Material and Methods

Materials

Copper(II) sulfate pentahydrate (99.999%) and N-acetyl-tryptophanamide were purchased from Sigma-Aldrich and used without further purification.

Protein Expression and Purification

The wild-type human α-syn expression plasmid (pRK172) was provided by M. Goedert (Medical Council Research Laboratory of Molecular Biology, Cambridge, U.K.). A fluorescent Trp residue was introduced at an aromatic-residue position (F4) by site-directed mutagenesis. The reported Cu(II) binding ligand, His50 was removed and substituted with a Ser residue. All site-directed mutagenesis reactions were performed using a QuickChange kit (Stratagene). All mutations were confirmed by DNA sequencing (Caltech DNA Sequencing Core Facility).Bacterial cells (BL-21(DE3)pLysS, Invitrogen) were chemically transformed with plasmids containing desired mutations. All bacterial growths were under the selective pressure of 34 mg/L chloramphenicol and 100 mg/L ampicillin. Starter culture (25 mL) was inoculated with a freshly transformed single colony of bacterial cells, grown in LB media overnight at 30 °C, and used further to inoculate 1-L LB medium. As soon as the culture reached an $OD_{600\ nm}$ ~ 0.6-0.8, protein expression was induced with 0.5 mM ITPG for 6 h at 30 °C.Recombinant α-syn was purified according to published procedures.[1] Protein concentrations were determined using a molar extinction coefficient estimated on the basis of amino-acid content: $\varepsilon_{280\ nm}$ = 10,810 $M^{-1}cm^{-1}$ (F4W and F4W/H50S). The purity of all protein samples was assessed by SDS-PAGE on a Pharmacia Phastsystem visualized by silver-staining methods. The protein molecular weights were confirmed by ESI-MS (Caltech Protein/Peptide Microanalytical Laboratory). Absorption and luminescence spectra were measured on a Hewlett-Packard 8452 diode array spectrophotometer and a Spex Fluorolog3 spectrofluorimeter, respectively. All purified proteins were concentrated using Centriprep YM-3(MWCO 3kD) (Millipore) and stored at −80 °C.

Copper(II) Titrations

Copper(II) concentrations were determined spectrophotometrically ($\varepsilon_{710\ nm}$ = 12 $M^{-1}cm^{-1}$). Prior to experiments, all protein samples were filtered through Microcon YM-100 (MWCO 100kD) (Millipore) spin filter units to remove oligomeric material and exchanged into the appropriate buffer (20 mM MOPS, 100 mM NaCl, pH 7) using gel filtration chromatography (PD-10 column, Amersham-Biosciences). All titrations were performed on deoxygenated samples to avoid deleterious metal-oxygen chemistry and photobleaching. Buffer solutions were filtered (0.22 μm) to remove any particulate matter. Tryptophan was excited at 295 nm and fluorescence was monitored from 300 to 600 nm. All experiments were conducted at 25 °C using a temperature-controlled cuvette holder.

Time-resolved Fluorescence Measurements

Fluorescence decay kinetics measurements were carried out as previously described.[1] Protein samples were deoxygenated by repeated evacuation/Ar-fill cycles on a Schlenk line. Buffer solutions were filtered (0.22 μm) to remove any particulate matter.

A polarized laser pulse (35° from vertical) from the third harmonic (292 nm) of a regeneratively amplified femtosecond Ti:sapphire laser (Spectra-Physics) was used as an excitation source and a picosecond streak camera (Hamamatsu C5680) was used in the photon-counting mode for detection. Trp emission was selected by using a combination of dielectric and color filters ($325 \leq \lambda \leq 400$ nm). All experiments were conducted at 25 °C using a temperature-controlled cuvette holder. All protein samples were filtered through Microcon YM-100 (MWCO 100kD) (Millipore) spin filter units to remove oligomeric material prior to experiments.

Data Analysis

Fluorescence Decay Kinetics

We describe the Trp fluorescence decay kinetics with a discrete distribution of exponential decay rate contants:

$$I(t) = \sum_k P(k) e^{-kt}$$

where $P(k)$ is the probability of finding a W fluorophore in the protein ensemble with a decay rate constant of k.

We define average excited state lifetimes as the integral over the normalized fluorescence decay curves:

$$\langle \tau \rangle = \int \frac{I(t)}{I(t=0)} dt = \sum_k \frac{P(k)}{k}$$

We have fit kinetics data by using a MATLAB (Mathworks, Natick, MA) algorithm (LSQNONNEG) that minimizes the sum of the squared deviations (χ^2) between observed and calculated values of $I(t)$, subject to a nonnegativity constraint.

