

Direct Observation of the Interconversion of Normal and Toxic Forms of α -Synuclein

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SUMMARY

Here, we use single-molecule techniques to study the aggregation of α -synuclein, the protein whose misfolding and deposition is associated with Parkinson's disease. We identify a conformational change from the initially formed oligomers to stable, more compact proteinase-K-resistant oligomers as the key step that leads ultimately to fibril formation. The oligomers formed as a result of the structural conversion generate much higher levels of oxidative stress in rat primary neurons than do the oligomers formed initially, showing that they are more damaging to cells. The structural conversion is remarkably slow, indicating a high kinetic barrier for the conversion and suggesting that there is a significant period of time for the cellular protective machinery to operate and potentially for therapeutic intervention, prior to the onset of cellular damage. In the absence of added soluble protein, the assembly process is reversed and fibrils disaggregate to form stable oligomers, hence acting as a source of cytotoxic species.

INTRODUCTION

α -synuclein (α S) is a 140 residue protein expressed abundantly in the brain, where it can account for up to 1% of all proteins in the neuronal cytosol (Bonini and Giasson, 2005). It does not appear to be an essential protein (Abeliovich et al., 2000) and has multiple proposed functions interacting specifically with numerous proteins involved in signal transduction, vesicular trafficking, synaptic behavior, the regulation of oxidative stress, and mitochondrial function (Bonini and Giasson, 2005). It is, however, the major constituent of intracellular protein-rich inclusions, Lewy bodies and Lewy neurites, the hallmark lesions

of Parkinson's disease (PD) (Spillantini et al., 1998). These inclusions share common structural characteristics, including a high β sheet content and a distinctive cross- β X-ray diffraction pattern, also observed for fibrillar deposits associated with other degenerative disorders, including Alzheimer's disease, and commonly described as amyloid fibrils (Geddes et al., 1968; Chiti and Dobson, 2006).

The formation of amyloid fibrils in vitro is a common phenomenon and is usually monitored through measurements of turbidity or by means of fluorometric dyes, such as Thioflavin T. These experiments suggest that fibril formation follows a nucleation-polymerization model (Jarrett and Lansbury, 1992), where soluble species undergo a nucleation process that generates oligomeric species that are then able to grow through further monomer addition, thereby forming protofilaments and eventually mature fibrils. The characteristic sigmoidal growth profile reflects the greater ease of addition of monomers onto existing aggregates compared with the de novo formation of new oligomers directly from monomers alone. The overall reaction rate therefore accelerates when significant numbers of aggregates are present in solution, resulting in an initial lag phase followed by a growth phase during which the overall conversion is accelerated before a plateau region is reached when the monomer concentration is depleted (Figure 1A). Recently, however, it has become evident that the kinetics of fibril growth can often be dominated by secondary nucleation events, such as fibril fragmentation (Cohen et al., 2011; Knowles et al., 2009), adding further elements of complexity to the kinetic process.

Both experimental and theoretical studies of the kinetics of fibril formation have given important insights into the overall mechanism of amyloid assembly, but little is known in any detail about the oligomeric species that not only represent the crucial first steps of the self-association process but have also been implicated as key species in the pathogenesis of protein misfolding and deposition diseases (Bucciantini et al., 2002; Kaye et al., 2003; Lashuel et al., 2002; Luheshi et al., 2007; Tokuda et al., 2010; Winner et al., 2011). A variety of distinct morphologies of α S oligomers have been observed using

