*Supporting Information*

*for*

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

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**Materials and Methods.**

Protein (wild-type and F4W) was expressed and purified as previously described.[1,2] Sample homogeneity was verified by SDS-PAGE gel analyses visualized by silver-staining methods (Phastsystem, Amersham Biosciences). All purified proteins were concentrated using a Millipore Amicon Concentrator (MWCO 3k), stored at −80 °C, and were filtered through Millipore Microcon YM-100 (MWCO 100k) spin filter units to remove any oligomeric material prior to experiments. Protein concentrations were determined using a value for molar absorptivity estimated on the basis of amino-acid content: $\varepsilon_{280\ nm}$ = 5,120 $M^{-1}cm^{-1}$ (wild-type) and $\varepsilon_{280\ nm}$ = 10,810 $M^{-1}cm^{-1}$ (F4W). Absorption data was collected on a Cary 300 Bio or Agilent 8453 spectrophotometer.

Copper(II) sulfate pentahydrate ($Cu^{II}SO_4$ • 5 $H_2O$; Sigma-Aldrich 99.999 %) was stored in a dessicator. Deionized water for anaerobic copper stock solutions[3] and pH 7.0 buffer solutions containing 20 mM 3-{N-morpholino}propanesulfonic acid (MOPS; SigmaUltra > 99.5 %) and 100 mM sodium chloride (NaCl ; Sigma 99.5 %) was sterile filtered (0.22 μm) and thoroughly deoxygenated *via* standard Schlenk techniques. Buffer solutions and $Cu^{II}$ stocks were stored under an inert atmosphere (10 % $H_2$, 90% $N_2$) within a glovebox (Coy Laboratory Products; Grass Lake, Michigan).

Fluorescence measurements were conducted using a Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter using a 1 cm quartz cuvette. Data were analyzed as previously published using the program IGOR 6.01 (Wavemetrics).[1] Dityrosine crosslinking was confirmed by 310 nm excitation (slit widths = 2 nm) of the Cu-α-syn fibrillar samples and monitoring emission in the 320 to 500 nm range. Metal extraction was confirmed by addition of ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich 99+ %) to $Cu^{II}$-F4W fibrils resulting in restoration of Trp emission ($\lambda_{ex}$ = 295 nm, $\lambda_{obs}$ = 300 – 500 nm, slit widths = 2 nm). Fibrillar material was confirmed by addition of 20 μM thioflavin T (ThT; Sigma-Aldrich ~75 %) to 15 μM α-syn samples in 1 cm disposable cuvettes ($\lambda_{ex}$ = 400 nm, $\lambda_{obs}$ = 450 – 680 nm, slit widths = 2 nm).[4] Circular dichroism (CD) spectra were collected on a Jasco J-715 spectropolarimeter using a 0.2 mm quartz slide or 1 mm quartz cuvette ($\lambda_{obs}$ = 200 – 260 nm; bandwidth = 1 – 2 nm; scan rate = 200 nm/min; accumulation = 5).

We would like to thank Dr. Duck-Yeon Lee (NHLBI Protein Analysis Facility) for maintaining the anaerobic glovebox and Dr. Grzegorz Piszczek (NHLBI Biophysical Facility) for help with the CD measurements.