Cu(II) binding curves

To estimate a binding constant for the dynamic quenching process, we assume a two state binding model:

$$PCu \rightleftharpoons P + Cu \qquad K_d = \frac{[P][Cu]}{[PCu]}$$

where $P \equiv$ free α-syn, $Cu \equiv$ free copper, $PCu \equiv$ 1:1 complex of copper and α-syn.

Mass balance gives:

$$[P] = [P]_\circ - [PCu]$$

$$[Cu] = [Cu]_\circ - [PCu]$$

Substitute into expression for K_d:

$$K_d = \frac{([P]_\circ - [PCu])([Cu]_\circ - [PCu])}{[PCu]}$$

Rearrange:

$$[PCu]^2 - ([P]_\circ + [Cu]_\circ + K_d) + [P]_\circ[Cu]_\circ = 0$$

Solve for concentration of copper-bound α-syn:

$$[PCu] = \frac{1}{2}([P]_\circ + [Cu]_\circ + K_d) - \frac{1}{2}\sqrt{([P]_\circ + [Cu]_\circ + K_d)^2 - 4[P]_\circ[Cu]_\circ}$$

The total time-resolved fluorescence intensity from *P* and *PCu* is given by the following relation:

$$I(t) = \frac{[P]}{[P]_\circ}\sum_k P(k)_{[P]_\circ} e^{-kt} + \frac{[PCu]}{[P]_\circ}\sum_k P(k)_{[PCu]} e^{-kt} = \sum_k \left[\frac{[P]}{[P]_\circ} P(k)_{[P]_\circ} + \frac{[PCu]}{[P]_\circ} P(k)_{[PCu]} \right]$$

The average decay time is given by:

$$\langle\tau\rangle = \int \frac{I(t)}{I(t=0)} dt = \frac{[P]}{[P]_o}\sum_k \frac{P(k)_{[P]_\circ}}{k} + \frac{[PCu]}{[P]_o}\sum_k \frac{P(k)_{[PCu]}}{k} = \frac{[P]}{[P]_o}\langle\tau\rangle_\circ + \frac{[PCu]}{[P]_o}\langle\tau\rangle_{[PCu]}$$

Substituting for [*P*] and rearranging gives:

$$\frac{\langle\tau\rangle}{\langle\tau\rangle_\circ} = 1 + \left(\frac{\langle\tau\rangle_{[PCu]}}{\langle\tau\rangle_\circ} - 1 \right)\frac{[PCu]}{[P]_\circ} = 1 + \left(\frac{\langle\tau\rangle_{[PCu]}}{\langle\tau\rangle_\circ} - 1 \right)\frac{\frac{1}{2}([P]_\circ + [Cu]_\circ + K_d) - \frac{1}{2}\sqrt{([P]_\circ + [Cu]_\circ + K_d)^2 - 4[P]_\circ[Cu]_\circ}}{[P]_\circ}$$

where $\langle\tau\rangle_{[PCu]}$ is the average fluorescence lifetime of pure [*PCu*].

To estimate a binding constant for the static quenching process, we used time-resolved fluorescence data collected with comparable protein concentrations and excitation intensities. We define the fractional quenching using the following expression:

$$\chi([Cu]_\circ) = \frac{I(t=0,[Cu]_\circ = 0) - I(t=0,[Cu]_\circ)}{I(t=0,[Cu]_\circ = 0)}$$

An apparent dissociation constant for the static quenching process is given by:

$$\chi([Cu]_\circ) = \chi_{[PCu]}\frac{[PCu]}{[P]_\circ} = \chi_{[PCu]}\frac{\frac{1}{2}([P]_\circ + [Cu]_\circ + K_d) - \frac{1}{2}\sqrt{([P]_\circ + [Cu]_\circ + K_d)^2 - 4[P]_\circ[Cu]_\circ}}{[P]_\circ}$$

where $\chi_{[PCu]}$ is the fractional quenching for pure [*PCu*].

Data were fit to the above equation using Igor Pro 6.01 (Wavemetrics, Inc. Oregon).

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[3]Livesey, A. K., Delaye, M., Licinio, P. & Brochon, J. C. (1987) *Faraday Discussions***,** 247-258.
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