2\ \alpha\text{-syn(20–50)-}Cu^{+} + O_2 + 2H^{+} = 2\ \alpha\text{-syn(20–50)-}Cu^{2+} + H_2O_2$$

3.4 The detection of H_2O_2 generation at electrolyses of α -syn(20–50)-Cu^{2+}

Notice from reaction the H_2O_2 generation requires that the Cu^{2+} center(s) to be pre-reduced to Cu^{+}. We therefore carried out electrolyses of α -syn(20–50)-Cu^{2+} in aerated solutions for different times and subsequently determined the amounts of H_2O_2 generated. Quantification of H_2O_2 produced from the solution was accomplished by using a wired-HRP electrode. A plastic thin-layer flow cell (CH Instrument) housing a 3-mm-diameter glassy carbon electrode (Bioanalytical System Inc.) was used for the detection of H_2O_2. A stainless steel tube, positioned downstream of the flow cell, served as the auxiliary electrode, and the reference electrode was a Ag/AgCl reference electrode. Coating of the glassy carbon electrode with the hydrogel matrix containing horse radish peroxidase (HRP) and the osmium bipyridine ($[Os(bpy)_2Cl]^{3+}$) complex followed the manufacture's procedure. The scheme for this H_2O_2 detection is as follows:



Briefly, the electrode potential was held at 0.1 V to reduce $[Os(bpy)_2Cl]^{3+}$ incorporated into the poly(vinylpyridine) (PVP) matrix to $[Os(bpy)_2Cl]^{2+}$. $[Os(bpy)_2Cl]^{2+}$ is in turn oxidized by HRP(ox), and the reduced form of HRP, HRP(red), can be readily oxidized by H_2O_2 permeated from the solution into the hydrogel. The complex of Cu^{2+} formed with α -syn(20–50) was electrolyzed at 0.04 V (vs. Ag/AgCl) for predefined times. The potential of 0.04 V was chosen to avoid possible H_2O_2 generation by free Cu^{2+}. The final solution was 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. As can be seen from Figure 4, the amounts of H_2O_2 generated from α -syn(20–50)-Cu^{2+}, whilst a prolonged electrolysis leads to a greater production of H_2O_2. We conducted a control experiment by holding the electrode potential at 0.04 V in a Cu^{2+} only solution and detected little H_2O_2. This is conceivable since free Cu^{+} is not stable in an aqueous solution. In addition, no H_2O_2 was detected if the Cu^{2+} center(s) in the complex was not reduced.

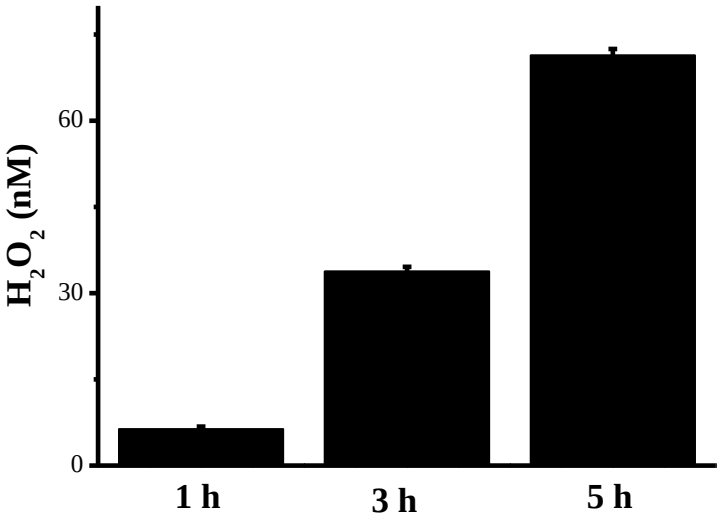


Figure 4. Concentrations of H_2O_2 generated after electrolyses at 0.04 V of solutions containing□ □-syn(20–50) and Cu^{2+} at 100 μM for different times (1, 3, and 5 h).

4. CONCLUSIONS

In this paper, the complex of α -syn(20–50) with copper was investigated by electrospray-mass spectrometry and electrochemistry. The redox potential of copper complex of α -syn(20–50) has been measured voltammetrically. Both ES-MS and voltammetry of the complex indicate that the amino acid residues of α -syn(20–50) remain intact upon binding to Cu^{2+}. The Cu^{2+} center can be reduced to Cu^{+} at relatively facile rates and are well accessible to solution species and the H_2O_2 was produced in the

present of O_2, the result suggested the copper complex could react with the redox molecules to induce the oxidative stress and could lead the dopaminergic cell damage.

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