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Binding of α -Synuclein with Fe(III) and with Fe(II) and Biological Implications of the Resultant Complexes

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Abstract

Parkinson's disease (PD) is hallmarked by the abnormal intracellular inclusions (Lewy bodies or LBs) in dopaminergic cells. Amyloidogenic protein α -synuclein (α -syn) and iron (including both Fe (III) and Fe(II)) are both found to be present in LBs. The interaction between iron and α -syn might have important biological relevance to PD etiology. Previously, a moderate binding affinity between α -syn and Fe(II) ($5.8 \times 10^3 \text{ M}^{-1}$) has been measured, but studies on the binding between α -syn and Fe(III) have not been reported. In this work, electrospray mass spectrometry (ES-MS), cyclic voltammetry (CV), and fluorescence spectroscopy were used to study the binding between α -syn and Fe(II) and the redox property of the resultant α -syn-Fe(II) complex. The complex is of a 1:1 stoichiometry and can be readily oxidized electrochemically and chemically (by O_2) to the putative α -syn-Fe(III) complex, with H_2O_2 as a co-product. The reduction potential was estimated to be 0.025 V vs. Ag/AgCl, which represents a shift by -0.550 V vs. the standard reduction potential of the free Fe(III)/Fe(II) couple. Such a shift allows a binding constant between α -syn and Fe(III), $1.2 \times 10^{13} \text{ M}^{-1}$, to be deduced. Despite the relatively high binding affinity, α -syn-Fe(III) generated from the oxidation of α -syn-Fe(II) still dissociates due to the stronger tendency of Fe(III) to hydrolyze to Fe(OH)₃ and/or ferrihydrite gel. The roles of α -syn and its interaction with Fe(III) and/or Fe(II) are discussed in the context of oxidative stress, metal-catalyzed α -syn aggregation, and iron transfer processes.

Keywords

α -Synuclein; iron; binding affinity; oxidative stress; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease (next only to Alzheimer's disease or AD) and affecting more than 1% of the people over the age of 65 in the

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United States.[1] Pathologically, PD is characterized by a progressive loss of the dopaminergic cells in the *substantia nigra*, a small brain region producing dopamine.[2] The surviving dopaminergic cells of PD patients contain cytosolic filamentous inclusions known as the Lewy bodies (LBs),[3] within which α -synuclein (α -syn) is a major component.[3,4] α -Syn comprises 140 amino acids and its exact function is still unknown.[5] Mounting evidence has shown that α -syn can misfold and subsequently aggregate and the resultant aggregates are neurotoxic.[6] It is also reported that abnormal iron homeostasis appears to exacerbate with PD progression. For example, unusually high concentrations of iron and a greater Fe(III)/Fe(II) ratio have been found in the *substantia nigra* of PD patients.[7] In addition, postmortem studies have revealed Fe(III) and Fe(II) presence in the LBs.[8,9] The role in PD pathology could be at least two-fold. On one hand, *in vitro* studies have shown that iron can accelerate the α -syn aggregation and microscopic techniques (i.e., the atomic force microscopy and scanning electron microscopy) have shown that α -syn also forms fibrous aggregates in the absence and presence of iron.[6,10-12] On the other hand, iron is a redox active metal and its interaction with α -syn could induce or be involved in oxidative stress. Lending support to the second aspect is the redox metal-induced oxidative stress associated with other neurodegenerative diseases such as AD.[13] Metal ions (e.g., Cu(II), Fe(II), and Fe(III)) can complex with amyloid- β (A β) peptides and the resultant complexes react with other cellular redox molecules to generate reactive oxygen species (ROS).[13,14] The ROS generated can subsequently cause damages to a variety of molecules and organelles (DNA, protein, lipid, and mitochondrial), leading eventually to neuronal cell death.