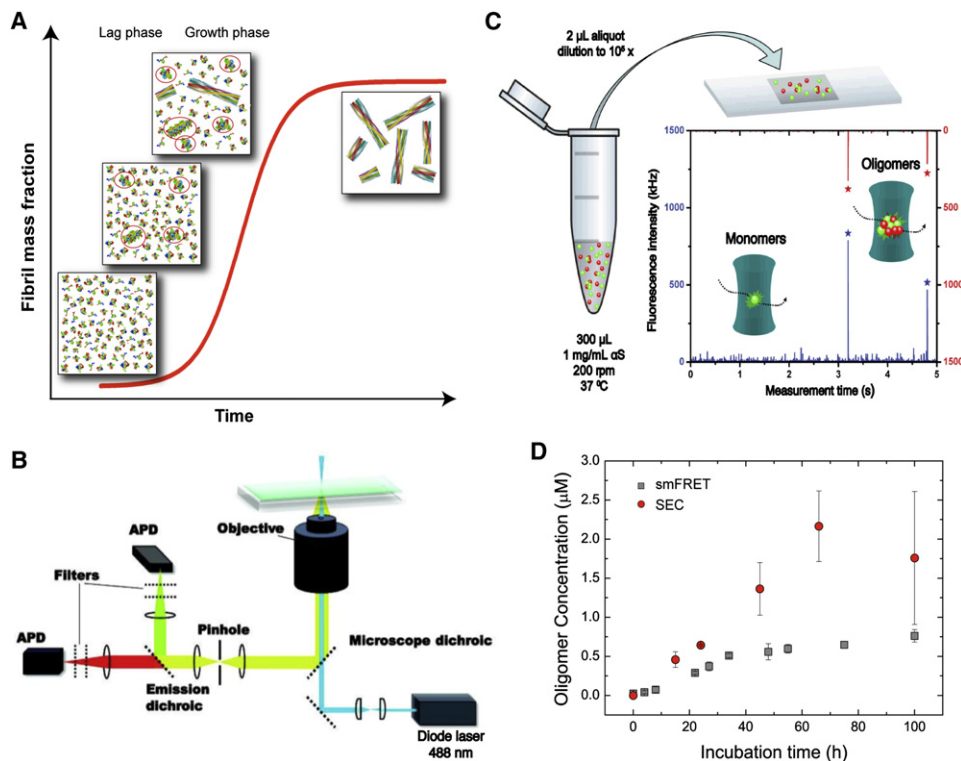


Figure 1. Experimental Protocol

(A) Example of the kinetics of amyloid fibril formation, including hypothetical snapshots of the ensemble of α S species present at different phases of the aggregation process, wherein the oligomeric species present in the sample are highlighted with red circles.

(B) Schematic representation of the instrument used for smFRET measurements.

(C) Schematic description of the experimental protocol for aggregation experiments. Bursts of fluorescence coincident in both channels indicate the presence of FRET-positive oligomeric species (marked as asterisks). Noncoincident bursts can be attributed to monomers and are normally much less bright than those corresponding to oligomers.

(D) Comparison between the kinetics of oligomer formation under bulk conditions obtained by quantitative SEC analysis (red circles; see also Figure S1G) and from smFRET experiments, after extrapolating the concentrations from sm to bulk conditions (gray squares). The data reported correspond to the mean and standard errors of two repetitions in the case of the SEC data and five repetitions for the sm data. See Figure S1 for a detailed characterization of the effect of the fluorophores on α S aggregation.

imaging techniques, notably, atomic force microscopy or transmission electron microscopy (see, e.g., Conway et al., 2000; Ding et al., 2002; Lashuel et al., 2002; Hoyer et al., 2004). Structural studies on α S oligomers have also been carried out using FTIR, Raman, CD, and fluorescence spectroscopy (see, e.g., Apetri et al., 2006; Goldberg and Lansbury, 2000; Hong et al., 2008; Nath et al., 2010; Thirunavukkuarasu et al., 2008), which have revealed the formation of various oligomeric structures during α S aggregation, consistent with a progressive increase in β sheet structure occurring concomitantly with the formation of more ordered aggregates.

In order to define in more detail the types of oligomers formed during α S fibril formation and the rates at which they develop, it is necessary to find the means of overcoming the challenges inherent in studying these heterogeneous and frequently transient intermediate species. Recently, techniques have been developed that are able to observe individual molecular species in solution (Chiou et al., 2009; Kostka et al., 2008; Orte et al., 2008), and in the present work we describe experimental and theoretical approaches that enable us to define in microscopic

detail the assembly of α S into amyloid fibrils. As we discuss below, the results obtained shed new light on the underlying molecular processes involved in α S oligomerization and fibril formation and, in conjunction with cell biology experiments, provide crucial information on their link with pathogenesis.

