J|A|C|S COMMUNICATIONS

Published on Web 04/27/2010

Evidence for Copper-dioxygen Reactivity during α-Synuclein Fibril Formation

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Amyloid formation of the presynaptic protein α-synuclein (α-syn), a process that has been implicated in the pathogenic mechanism of Parkinson's disease,[1] has been shown to accelerate in the presence of copper.[2,3] In fact, Cu/protein interactions also are associated with other neurodegenerative ailments such as Alzheimer's and prion diseases.[4−9] Cu is a redox-active metal ion that can activate O_2 leading to the generation of reactive oxygen species (ROS) such as $O_2^{-\bullet}$, $HO_2^{-\bullet}$, $HO^{-\bullet}$, and H_2O_2, which can in turn lead to enhanced oxidative stress and/or protein damage. In addition, metal coordination can perturb protein structure or initiate protein misfolding events, both of which could lead to altered function. In this work, the Cu^{II} coordination site of α-syn (Figure 1) is explored in the soluble and fibrillar states and evidence of Cu^{I}/O_2 chemistry is reported.

α-Syn fibrils were formed by incubating equimolar $Cu^{II}SO_4$ and α-syn (70 − 180 μM) in pH 7.0 buffer (20 mM MOPS, 100 mM NaCl) at 37 °C with agitation (450 rpm). The aged samples were then analyzed after one week. Amyloid formation (Figure 2) was confirmed by (1) the characteristic secondary structural change from unfolded to β-sheet monitored by circular dichroism (CD) spectroscopy, (2) a thioflavin T (ThT) fluorescence assay with subsequent quantum yield increase in the presence of α-syn fibrils,[10] and (3) transmission electron microscopy (TEM). In all instances (1−3), the metal chelator ethylenediaminetetraacetic acid (EDTA) was added to the aged samples to probe changes in the fibril morphology following Cu extraction; however, no apparent differences were detected.[11]

Extraction of the metal was confirmed by employing the Trp-containing variant, F4W. On the basis of fluorescence quenching experiments, F4W is the most responsive Cu^{II} reporter among a series of site-specific Trp substitutions (Figure 1).[12] Coordination of Cu^{II} to F4W results in full quenching of the Trp emission. Metal extraction can then be accomplished by addition of EDTA, which restores Trp emission in both soluble and fibrillar forms.[11] Our result also suggests that the Cu site in fibrils is solvent-accessible (outside of the amyloid core), consistent with previously proposed fibril models where the amyloid core spans residues 32−100.[13−15]

To examine local conformational changes in Cu^{II}-α-syn upon fibril formation, we measured the respective Cu K-edge X-ray absorption (XAS) spectra. Notably, the Cu K-edge spectrum of the soluble Cu-bound α-syn (150−180 μM) exhibited an estimated 20% metal reduction (based on a four-coordinate Cu^{I} model) with the appearance of a characteristic Cu^{I} absorption feature at lower energy (~8983 eV) (Figure 3A).[11] In contrast, after aging and forming fibrils, the edge absorption spectrum showed that the bound Cu ions are more oxidized than the soluble sample indicating that metal oxidation occurred during aggregation. We ensured that this result is reproducible and not attributable to photoreduction.[11]

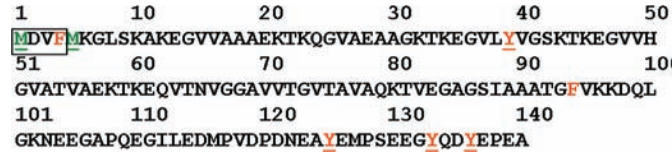

Figure 1. Primary amino acid sequence of human α-syn with all Tyr and N-terminal Met residues underlined. The primary Cu^{II} binding site is boxed and all other aromatic residues are colored orange to note available Trp probes.