A better understanding of the roles of iron in PD pathology can be gained from studies of the interaction between iron and α -syn. Although binding between Fe(II) and α -syn has been studied and a binding constant of $5.8 \times 10^3 \text{ M}^{-1}$ was reported,[15] any description of the functions of iron is incomplete without the knowledge about the binding between α -syn and Fe(III). Specifically, important questions such as the effect of iron (including Fe(II) and Fe(III)) on α -syn aggregation, possible redox reactions of the α -syn-iron complexes, and the role of α -syn in iron transfer, cannot be elucidated. The lack of the knowledge about the binding between α -syn and Fe(III) largely stems from the fact that *in vitro* studies are hampered by the extensive hydrolysis of Fe(III) at physiological pH (i.e., pH 7.4). Particularly intriguing is whether Fe(III) complexation by α -syn would inhibit the Fe(III) hydrolysis. Of equal interest is whether α -syn-Fe(II) and the putative α -syn-Fe(III) complex can react with cellular redox species. As aforementioned, such reactions could potentially produce ROS that will impose oxidative stress to neuronal cells.[13,14]

We wish to report our mass spectrometric, fluorescence, and electrochemical studies of the interaction between iron and α -syn. Cyclic voltammetry (CV) is a simple and accurate technique to measure the redox potential of many biologically important species. We have successfully utilized CV to determine the binding affinity of A β to Fe(III) or Fe(II) by comparing the experimentally measured redox potentials of the complexes to the standard reduction potential of the free Fe(III)/Fe(II) couple.[14] Extension of this approach to the studies of the putative α -syn-Fe(III) complex not only yields the binding constant, but also enables us to gain insight into the possible roles of α -syn in iron transport and ROS generation. The possible effects of the oxidation of α -syn-Fe(II) on the α -syn aggregation process are also discussed.

2. Materials and Methods

2.1. Materials

Sodium dihydrogen phosphate, sodium hydroxide, sodium sulfate, ammonium sulfate, trifluoroacetic acid, ferrous ammonium sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$), Trizma base (Tris), isopropyl β -D-thiogalactopyranoside (IPTG), and acetonitrile were purchased from Thermo

Fisher Scientific Inc. (Pittsburgh, PA). All aqueous solutions were prepared using Millipore water (18 MΩ•cm). *E. coli* BL21 (DE3) and lysozyme were purchased from Invitrogen Corp (Carlsbad, CA) and EMD Inc. (Gibbstown, NJ), respectively. Tris buffer was made by mixing 20 mM Tris solution with concentrated HCl solution to a final pH 7.4.

2.2. Expression and Purification of α -Syn

The expression of α -syn followed the published procedure.[16] In brief, *E. coli* BL21 (DE3) were transfected with pRK172/ α -synuclein plasmids kindly donated by Prof. P. Lansbury (Harvard University). 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, the lysate was enriched by two sequential ammonium sulfate precipitation steps. The resultant pellet was resuspended in 10 mM phosphate buffer solution (pH 7.4). The crude α -syn was purified by semi-preparative reversed-phase (RP) HPLC (Shimadzu 6AD, Columbia, MO) with a column (Jupiter-10-C18-300, 10 mm i.d. $\times$ 250 mm) from Phenomenex (Torrance, CA). The mobile phases were 0.1% trifluoroacetic acid in water (v/v, mobile phase A) and 0.1% trifluoroacetic acid in acetonitrile (v/v, mobile phase B). The elution gradient was 25–75% B for 20 min at a flow rate of 4.7 mL/min.

2.3. Mass Spectrometric Measurement

Electrospray mass spectrometry (ES–MS) was conducted on a Thermal Fisher LTQ linear ion-trap mass spectrometer (San Jose, CA). $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and α -syn were dissolved in water at 1 mM and 100 μM , respectively. Aliquots of the $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ solution were added into the α -syn solution and the resultant solutions were subsequently diluted with a water/methanol (v/v = 1:1) solution to a final concentration of 10 μM for each species. The sampler capillary of the LTQ mass spectrometer was kept at 200 °C and all the mass spectra were collected in the positive ion mode.

2.4. Electrochemical Measurement

Voltammetric measurements of the α -syn and its ferrous complex were carried out on a CHI832 electrochemical workstation (CH Instruments, Austin, TX). The three-electrode system comprised 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 Tris buffer (pH 7.4) containing 0.1 M Na_2SO_4 and all the potentials were measured against Ag/AgCl. The potential of the Ag/AgCl reference electrode is 0.196 V with respect to the normal hydrogen potential (NHE). Thus, when 0.196 V is added to all the potentials reported in the work, the resultant values will correspond to those versus the NHE. The electrochemical workstation and the cell were housed in a N_2 -purged glove box (Plas Lab, Lansing, MI).