RESULTS AND DISCUSSION

Single-Molecule Experiments Reveal the Presence of Distinct Oligomeric Species during α S Fibril Formation

We have previously demonstrated, using a model amyloidogenic protein, that it is possible to quantify the fraction of oligomers in a sample and estimate their size distribution using the two-color coincidence detection (TCCD) method in diffusion single-molecule (sm) fluorescence experiments (Orte et al., 2008). Here, we have applied the same principles to α S and have selected residue 90, at the C-terminal end of the central hydrophobic NAC region of α S, as an appropriate position to incorporate fluorophores (see Figure S1 available online). Although this residue has previously been tagged with

fluorescent probes to investigate the aggregation of α S (Thirunavukkuarasu et al., 2008), we nevertheless compared the aggregation behavior of α S tagged with the two fluorophores used in the TCCD experiments, Alexa Fluor 488 (AF488) and Alexa Fluor 647 (AF647), to that of the unlabeled protein and established that the presence of the dye molecules does not alter the fundamental features of the aggregation reaction or the appearance of the resulting fibrillar aggregates (see [Extended Experimental Procedures](#) for details).

We next performed a series of smTCCD experiments at different time points during the α S aggregation process. Equimolar mixtures of α S labeled with either AF488 or AF647 were incubated under conditions used previously to form fibrils in vitro (Wood et al., 1999), and aliquots were taken at a series of time points and then diluted rapidly by a factor of 10^5 to a final concentration of ~ 500 pM, which is suitable for sm analysis. In TCCD, the confocal volume is excited by the overlapped red (633 nm) and blue (488 nm) lasers, and oligomers are detected as coincident bursts of fluorescence and readily distinguished from single-label monomers once account is taken of chance coincidental background events (Orte et al., 2006). In this way, we were able to estimate the fraction of oligomeric species along the α S aggregation process, as well as track the time evolution of their size distribution.

To obtain additional information on the nature of the oligomers formed in the aggregation process, we explored the use of intermolecular fluorescence resonance energy transfer (FRET) measurements. The samples were irradiated by the blue laser alone (Figure 1B), that is, only AF488-labeled molecules were excited directly. We observed, however, that the AF647 dye was excited indirectly by FRET from adjacent AF488 fluorophores within the aggregated species, generating coincident fluorescence events in both the AF488 and AF647 emission channels (Figures 1C and S2A); monomers, by contrast, can only give rise to bursts of fluorescence in the AF488 emission channel. This approach therefore allowed us to quantify the relative number of oligomeric and monomeric species in each sample and explore if there are any differences in the FRET efficiency that would indicate differences in the oligomer structure.

This analysis was then applied to samples taken at different incubation times during the aggregation reaction, enabling us to characterize the populations of α S oligomeric species as a function of their FRET efficiency and size. Because size is determined indirectly from fluorescence measurements in confocal single-molecule techniques applied in solution, we refer to the apparent oligomer size throughout the paper, which is only an estimate of the real oligomer size, in part due to the different paths that an oligomer can take through the confocal volume, but it can be used to determine the differences in relative sizes between oligomeric species. Based on our previous method (Orte et al., 2008), we determined the apparent size of α S oligomers by comparing the intensity in the AF488 emission channel of the fluorescence burst due to a given oligomer with the average intensity value for the AF488-labeled monomer (fluorescence lifetime measurements confirm that there is no detectable fluorophore quenching in monomers or soluble oligomers; see [Extended Experimental Procedures](#)) after correcting for the average FRET efficiency of that particular oligomer (see [Experi-](#)

[mental Procedures](#)). Both the fraction of oligomers and their size distribution during aggregation were in very good agreement with those obtained by smTCCD measurements.