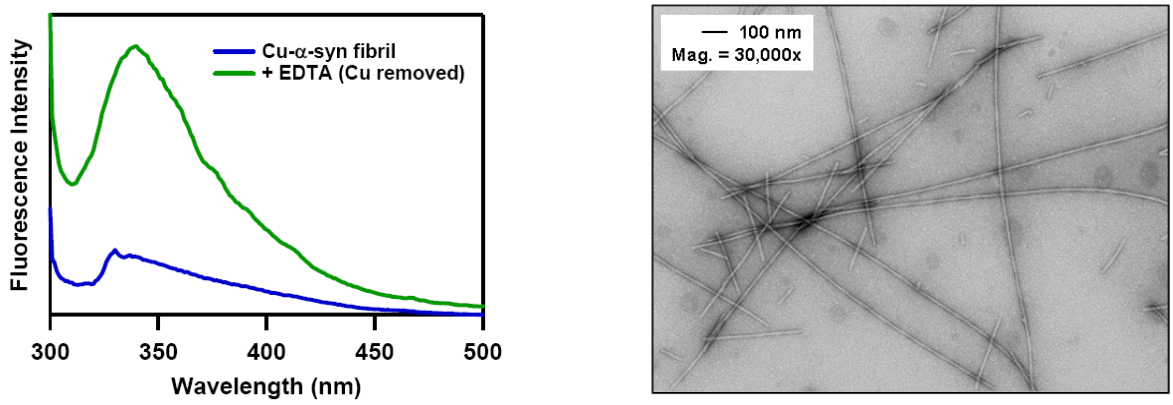


Figure 1. *Left*: Fluorescence spectra representing metal extraction from Cu-F4W fibril. *Right*: TEM image of wild-type α-syn fibril after removal of copper.

**Transmission Electron Microscopy.** (Myoung-Soon Hong, NHLBI Laboratory of Cell Biology)

One drop of aged α-synuclein (0.1 to 0.5mg/ml) sample was adsorbed on to a formvar/carbon coated 400 mesh Cu EM grid that had been previously glow discharged for 30 seconds in an EMScope TB500 (EMSCOPE Laboratories, England). After 1 min, the excess was drained off with filter paper. Then the grid was washed and stained with a drop of 0.5 % aqueous uranyl acetate for 30 seconds. The stain was removed with filter paper. The sample was observed in a JEM-1200EX II transmission electron microscope (JEOL, Japan) and images were taken using an AMT CCD camera system (Advanced Microscopy Techniques, Danvers, MA USA).

**Preparation of Cu-α-syn fibrils for XAS.** Freshly desalted α-syn (150 – 180 μM) and pre-filtered $Cu^{II}$-$SO_4$ (135 – 162 μM) were added to a 2 mL siliconized screw-cap tube (600 μL total sample volume), parafilmed, and placed in an incubator (37 $^{o}$C ; 450 RPM); less than 1 eq. $Cu^{II}$ was added in order to prevent interference from unbound $Cu^{II}$ during data collection and analysis. After one week, each sample was centrifuged at 4000 RPM in triplicate for 45 mins. After each centrifugation, the supernatant (450 μL) was removed and the pellet was resuspended with 450 μL buffer and vortexed before the next centrifugation. After removal of the third supernatant fraction, only 75 μL buffer was added. Analysis of supernatant by UV-visible spectroscopy allowed for the final sample concentration to be estimated (120 – 165 μM $Cu^{II}$-α-syn). The final sample (140μL) was then vortexed and transferred to an XAS cell (12.2 mm Lucite with 38 micron Kapton windows). Anaerobic samples were prepared in an identical manner except all steps were conducted in the glovebox. For oxygenated samples, buffer was pre-saturated with 100% $O_2$ prior to sample preparation. All Cu-α-syn samples were frozen in liquid $N_2$ within the XAS cells and shipped in dry-ice to the Stanford Synchrotron Radiation Lightsource at Stanford University and analyzed by Dr. Serena DeBeer George (Cornell University, Department of Chemistry).

**X-ray Absorption Spectroscopy.** (Serena DeBeer, Cornell University)

XAS data were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL) on focused beam line 9-3, under ring conditions of 3 GeV and 60-100 mA. A Si(220) double-crystal monochromator was used for energy selection and a Rh-coated mirror (set to an energy cutoff of 13 keV) was used for harmonic rejection. Internal energy calibration was performed by assigning the first inflection point of the Cu foil spectrum to 8980.3 eV. All samples were maintained at 10 K during data collection using an Oxford Instruments CF1208 continuous flow liquid helium cryostat. Data were measured in fluorescence using a Canberra Ge 30-element array detector.