2.5. Detection of Hydrogen Peroxide

The hydrogen peroxide generation was monitored by the Fluoro H_2O_2 detection kit (Cell Technology Inc., Mountain View, CA), as previously reported.[13] When H_2O_2 is present, 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) is oxidized to resorufin, which emits fluorescence around 585 nm. The oxidation of ADHP is catalyzed by horse radish peroxidase (HRP). Sample solution (50 μL aliquot) was added to a reaction cocktail, which contained 100 μL of 10 mM ADHP, 200 μL of HRP (10 units/mL), and 4.7 mL of the reaction buffer. The mixture was incubated at room temperature for 10 min and the fluorescence of resorufin was measured using the procedure described below.

2.6. Fluorescence Spectroscopy

The fluorescence spectra were carried out at room temperature using a Cary Eclipse spectrofluorimeter (Varian Inc, Palo Alto, CA). The excitation wavelength was set at 280 nm

and the emission spectra were recorded from 290 to 450 nm for the tyrosine residue in α -syn. For the detection of H_2O_2 , the excitation wavelength was set at 550 nm while the emission spectra were recorded from 565 to 690 nm.

3. Results

3.1. Identification of α -Syn-Fe(II) by ES–MS

We first attempted to identify the α -syn-Fe(II) complex by ES–MS. Fig. 1 depicts an ES–MS spectrum of a mixture of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and α -syn. The multiply charged (protonated to 10–18) α -syn peaks are easily discernable. Next to each α -syn peak is one whose m/z value corresponds to the α -syn-Fe(II) complex (identified by an asterisk in Fig. 1). We should note that each of the complex peaks contains one Fe(II) and two Na^+ ions (given rise by Na^+ in the phosphate buffer used for the α -syn expression and buffer preparation). Table 1 lists the m/z values for α -syn and α -syn-Fe(II) peaks bearing various numbers of protons and the two Na^+ ions. These experimentally determined m/z values are in excellent agreement with the molecular weights of α -syn and the α -syn-Fe(II) (upon exclusion of the protons and Na^+ ions). From our ES–MS measurements, it is clear that the binding stoichiometry between α -syn and Fe(II) is 1:1, which correlates well to the previous report showing position 121 as the major binding site for Fe(II).[17] Note that the peak heights of α -syn are greater than those of the complex, suggesting that the binding between α -syn and Fe(II) is not particularly strong. This is in line with the relatively low binding constant reported before ($5.8 \times 10^3 \text{ M}^{-1}$)[15]. Interestingly, we found that α -syn-Fe(II) in an aerated solution is not stable after 2 h, as the mass spectrum of the resultant solution shows only peaks corresponding to α -syn. This point will be discussed in connection with our voltammetric data (*vide infra*).

3.2. Redox Potential of the α -Syn-Fe(III)/ α -Syn-Fe(II) Couple

We carried out voltammetric experiments to gauge the oxidation potential of α -syn-Fe(II). Fig. 2 overlays the CVs of α -syn acquired in the absence (dotted line curve) and presence (solid line curve) of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ under a N_2 atmosphere. When $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was absent, only a small irreversible oxidation peak at 0.730 V was observed (dotted line curve). This irreversible peak can be attributed to the oxidation of one or more of the tyrosine residues (at positions 39, 125, 133, and 136).[13] Interestingly, this peak disappeared when Fe $(\text{NH}_4)_2(\text{SO}_4)_2$ was present (solid line curve). We postulate that complexation of α -syn with Fe (II) might have altered the secondary structure near the C-terminus, making some or all of the tyrosine residues less accessible to electrooxidation. In the same voltammogram, a pair of new peaks emerged, with the oxidation and reduction peak potentials at 0.143 and -0.093 V , respectively. We assign these peaks to the oxidation of α -syn-Fe(II) and reduction of α -syn-Fe(III), respectively. Notice that a weak shoulder peak is observable at ca. -0.075 V (identified by the dashed arrow in Fig. 2), which is close to that of the free ammoniated Fe(II) in a Fe $(\text{NH}_4)_2(\text{SO}_4)_2$ solution (*cf.* the inset showing a CV of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ with an oxidation potential of -0.021 V). It is likely that the reduction peak of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ shown in the inset (-0.167 V) has overlapped to some extent with that of the α -syn-Fe(III) complex. The coexistence of the α -syn-Fe(III)/ α -syn-Fe(II) peaks with the ammoniated Fe(II) peaks is indicative of the incomplete complexation of α -syn by Fe(II). With the α -syn concentration fixed at $200 \mu\text{M}$, we varied the $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ concentrations and performed differential pulse voltammetry (DPV). DPV (Fig. 2B) more clearly resolved the redox peaks of the complex (at 0.121 V) and free (ammoniated) Fe(II) (at 0.043 V) than CV (Fig. 2A). Notice that (1) the positions of the two peaks vary slightly with the $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ concentration and (2) the peak at the more negative potential increases with the $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ concentration, suggesting that the fraction of the free ammoniated Fe(II) has become greater at a higher Fe $(\text{NH}_4)_2(\text{SO}_4)_2$ concentration. Overall, it is evident that the presence of the ammoniated Fe(II) does not affect the determination of the redox potential of the complex. The quasi-reversibility