A representative set of smFRET results is shown in Figure 2A. The data have been analyzed to generate two-dimensional (2D) histograms, in which the distribution of apparent oligomer sizes is represented as a function of FRET efficiency. Incubation of monomeric α S under the aggregation-promoting conditions used here initially results in the formation of a series of small oligomers ($n \leq 10$) and then, after a period of a few hours, in a wide distribution of oligomers, ranging in size from dimers to species containing more than 100 monomers. Despite the highly heterogeneous size distribution of the oligomeric species, a relatively small range of FRET efficiency values is observed. From a visual inspection of the data (Figure 2B, top), the distribution of FRET values appears to vary with the size of the oligomers, revealing two dominant populations, one corresponding to those of small size with medium FRET values (0.4–0.7) and the other to larger species with higher FRET values (0.6–0.9). To analyze these populations in more detail, we classified the oligomers into three broad size groups, small (~ 2 –5 mers), medium (~ 5 –15 mers), and large (~ 15 –150 mers) (larger oligomers were excluded from the analysis, because too few were encountered to generate significant statistics), and fitted the FRET histograms for each group as Gaussian distributions (see [Extended Experimental Procedures](#) for a detailed description of the method).

Using this classification we detected four distinct distributions of oligomers, denoted as A_{small} , A_{med} , B_{med} , and B_{large} , where A and B are used to distinguish between species with mid- and high-FRET values, respectively, and the subscripts are used to distinguish between the different oligomer size groups. Further examination of the data revealed that the mean and the width of the FRET distributions for each oligomer group remained unchanged during the incubation time, allowing the data sets at the different times to be fitted globally (Figure 2C). To test the robustness of this approach, five repetitions of the entire aggregation experiment were carried out, and the same four populations with almost identical Gaussian parameters defining their FRET distributions were obtained in each case (see Table 1 and error bars in Figure 3A).

We also analyzed the formation of oligomers at a protein concentration (6 μ M) similar to that found under physiological conditions (Iwai et al., 1995). Interestingly, the two major structural types of oligomers detected at a concentration of 70 μ M were also found in the experiments carried out at this lower concentration (Figure S2B), although their absolute levels were much lower and no fibrils were detectable by transmission electron microscopy even after 7 days of incubation. Crucially, this finding suggests that at physiologically relevant concentrations (Iwai et al., 1995), α S is able to form potentially toxic oligomeric species.

Next, we set up a series of smFRET experiments on samples diluted in the presence of unlabeled α S and compared the number and type of oligomers detected with those found in samples diluted into buffer alone. The relative difference in the number of oligomers detected in the two cases provides an estimate of the relative kinetic stabilities of the oligomeric species upon dilution to single-molecule conditions. We