XAS data were measured to k = 11 $Å^{-1}$ for solution samples. The truncated data range is due to the presence of ice diffraction at high k. Only those scans which showed no evidence of photoreduction were used in the final average.

The data were calibrated and averaged using EXAFSPAK.[5] Pre-edge subtraction and splining were carried out using PYSPLINE.[6] A three-region cubic spline of order 2, 3, 3 was used to model the smooth background above the edge. Normalization of the data was achieved by subtracting the spline and normalizing the post-edge region to 1. The resultant EXAFS was k3-weighted to enhance the impact of high-k data.

Theoretical EXAFS signals χ(k) were calculated using FEFF (version 7.0)[7,8] and fit to the data using EXAFSPAK.[5] The non-structural parameter E0 was also allowed to vary but was restricted to a common value for every component in a given fit. The structural parameters varied during the refinements were the bond distance (R) and the bond variance ($\sigma^2$). The $\sigma^2$ is related to the Debye-Waller factor, which is a measure of thermal vibration and to static disorder of the absorbers/scatterers. Coordination numbers were systematically varied in the course of the analysis, but they were not allowed to vary within a given fit.

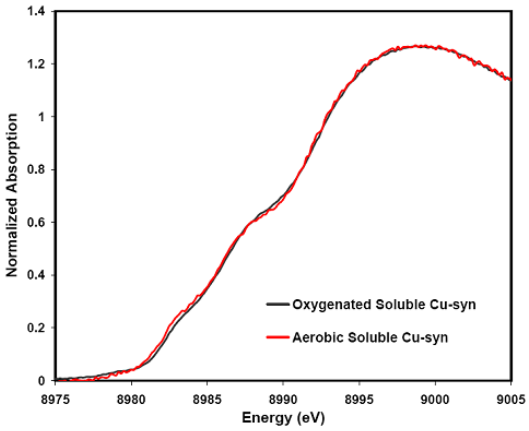


Figure 2. Similar Cu K-edge spectra result when Cu-α-syn samples are prepared aerobically (red) and in $O_2$ saturated buffer (black).

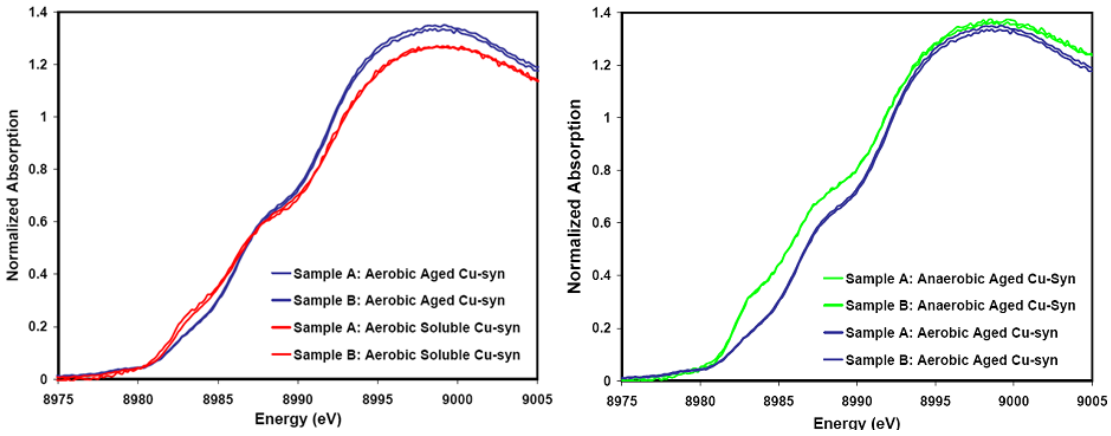


Figure 3. Cu K-edge spectra to demonstrate that our Cu-α-syn samples results are consistent after multiple experiments.

**References.**


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