of the redox peaks in the solid line curve indicates that the α -syn-Fe(II) complex can undergo a relatively facile electron transfer reaction. Remarkably, when the solution was exposed to air for ca. 2 h, the peaks in the solid line curve were completely diminished (*cf.* dashed line curve). Although the putative α -syn-Fe(III) is observable on the short CV time scale, it does not appear to be stable over a longer period of time in an aerated solution. This is consistent with the abovementioned ES–MS results.

3.3. Fluorescence Spectra of α -Syn-Fe(II) in Deaerated and Aerated Solutions

Previously Gray's group has shown that quenching of the α -syn tyrosine fluorescence occurs upon Cu(II) complexation.[18] Using a similar approach, Golts et al. determined the binding constant between α -syn and Fe(II) to be $5.8 \times 10^3 \text{ M}^{-1}$. [15] Consistent with literature, we found that the fluorescence peak at 305 nm decreases upon binding to Fe(II) (see the contrast between the solid and dashed curves in Fig. 3). Interestingly, when the α -syn-Fe(II) solution was purged with air for 2 h, the fluorescence intensity became fully recovered (*i.e.*, compare the dashed and dotted curves). Based on the oxidation potential of α -syn-Fe(II) deduced from Fig. 2, it is evident that O_2 , whose potential is 0.484 V vs Ag/AgCl,[19] can easily oxidize α -syn-Fe(II) to α -syn-Fe(III). As aforementioned, ES–MS of α -syn-Fe(II) solution that had been exposed to air for more than 2 h showed only α -syn peaks. Again, the fluorescence experiment clearly confirms that the α -syn-Fe(III) complex is not stable in a fully aerated solution. As will be seen from the derivation described in the Discussion Section, the binding constant between Fe(III) and α -syn is not sufficiently high to prevent the extensive hydrolysis of Fe(III) in solution at physiological pH.

3.4. Hydrogen Peroxide Generation

If α -syn-Fe(II) can be oxidized by O_2 , one of the possible products could be H_2O_2 . Previously we and others have shown that $\text{A}\beta$ -Cu(I) can reduce O_2 to H_2O_2 . [13,20] Recently, we reported that a ternary complex formed among $\text{A}\beta$, Fe(II), and nitrilotriacetic acid (NTA) can also reduce O_2 to H_2O_2 . [14] We therefore used the same H_2O_2 detection kit to verify whether H_2O_2 is present in an α -syn-Fe(II) solution that had been exposed to air for 2 h. As shown in Fig. 4, the peak at 585 nm corresponds to the fluorescence spectrum of resorufin, which is the product of the ADHP oxidation by H_2O_2 . [13,14] The free (ammoniated) Fe(II) (200 μM) generates the most H_2O_2 (solid line) whereas no H_2O_2 was detected when only α -syn was present in an aerated solution (dotted line). This indicates that it is Fe(II), not α -syn, which reduces O_2 to H_2O_2 . Notice that the mixture of 200 μM α -syn and 200 μM Fe(II) also generated H_2O_2 (dashed line curve) but the amount of H_2O_2 is less than that produced by 200 μM free ammoniated Fe(II). To exclude the possibility that H_2O_2 detected in the mixture is generated solely by the free Fe(II) remaining in the solution, we further measured the level of H_2O_2 in a solution containing 118 μM ammoniated Fe(II), a concentration predicted from the equilibrium constant (K_A') deduced in the Discussion Section for a mixture of 200 μM α -syn and 200 μM Fe(II). Evidently, from the dash-dotted curve, the level of H_2O_2 generated by 118 μM ammoniated Fe(II) is less than the mixture, demonstrating that α -syn-Fe(II) can also reduce O_2 to H_2O_2 , albeit more slowly than the free (ammoniated) Fe(II).

