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Supporting Information

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Supporting Information

for

Single-Molecule FRET Reveals Structural Heterogeneity of SDS-Bound α-Synuclein

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Materials, Methods and Results

Chemicals and reagents: All chemicals were from Sigma-Aldrich and of the highest purity grade available. Thiol-reactive maleimide derivatives of Alexa Fluor 488 (AF488) and Alexa Fluor 568 (AF568) were from Invitrogen (Carlsbad, CA). Solutions and buffers were prepared in 0.2 μm filtered Milli-Q water (Millipore, Bedford, MA).

Purification and labeling of α-Synuclein mutants: Single-Trp and single- and double-cysteine mutants of αSyn were engineered with tryptophans or cysteines at amino acid positions 9 and/or 69, resulting in αSyn-9W, αSyn-69W, αSyn-9C, αSyn-69C and αSyn-9C/69C. Expression and purification of αSyn mutants was performed essentially as described,[1] with some minor modifications. During purification of cysteine variants,1 mM freshly prepared DTT was added to all buffers. Purified proteins were stored at a concentration of ~200 μM in 10 mM Tris-HCl, pH 7.4 at -80 °C, supplemented with 1 mM DTT as required. For labeling, typically 0.5 mL of 200 μM αSyn was used. Prior to labeling, the cysteines were reduced with 1 mM freshly prepared DTT and incubated for 30 min at room temperature. Immediately after removal of excess DTT using Zeba Spin desalting columns (Pierce Biotechnology), a 3× molar excess (based on cysteine content) of AF488 or AF568 was added to single-cysteine mutants and an equimolar amount of AF488 to the double-cysteine mutant. Alkylation was carried out for 1 hour at room temperature in the dark followed by quenching with 10× molar excess of DTT. For the single-cysteine mutants, free dye was removed by using two subsequent desalting steps, and any precipitated protein was re-

moved by using Microcon YM100 filters (Millipore). Stoichiometry of labeling was determined as described below. For the double-cysteine mutant, unreacted AF488 and DTT were removed using a desalting step followed by immediate incubation with 330 mg prewashed Thiopropyl Sepharose 6B (GE Healthcare Life Sciences), according to manufacturer procedures. This step separates doubly-labeled αSyn, not binding to the resin, from single or unlabeled αSyn. The binding reaction was allowed to proceed for at least 1 hour, rotating in the dark at 4 °C. Subsequently, the resin was washed with several column volumes of 10 mM Tris-HCl, pH 7.4 buffer, followed by elution of single-labeled and/or unlabeled αSyn using 10-15 mL of buffer containing β-mercaptoethanol at concentrations according to manufacturer procedures. Eluted fractions were pooled, concentrated to about 0.5 mL and desalted. In the case of the double-cysteine mutant, a 2-3× molar excess of the second dye (AF568) was added immediately after elution. After incubation for 1 hour at room temperature, free dye was removed using two desalting steps and the solution was filtered through a Microcon YM100 filter (Millipore, Bedford, MA).

For AF488-labeled proteins (both single and double-cysteine), the stoichiometry of labeling was determined from measured absorption spectra using manufacturers extinction coefficients for the dyes and a calculated extinction coefficient of 5960 $L \cdot mol^{-1} \cdot cm^{-1}$ for αSyn at 280 nm (according to ref. [2]). For AF488-labeled proteins this is a suitable method as the contribution of AF488 to the absorbance at 280 nm is only about 10% of the total absorption of the dye at 495 nm, allowing accurate protein concentration determination. AF568 absorbs strongly in the UV region, making estimates of the protein concentration, and thus stoichiometry, difficult. For the double-cysteine mutant the amount of protein (and thus the amount of cysteines) and occupied cysteines by AF488 could be accurately determined. Therefore, the amount of remaining cysteines that could be alkylated by AF568 was also known and was experimentally always in perfect agreement with the observed amount of AF568 attached to the protein, as determined from the ratio between the absorbances of AF488 and AF568. Generally, a labelling efficiency of AF488 of >90% was observed and an AF568 labelling efficiency up to 110%, resulting in nearly each αSyn molecule having both a donor and acceptor attached.

Since for labeling of the single-cysteine mutants an excess of dye was used and both AF488 and AF568 have the same reactive group, it is fair to expect for single-cysteine αSyn-AF568 a stoichiometry similar to αSyn-AF488.