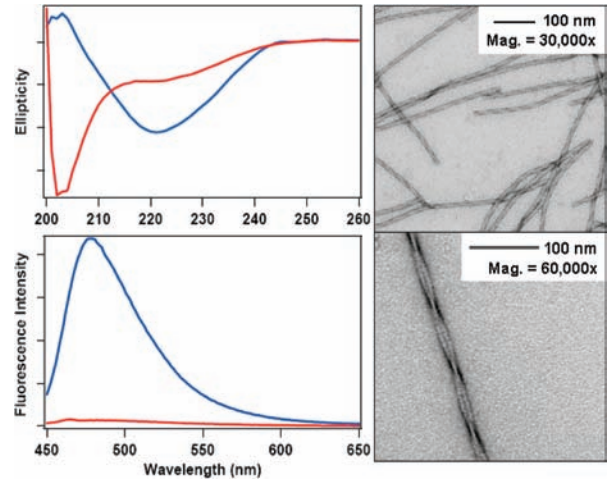

Figure 2. CD spectra (upper left) and ThT fluorescence assay (lower left) of Cu^{II}-α-syn in soluble (red) and β-sheet fibrillar (blue) forms. TEM images (right) of fibrils are shown at different magnifications.

The direct observation of Cu^{I} is in line with our previous study where we found enhanced Cu^{II} binding to F4W in the presence of dioxygen.[16] As a result, we hypothesized that the differences in metal-protein affinity may be due to autoreduction ($Cu^{II} \rightarrow Cu^{I}$) that is initiated by Met oxidation (Met → Met-O). The primary Cu^{II} binding motif of α-syn is at the N-terminus (MDVFMK) containing two nearby Met residues, which are highly susceptible to oxidation.[17,18] Furthermore, the reduction of $Cu^{II} \rightarrow Cu^{I}$ has been suggested for Aβ, the amyloidogenic peptide implicated in Alzheimer's disease.[4,9] Accordingly, a similar electron transfer pathway is feasible for $Cu^{II/I}$-α-syn. For both systems, Aβ and α-syn, further work is necessary to confirm this claim since many possible electron donors are present in aqueous buffer solution, including water.

To assess the role of O_2-chemistry, the Cu K-edge measurements were conducted on samples prepared under anaerobic conditions. We find higher percentages of metal reduction ($Cu^{II} \rightarrow Cu^{I}$) for both soluble and aggregated samples. Similar experiments also were conducted under 100% O_2 and a comparable extent of metal reduction was observed as under aerobic conditions (air ~21% O_2).[11] Taken together,

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10.1021/ja101756m

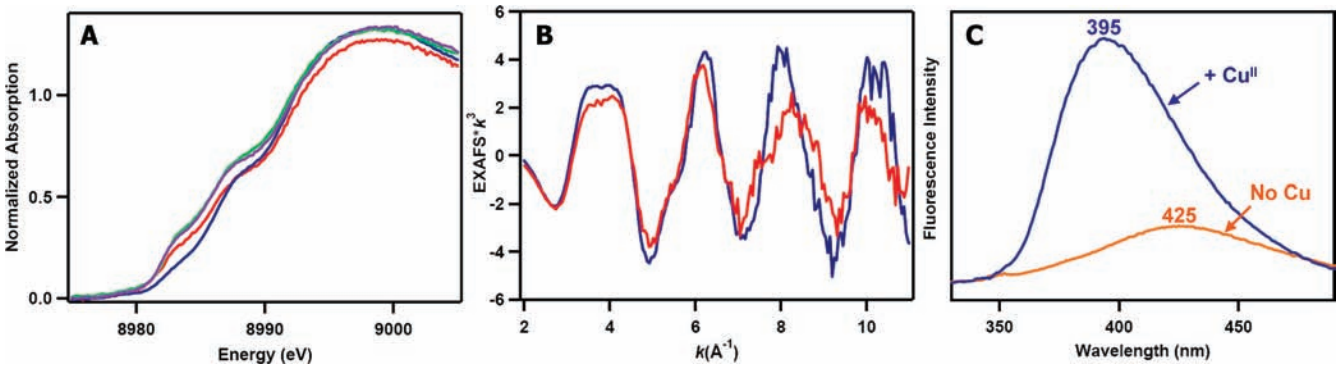

Figure 3. (A) Cu K-edge data of soluble (aerobic, red; anaerobic, purple) and aged (aerobic, blue; anaerobic, green) Cu^{II}-α-syn. (B) EXAFS data of aerobic soluble and aged Cu^{II}-α-syn. (C) Fluorescence detection of dityrosine cross-links in fibrillar α-syn both in the presence (blue) and absence (orange) of Cu^{II} under aerobic conditions.