4. Discussion

Our ES–MS results confirmed the formation of the α -syn-Fe(II) complex (Fig. 1) and the dissociation of Fe(III) from the complex after being oxidized by O_2 . The CV (Fig. 2A) and fluorescence data also showed the loss of Fe(II) when the solution was exposed to air. Fluorescence studies verified that, similar to the complexes formed between $\text{A}\beta$ and redox metal ions, the reaction between α -syn-Fe(II) and O_2 can generate H_2O_2 (Fig. 4). This is reasonable because the potential of the α -syn-Fe(III)/ α -syn-Fe(II) couple is negative with respect to that of the $\text{O}_2/\text{H}_2\text{O}_2$ couple.

As mentioned in the Introduction, owing to the strong tendency of Fe(III) to hydrolyze at physiological pH (K_{sp} of $\text{Fe}(\text{OH})_3 = 1.0 \times 10^{-38}$), *in vitro* formation of complexes between Fe (III) and amyloidogenic proteins/peptides has not been quantitative. $\text{Fe}(\text{OH})_3$ hydrolyzed from Fe(III) eventually produces the ferrihydrite gel:[21]

where m and n are stoichiometric numbers of $\text{Fe}(\text{OH})_3$ and the intermediate that lead to the ferrihydrite gel formation, respectively. The dissociation of the α -syn-Fe(III) complex suggests that the binding affinity between α -syn and Fe(III) is not sufficiently high to prevent equations (eqs.) 1 and 2 from occurring. The binding constant between α -syn and Fe(III) (K_A) is described by the following equation:

$$K_A = [\alpha - \text{syn} - \text{Fe}(\text{III})]_{\text{eq}} / [\text{Fe}(\text{III})]_{\text{eq}} [\alpha - \text{syn}] \quad (3)$$

whereas binding constant (K_A') between α -syn and Fe(II) has been determined by Golts et al.: [15]

$$K_A' = [\alpha - \text{syn} - \text{Fe}(\text{II})]_{\text{eq}} / [\text{Fe}(\text{II})]_{\text{eq}} [\alpha - \text{syn}] = 5.8 \times 10^3 \text{ M}^{-1} \quad (4)$$

K_A can be estimated from the shift of the redox potential of the free Fe(III)/Fe(II) couple upon complexation with α -syn. For the redox reaction $\text{Fe(III)} + e^- \leftrightarrow \text{Fe(II)}$, the standard Fe(III)/Fe (II) reduction potential will be shifted in the presence of a ligand (e.g., a protein). Dhungana et al. have correlated the shift in standard potential to the binding constant values of transferrin to Fe(III) and to Fe(II).[22] We have also determined the binding constant between A β and Fe (III) using the same approach.[14] Thus, the shift in the potential of the Fe(III)/ Fe(II) couple in the presence of α -syn, (ΔE^0), is given by:

$$\Delta E^0 = E_{\alpha\text{-syn-Fe(III)/}\alpha\text{-syn-Fe(II)}}^0 - E_{\text{Fe(III)/Fe(II)}}^0 = \frac{RT}{F} \ln\left(\frac{K_A'}{K_A}\right) \quad (5)$$

where $E_{\alpha\text{-syn-Fe(III)/}\alpha\text{-syn-Fe(II)}}^0$ is the standard reduction potential of α -syn-Fe(III)/ α -syn-Fe(II) and $E_{\text{Fe(III)/Fe(II)}}^0$ is that of Fe(III)/Fe(II) (0.575 V vs AgCl/Ag[19]). T, F, and R are temperature (298 K), the Faraday's constant, and the gas constant, respectively.