Steady-state fluorescence spectroscopy: All steady-state fluorescence measurements were performed on a Fluoromax-4 instrument (Horiba Jobin Yvon, Longjumeau, France). Excitation of single-Trp mutants αSyn-9W and αSyn-69W was at 295 nm with emission recorded from 305-500 nm. Excitation of AF488 and AF488-labeled proteins was at 488 nm (identical to the laser-line used in single-molecule experiments); excitation of AF568 and AF568-labeled proteins was at 575 nm. Emission was recorded up to 700 nm. Concentrations for single-Trp mutants were 10 μM; for dye-labeled proteins typically ~100 nM in 10 mM Tris-HCl, pH 7.4, supplemented with 0.005% (*v/v*) Tween-20 (see below). Emission spectra were corrected for background fluorescence of the buffer components and for instrument response according to the manufacturer.

Figure S1 indicates that, in ensemble measurements, AF488 and AF568 labeled αSyn-9C/69C displayed pronounced acceptor fluorescence and hence FRET-signal, that is completely abolished upon addition of trace amounts of trypsin, digesting αSyn-9C/69C at multiple positions, also between amino acids 9 and 69. This result indicates that the FRET-signal is originating from proteins that have both dyes attached.

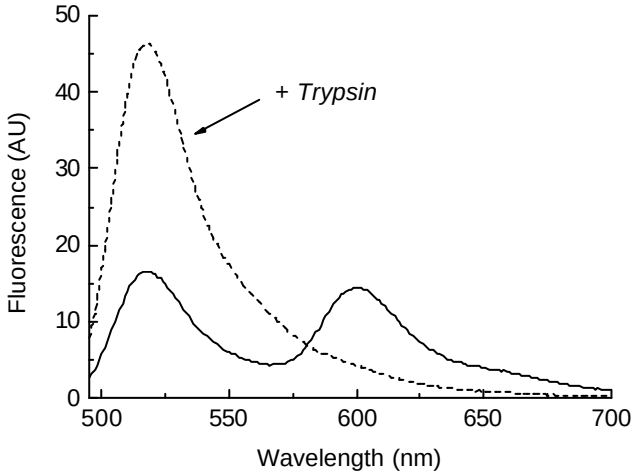

Figure S1. Fluorescence emission spectra of AF488 and AF568 labeled αSyn-9C/69C in the absence (solid line) and presence (dashed line) of Trypsin. Excitation was at 488 nm. Spectra were corrected for a small contribution of direct acceptor-excitation.

To check for sufficient rotational freedom of the attached dyes at the selected positions, steady-state fluorescence emission anisotropy was collected at the peak emission maximum of the various samples until the standard deviation was <2% (according to manufacturer's procedures). αSyn-9C and αSyn-69C labeled with only AF488 donor exhibited steady-state anisotropy values of 0.06 and 0.08, respectively that did not alter significantly upon addition of SDS (Figure S2). For αSyn-9C and αSyn-69C labeled with the acceptor AF568, initial anisotropy values of 0.09 and 0.11 were measured. Upon addition of SDS, these values changed to 0.16 and 0.23, respectively (see Figure S2). The increase in anisotropy during SDS titration was also observed for acceptor ATTO565 (Sigma-Aldrich) attached to the protein, but not for any of the free dyes (data not shown). Surprisingly, the transition to somewhat higher anisotropy values occurred for both AF568-labeled mutants around 0.5 mM SDS, similar to the blue-shifts observed with Trp-fluorescence (Figure 4b Communication). This indicates binding or association of SDS molecules to αSyn, apparently somewhat affecting the motion of AF568(like)-dyes. The constant fast rotation of the donor dye at any of the positions allows the assumption that dipole-orientational differences between donor and acceptor are not significant during the titration of SDS and do not affect the FRET-efficiency distribution. Furthermore, these relatively low anisotropy values have been indicated before to be no significant issue for SM-FRET regarding dipole orientation.[3, 4]

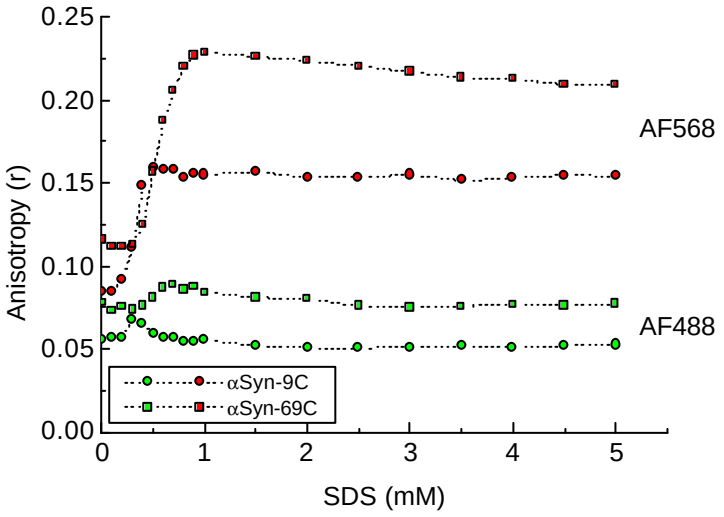

Figure S2. Steady-state anisotropy of αSyn-9C (circles) and αSyn-69C (squares) labeled with either AF488 (green symbols) or AF568 (red symbols). Dotted lines are shown for visibility.