our results indicate that metal reduction of $Cu^{II} \rightarrow Cu^{I}$ occurs in the absence of dioxygen and in the presence of O_2 reoxidation follows ($Cu^{I} \rightarrow Cu^{II}$) suggesting generation of ROS.

Evidence of ROS formation and presumably Cu^{I}/O_2 reactivity was gained by the observation of a new emission feature at 395 nm following fibril formation, which we attribute to the formation of dityrosines (Figure 3C).[19] Interestingly, protein samples aged in the absence of Cu exhibited a less pronounced and significantly red-shifted emission at 425 nm (Figure 3C), which we also associate with the presence of dityrosine chromophores.[20,21] Importantly, neither of these emission bands were detected from samples aged anaerobically. Therefore, dityrosine formation occurs in the presence of O_2 and is greatly enhanced by coordinated Cu. Furthermore, the 30 nm spectral shift may indicate the involvement of different Tyr residues and possibly differences between inter- versus intramolecular cross-links (Figure 1).

Surprisingly, the extended X-ray absorption fine structure (EXAFS) data (Figure 3B) show only modest differences between soluble versus aggregated Cu^{II}-α-syn despite the clear differences in secondary structure content as indicated by our CD data (Figure 2). Moreover, this result was unexpected because of the intrinsically disordered nature of α-syn; it is reasonable to anticipate a perturbation to the Cu coordination during global polypeptide rearrangement, which is necessary for amyloid formation. However, our data support coordination of three-to-four N- or O-containing ligands in both protein structures with approximate bond distances of 1.96 Å.

A tetra-coordinate Cu^{II} binding site is consistent with that proposed by Fernández and co-workers as well as our own group.[17,22] In previous work, we identified the first four N-terminal residues, MDV(F/W), as the minimal Cu^{II} binding site based on Trp fluorescence measurements on synthetic peptide models.[17] The free N-terminal nitrogen (NH_2) is the anchoring residue and the remainder of the coordination site is thought to consist of two deprotonated backbone amides (N^-) and either the Asp-2 carboxylate or an exogenous H_2O molecule. Notably, we recently have reported an intense minimum observed by CD spectroscopy that points to the possible existence of a Cu^{II}-(Phe/Trp) cation-π interaction in α-syn based on our synthetic N-terminal peptide models;[16] further work is underway.

For the first time, we show that upon coordination of Cu^{II} to α-syn, metal reduction from $Cu^{II} \rightarrow Cu^{I}$ occurs in the absence of dioxygen. In the presence of O_2, reoxidation of $Cu^{I} \rightarrow Cu^{II}$ takes place through the generation of ROS and over time results in dityrosine cross-linking. The XAS data indicate that the Cu^{II} coordination site of α-syn exhibits little change after amyloid formation and TEM analyses further show that the fibrils remain intact with removal of Cu. Overall, the results underscore that coordination of Cu to α-syn promotes oxidative stress, a factor largely associated with age-related diseases.[2,4,23]

Acknowledgment. Supported by the Intramural Research Program at NIH, NHLBI (J.C.L.) and Cornell University (S.D.). XAS was carried out at SSRL, a DOE, BES facility. The SSRL SMB Program is supported by the DOE, BER and NIH, NCRR. We also thank Duck-Yeon Lee (NHLBI Protein Analysis Facility) and Grzegorz Piszczek (NHLBI Biophysical Facility).

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA101756M