If we assume $E_{\alpha\text{-syn-Fe(III)/}\alpha\text{-syn-Fe(II)}}^0$ to be the average of the oxidation (0.143 V) and reduction (−0.093 V) potentials determined from the CV experiment, i.e., 0.025 V, a difference of 0.550 V between $E_{\alpha\text{-syn-Fe(III)/}\alpha\text{-syn-Fe(II)}}^0$ and $E_{\text{Fe(III)/Fe(II)}}^0$ is obtained. Strictly speaking, determining E^0 using the oxidation and reduction peak potentials requires the redox reaction be reversible. Although the CV wave in Fig. 2 is quasi-reversible, we think that it is a reasonable assumption because

the reversibility of the α -syn-Fe(III)/ α -syn-Fe(II) wave might be even better considering that the redox wave is overlapped with that of the free ammoniated iron species (see the shoulder peak identified by the vertical dashed arrow in Fig. 2). Previously, using the CV wave of similar reversibility, K_A of the $A\beta$ -Fe-NTA ternary complex deduced was found to be in good agreement with that calculated from the known binding constants.[14] The shift in the standard reduction potential shown in eq. 5 enabled us to obtain a K_A'/K_A ratio of 5.0×10^{-10} . Substituting the reported K_A' value [15] into this ratio afforded a K_A value of $1.2 \times 10^{13} \text{ M}^{-1}$, which is a large value.

It is worth noting that both K_A and K_A' are conditional binding affinity constants, whose values are affected by factors such as solution pH and the presence of other ligands that can compete for iron binding. Golts et al. measured the quenching of α -syn fluorescence by Fe(II) at various pH values and measured the binding affinity of α -syn towards Fe(II) under physiological condition.[15] Because we carried out our measurements at physiological pH and in the absence of competing ligands, the above derivation for the binding (stability) constant of α -syn-Fe(III) complex is reasonable. In our derivation, the possible coexistence of Fe(II) and Fe(III) is also neglected. This is a valid approach since the voltammetric experiment was conducted under an oxygen-free atmosphere in a solution that does not contain any oxidant. In other words, the ammoniated Fe(II) will not be converted to Fe(III) in the absence of an oxidant.

With K_A determined, $[\text{Fe(III)}]_{\text{eq}}$ yielded from a given Fe(III) and α -syn concentrations can be estimated. For example, for a formal α -syn-Fe(III) concentration of 200 μM , $[\text{Fe(III)}]_{\text{eq}}$ would be $4.1 \times 10^{-9} \text{ M}$. Despite the rather large K_A , the product $[\text{Fe(III)}]_{\text{eq}}[\text{OH}^-]^3$ at the physiological pH, 2.6×10^{-31} , is still significantly greater than K_{sp} (1.0×10^{-38}). Thus, the α -syn-Fe(III) complex should have a strong tendency to dissociate and the released Fe(III) also has a strong tendency to hydrolyze. Even if the actual ΔE^0 were greater than 0.550 V (owing to the quasi-reversibility of the CV wave in Fig. 2), the uncertainty in estimating the K_A value would not change the above conclusion. For example, if $\Delta E^0 = 0.650 \text{ V}$, the product of $[\text{Fe(III)}]_{\text{eq}}[\text{OH}^-]^3$ would be 4.8×10^{-32} , which is still more than five orders of magnitude greater than the K_{sp} value. Now it becomes clear that, upon oxidation of the α -syn-Fe(II) complex, the binding constant between Fe(III) and α -syn is not sufficiently high to prevent Fe(III) from hydrolyzing.

Collectively, in the presence of O_2 , α -syn-Fe(II) gradually undergoes the following reaction:

The reaction is responsible for the recovery of α -syn tyrosine fluorescence (*cf.* Fig. 3) and the loss of the ES-MS and voltammetric signals (Fig. 3) of the α -syn-Fe(II) complex. Again, we emphasize that the hydrolysis is not significant on the timescale of CV experiment.