Circular dichroism spectroscopy: Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter operated at room temperature using a quartz cuvette with a 0.5 mm path length. The wavelength range was 190 – 250 nm with 1.0 nm bandwidth, 0.2 – 0.5 nm resolution and 0.5 s response time. All spectra shown are averages of at least 8 recordings and are corrected for background absorption. Protein concentrations were typically 10 µM in 10 mM Tris-HCl, pH 7.4 and 0.005% (*v/v*) Tween-20. No differences in structural changes dependent on the SDS concentration were observed between wild-type αSyn (not shown) and αSyn-9C/69C (Figure S3).

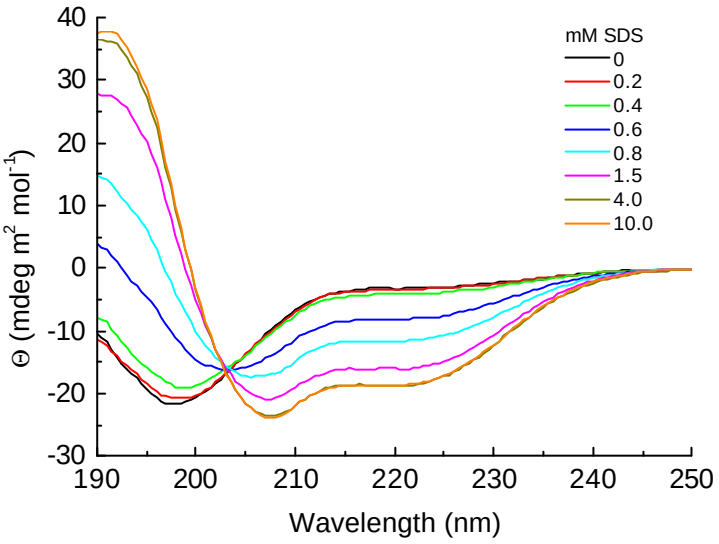

Figure S3. Structural changes in αSyn-9C/69C (unlabeled) induced by increasing concentrations of SDS measured with far-UV circular dichroism. For clarity, not all employed concentrations shown in Figure 4A, are presented here.

Isothermal titration calorimetry: The critical micelle concentration of SDS in 10 mM Tris-HCl, pH7.4 supplemented with 0.005% (*v/v*) Tween-20 was estimated from Isothermal Titration Calorimetry (ITC) experiments using a MicroCal VP-ITC microcalorimeter (MicroCal, Northhampton, MA), operated at 21°C (resembling the temperature conditions of all other experiments). Heat-changes were monitored upon 59 × 5 µL additions of 50 mM SDS in 10 mM Tris-HCl, pH7.4 and 0.005% (*v/v*) Tween-20 to the 1.345 mL calorimeter cell, containing the same buffer, without SDS. In Figure S4 a duplicate of the titration is shown. The CMC, reflected by the steep change in ΔH, was around 7 mM and in good agreement with the CMC for SDS in water.[5] The rea-

son this value is higher than reported by Ferreon & Deniz,[6] is most likely due to the presence of 200 mM NaCl in their buffer system, strongly affecting the CMC.[5]

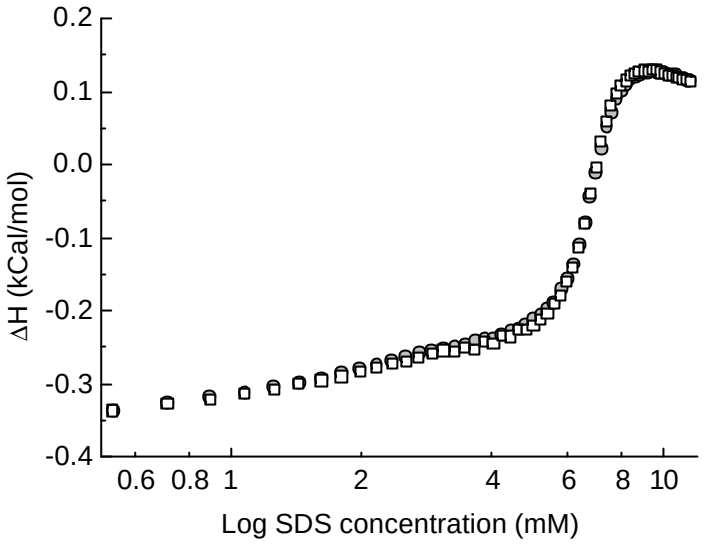

Figure S4. Isothermal titration calorimetry to estimate the critical micelle concentration of SDS in 10 mM Tris-HCl, pH 7.4 and 0.005% (*v/v*) Tween-20. Shown is a duplicate experiment (open squares and light grey circles).