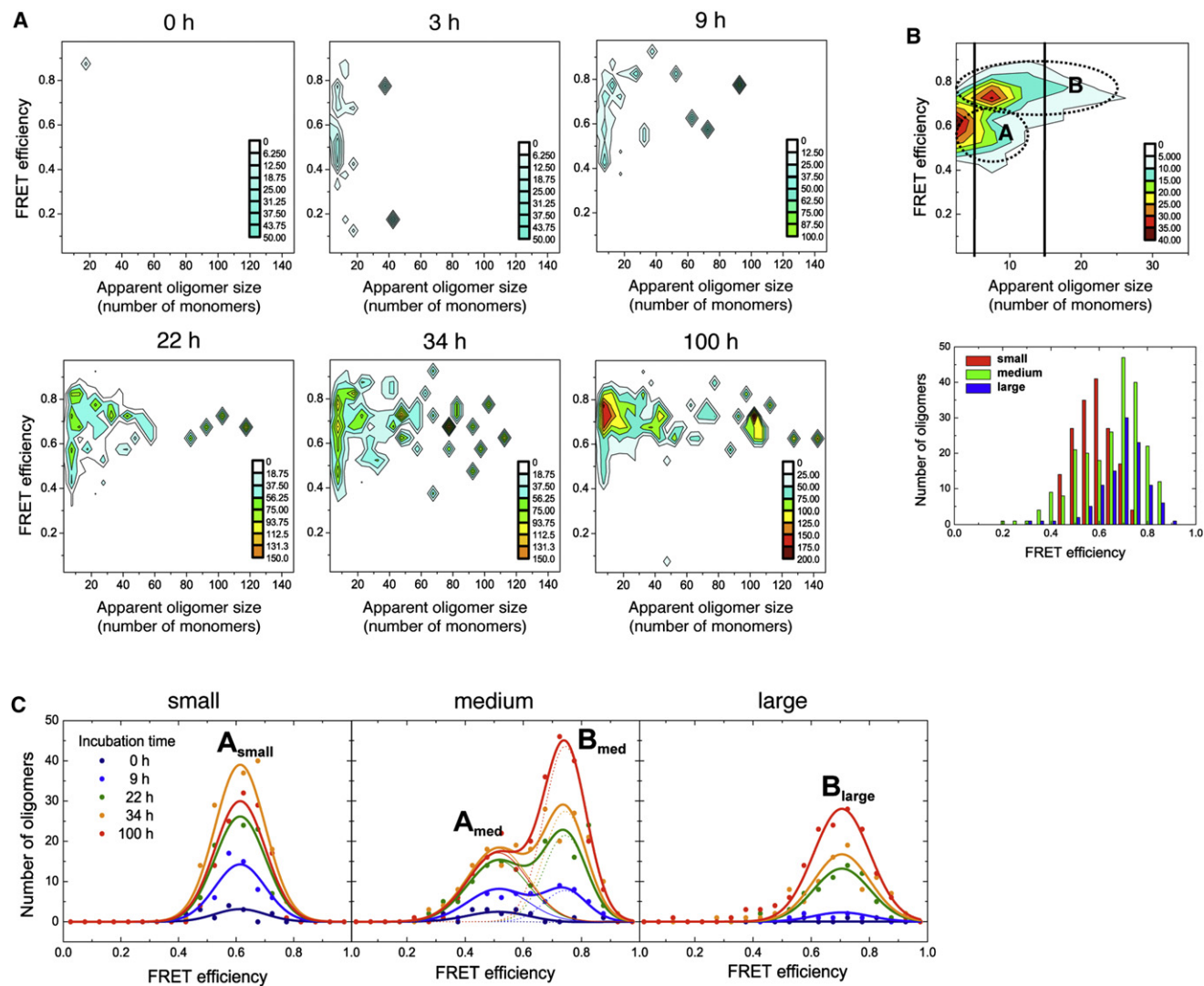


Figure 2. α S Oligomers Present during Fibril Formation

Aggregation of α S was followed by smFRET at a range of time points during incubation.

(A) 2D plots corresponding to the relative mass distribution of oligomers at different incubation times as a function of apparent oligomer size (x axis) and FRET efficiency (y axis); for a clearer visualization of the presence of large oligomers, the mass rather than the number distribution is represented.

(B) A representative 2D plot of the number distribution of oligomers after 60 hr of incubation illustrates the size-dependence of the FRET efficiency distributions (vertical lines corresponding to apparent oligomer sizes of 5- and 15-mer have been added as a visual guide), where two main FRET oligomer populations can be identified. The FRET-derived distributions based on small (red bars), medium (green bars), and large (blue bars) classification are plotted on the graph shown below.

(C) Representative global fits of the four size-derived FRET oligomer distributions to Gaussian functions as the incubation time varies. See also Figure S2.

detected the same FRET distributions and comparable numbers of oligomers in both types of experiments (data not shown), far from the difference of a factor of 10^5 expected from the dilution from bulk to single-molecule conditions if the oligomers were to dissociate rapidly at pM concentrations.