Previously we and others have shown that complexes formed between $A\beta$ and Cu(II) or Fe(III) can continuously react with a cellular redox species (e.g., ascorbic acid) in the following catalytic cycle:

Due to the high abundance of O_2 in brain ($>20 \mu\text{M}$ [23]), the net effect of the above catalytic cycle is that some essential cellular redox species will eventually be depleted and H_2O_2 , a ROS, will be produced. H_2O_2 is a highly mobile ROS and can permeate cell membrane to cause deleterious effects.[24] Interestingly, although $\alpha\text{-syn-Fe(II)}$ appears to react with O_2 in a similar fashion to eq. 8, the product, $\alpha\text{-syn-Fe(III)}$, is not stable and the catalytic cycle will be halted. In other words, the reaction (*cf.* eq. 6) will cease once all of the $\alpha\text{-syn-Fe(II)}$ has been oxidized. Therefore, the possible pathway of H_2O_2 -inflicted oxidative stress in AD might not be applicable to PD. However, because of the relatively low binding affinity between $\alpha\text{-syn}$ and Fe(II) (*vide supra*), H_2O_2 generated in eq. 6 could react with the uncomplexed Fe(II) in the following Fenton reaction to generate hydroxyl radical, a more reactive and potent ROS:

Therefore, the $\text{OH}^\bullet$ radicals could be a source for producing the oxidized lipids, damaged proteins, and covalently modified DNA[25], all of which have been found in PD.[26-28] It has also been noted from postmortem studies that the Fe(III)/Fe(II) ratio in the *substantia nigra* of PD patients increases.[7] This is consistent with the ease of $\alpha\text{-syn-Fe(II)}$ oxidation and the high tendency of $\alpha\text{-syn-Fe(III)}$ to hydrolyze.

In vitro studies have shown that at physiological pH, high (2 mM[29]) and low (10 μM [11]) Fe(III) concentrations both accelerate the $\alpha\text{-syn}$ aggregation to form the more pernicious oligomers/fibrils. Given the exceedingly small K_{sp} of Fe(OH)_3 , at neutral pH the major Fe(III) -containing species should be Fe(OH)_3 and/or ferrihydrite gel. It has been well accepted that heterogeneous surfaces (e.g., externally introduced seeds for *in vitro* investigations and cell membranes for *in vivo* studies) enhance the amyloidogenic protein/peptide aggregation.[6, 30] Thus, these precipitates might serve as nucleation sites for fusing $\alpha\text{-syn}$ monomers into oligomers and/or other higher-ordered aggregates (e.g., fibrils). In cellular milieu, the level of iron is present at micromolar level,[31] which has been shown by *in vitro* studies to be sufficient to induce $\alpha\text{-syn}$ aggregation/fibrillation.[11]

It has been suggested that transferrin is the major protein for transporting iron across neuronal cell membrane and upon internalization its Fe(III) centers are reduced to Fe(II) .[32] Transferrin has a much weaker affinity to Fe(II) than to Fe(III) . Its affinity constants are $1.6 \times 10^3 \text{ M}^{-1}$ and $3.1 \times 10^2 \text{ M}^{-1}$ for the first and second Fe(II) centers, respectively.[33] Consequently, Fe(II) could be readily released to the “labile iron pool (LIP)”.[34] Amyloidegic proteins/peptides (e.g., $A\beta$ for Fe(II) and prion protein for Cu(II)) have been implicated as redox metal chaperons.[14,35] For a protein to act as a Fe(II) chaperon, the affinity constant towards Fe(II) must be greater than that of transferrin.[14] Indeed, we have recently shown that the binding affinity of $A\beta$ towards Fe(II) is greater than that of transferrin. Notice that, although the binding constant between $\alpha\text{-syn}$ and Fe(II) is moderate, it is still greater than that between transferrin and Fe(II) . Such a moderate binding affinity suggests that a possible role of $\alpha\text{-syn}$ could be to chaperon Fe(II) . Because $\alpha\text{-syn}$ is near the cell membrane where endocytosis of transferrin takes place,[34] $\alpha\text{-syn}$ could be one of the first relay molecules in transporting Fe(II) . We should however caution that more concrete evidence is needed to consolidate such a suggestion.