Single-molecule fluorescence: Single-molecule measurements were performed on a custom-built confocal fluorescence microscope,[7-9] with some minor modifications. A laser-line at 488 nm was used to excite labeled αSyn molecules. Fluorescence emission collected by the objective, was passed through a 488 nm notch filter (Kaiser Optical Systems, Ann Arbor, MI) and separated by a dichroic beamsplitter (565DCXR, Chroma, Rockingham, VT) for APD detection of donor and acceptor emission in separate channels. For detection of donor emission a 525/50 band-pass filter was used and for acceptor emission a 617/73 band-pass filter (Semrock, Rochester, NY). We observed that αSyn has a high tendency to adhere to surfaces, e.g. of glass and plastics. Especially at low (sub)-nM concentrations, this resulted in a significant loss of signal (both in ensemble measurements as well as in a SM approach). Therefore, for SM experiments dimethyldichlorosilane treated Lab-Tek 8-well chambered coverglasses (Nalge Nunc, Rochester, NY) were used in combination with 0.005% (*v/v*) Tween-20 in all solutions, adequately preventing adhesion. Tween-20 did not interact with αSyn as no change in the SM-FRET histograms was observed upon addition of this low concentration of Tween-20 (not shown). SM-FRET experiments were typi-

cally performed at 50-100 pM Alexa Fluor 488 and Alexa Fluor 568 labeled αSyn-9C/69C in 10mM Tris-HCl, pH 7.4 supplemented with 0.005% Tween-20, and 10 mM DTT to suppress acceptor photobleaching. These extremely low concentrations of αSyn ensure that association of αSyn molecules is very unlikely to occur. The applied 488 nm excitation intensity was 50 µW, measured at the back aperture of the objective, resulting in an intensity of ~25 µW in the sample. For analysis the fluorescence emission intensities, measured with a time resolution of 12.5 µs, were binned to 1 ms time intervals and the FRET-efficiency (E_{obs}) was calculated according to Equation (S1), including only signals with summed donor and acceptor intensities higher than 40 counts:

$$E_{obs} = \frac{1}{1 + g\left(\frac{I_D}{I_A}\right)} \qquad \text{(S1)}$$

where I_D and I_A represent the number of counts collected within the time-bin for the donor and acceptor channel, respectively. Both I_D and I_A were corrected for background counts and I_A was corrected for leakage of donor emission into the acceptor channel, amounting to ~10%. The factor g accounts for differences in donor and acceptor quantum yields and detection efficiencies. Considering the quantum yields of the dyes as reported by the supplier and the differences in detection efficiencies of the detection channels, it is fair to assume g in Equation (S1) being close to unity. Furthermore, no significant changes in the quantum yields of both donor and acceptor dyes at either amino acid position 9 or 69 were observed during the titration with SDS, as judged from similar integrated intensities of the emission spectra measured at each SDS concentration with ensemble fluorescence spectroscopy (data not shown). Steady-state anisotropy further confirmed that no dipole orientational issues affected the SM-FRET histograms at any of the SDS concentrations (see above).

The resulting E_{obs}-data were plotted in histograms revealing the typical zero peak, originating from either donor-only labeled αSyn molecules or doubly labeled molecules with inactive acceptor dyes, as has been reported before in literature (e.g. ref. [10]). Distributions with E_{obs}-values > 0.15 were considered to originate from αSyn-9C/69C molecules with both donor and acceptor dyes active. The distributions reflecting αSyn conformers were fitted using Gaussian distribution profiles [Eq. (S2)]

with free parameters for the centre (x_c), FWHM [Eq. (S3)] and area (A) for each Gaussian:

$$f(x) = \frac{A}{s\sqrt{\frac{p}{2}}} \cdot e^{-2\frac{(x-x_c)^2}{s^2}} \qquad \text{(S2)}$$

with the FWHM according to:

$$FWHM = s \cdot \sqrt{\ln(4)} \qquad \text{(S3)}$$

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