We should add that it is likely that the binding affinity of $\alpha\text{-syn}$ towards Fe(II) is higher *in vivo* and therefore the abovementioned iron-transporting process might be more likely. Such a contention is made based on the following findings. First, $\alpha\text{-syn}$ in the LBs has been shown

to be extensively phosphorylated and a higher binding affinity of phosphorylated α -syn with metal ions has been demonstrated.[36,37] For example, complex of phosphorylated α -syn with Fe(III) has been detected by ES-MS and the hydrolysis of Fe(III) is suppressed due to the strong binding with phosphorylated α -syn. The binding affinities between other metal ions (e.g., Cu(II) and Fe(II)) and phosphorylated α -syn are also implied to increase significantly. [36] Second, near the presynaptic region concentrations of lipids or fatty acids are high. These molecules have been shown by *in vitro* studies to result in changes in the α -syn secondary structure,[38] which in turn could increase its binding to metal ions.[39] Finally, it was reported that the iron concentration in the *substantia nigra* of PD patients has increased by ~30%.[40] The iron concentration in the LIP of PD patient brains was also shown to be much higher than the normal level (i.e., ~1.5 μ M).[31] The increased Fe(II) concentration and the higher binding affinity of α -syn in the cytosol of the dopaminergic cell should favor the formation of a higher α -syn-Fe(II) concentration. However, we should add that *in vivo* there are other ligands (e.g., citrate and carbonate in the LIP) [34] that can compete with α -syn in binding with Fe(II). The result is that the conditional binding constant of α -syn is lowered. Again, whether α -syn can serve as an iron-chaperon awaits further investigation.

In summary, our work represents the first study showing the oxidation of α -syn-Fe(II) complex by O₂ to α -syn-Fe(III), a short-lived complex. Based on the binding constants of α -syn-Fe(II) and α -syn-Fe(III), this study sheds light on the possible roles of chaperoning Fe(II) within the cytosol by α -syn. These possible roles are discussed in the context of iron-induced oxidative stress and α -syn aggregation.

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Figure 1.
ES-MS spectrum of a solution containing 10 μ M α -syn and 10 μ M Fe(II) with peaks bearing 10–18 protons. The asterisks indicate the α -syn-Fe(II)-2Na⁺ adduct ions.

Figure 2.

(A) Cyclic voltammograms of Tris buffer (pH 7.4) containing 200 μM α-syn (dotted line curve) in ambient atmosphere, 200 μM α-syn and 200 μM Fe(II) (solid line curve) in a N₂-purged glovebox, and 200 μM α-syn and 200 μM Fe(II) after exposure to air for 2 h (dashed line curve). The scan rate was 20 mV/s and the solid arrow indicates the initial scan direction. The dashed arrow indicates a shoulder peak at -0.075 V. Inset: a CV of Fe(NH₄)₂(SO₄)₂. (B) Differential pulse voltammograms of 200 μM α-syn in the presence of various concentration of Fe (NH₄)₂(SO₄)₂: 200 μM (solid line curve), 400 μM (dotted line curve), 800 μM (dashed line curve), and 1000 μM (dash-dotted line curve).

Figure 3. Fluorescence spectra of 10 μM α-syn only (solid) and mixture of 10 μM α-syn and 500 μM Fe (II) before (dashed) and after (dotted) aeration in Tris buffer at pH 7.4.

Figure 4.

Fluorescence spectra of resorufin after exposing the following solutions to air for 2 h: 200 μM Fe(II) only (solid line curve), 200 μM Fe(II) and 200 μM α-syn (dashed), 118 μM Fe(II) only (dash-dotted), and 200 μM α-syn only (dotted).

Table 1
Measured m/z values of α -syn and α -syn-Fe(II) and their theoretical molecular weights

Charges	m/z of α -syn + protons	Calculated MW	m/z of α -syn-Fe(II) complex + protons + 2 Na^+	Calculated MW of the α -syn-Fe(II) complex + 2 Na^+
10	1447.00	14460.0	1456.70	14561.0
11	1315.61	14460.6	1324.53	14562.8
12	1205.95	14459.4	1214.18	14562.1
13	1113.26	14459.4	1120.85	14562.1
14	1033.82	14459.5	1040.92	14562.9
15	965.00	14460.0	971.55	14562.3
16	904.77	14460.3	910.89	14562.2
17	851.63	14460.7	857.34	14562.8
18	804.35	14460.7	809.76	14562.4
Average		14460.1		14562.2