This assumption was further strengthened by the close agreement between the concentrations of oligomers detected under bulk conditions by quantitative size-exclusion chromatography (SEC) and those estimated from our single-molecule experiments and extrapolated to bulk conditions at different incubation times (Figure 1D). In the SEC analysis under bulk conditions, we

were able to quantify directly the concentrations of oligomers present at different incubation times by exploiting the high extinction coefficients of the fluorophores in the visible region of the spectra (Figure S1G). The mass fraction of soluble aggregates was found at all times to be very low ($\leq 5\%$), in agreement with a previous report (Conway et al., 2000), and the kinetics of oligomer formation were in good agreement with extrapolations from the single-molecule experiments (Figure 1D). The discrepancies at long incubation times between the two set of data can be attributed to the increasing contribution to the mass fraction of large oligomers formed at long incubation times,

Table 1. Kinetic Analysis of the Formation of Different Oligomeric Populations Classified by Apparent Size and FRET Efficiency during α S Aggregation and Fibril Formation

Oligomer Type	Apparent Growth Rate (hr^{-1})	Lag Time (hr)	Average FRET Efficiency
A_{small}	0.047 ± 0.019	0.2 ± 1.7	0.61 ± 0.02
A_{med}	0.041 ± 0.013	0.1 ± 1.0	0.53 ± 0.05
B_{med}	0.013 ± 0.005	2.7 ± 1.2	0.74 ± 0.03
B_{large}	0.013 ± 0.003	3.3 ± 1.4	0.68 ± 0.05

The apparent growth rate and the lag time were obtained from the quantification of each oligomer type obtained by smFRET experiments by fitting the time dependence of the population of each oligomer class to the function $y = A \cdot (1 - \exp(-(x - x_0) \cdot r))$, where A is the amplitude, r is the apparent growth rate, and x_0 is the lag time. The data and the fits to this function are plotted in Figure 3A (see Extended Experimental Procedures for a detailed explanation of the analysis). The errors reported for the kinetic parameters correspond to the fitting errors. The average FRET efficiency was obtained from a global analysis of the FRET distributions at the different incubation times (see Figure 2C). The values reported correspond to the mean and standard error of five repetitions.

which were excluded from the analysis in our smFRET experiments because of inefficient sampling of these species.

α S Oligomers Undergo Structural Reorganization prior to Fibril Growth

We next determined the kinetics of formation of all four types of oligomers observed in the smFRET experiments (Figure 2C). For simplicity, we fitted a single-exponential function coupled with a lag time (Figure 3A); the parameters obtained from the fitting are shown in Table 1. Oligomers A_{small} and A_{med} show similar kinetics, as do oligomers B_{med} and B_{large} (see Table 1), suggesting that the different FRET efficiencies report on two different structural types of oligomers. A longer lag time was observed for the formation of type B oligomers, suggesting that these species could result from a reorganization of type A oligomers, in agreement with previous suggestions that fibril formation by α S involves the sequential appearance of discrete oligomeric intermediates (Conway et al., 2000).

To gain further insight into the different structures of A and B oligomers, we measured the susceptibility of each to proteinase K degradation relative to that of the monomeric and fibrillar forms of the protein. The core of α S fibrils, like that of other amyloid structures, is highly resistant to proteinase K degradation (Miake et al., 2002; Neumann et al., 2002). We found that the fibrils labeled with fluorophores were completely resistant to degradation when treated with 0.4 $\mu\text{g}/\text{ml}$ proteinase K for 20 min at 37°C but were gradually degraded when the proteinase K concentration was increased from 0.4 to 10 $\mu\text{g}/\text{ml}$ (Figure S3B), in agreement with the proteinase K degradation profiles reported previously for α S fibrils both in vitro and in vivo (Neumann et al., 2002). In contrast, the monomeric form of the protein is highly susceptible to degradation in agreement with expectations in the light of its intrinsically disordered nature.

The resistance to degradation of A- and B-type oligomers was assessed by smFRET experiments after similar incubations at increasing proteinase K concentrations (see Extended Experimental Procedures and Figure S3A). By comparing the concen-

trations of proteinase K at which 50% of each protein species is degraded (C_m), we can conclude (Figure 3B) that type-A oligomers are as sensitive to proteinase K as the monomeric protein ($C_m = 0.05 \pm 0.09 \mu\text{g}/\text{ml}$, and $0.05 \pm 0.01 \mu\text{g}/\text{ml}$, respectively), whereas type-B oligomers are substantially more resistant to degradation ($C_m = 0.27 \pm 0.02 \mu\text{g}/\text{ml}$) although not as highly resistant as the mature amyloid fibril ($C_m = 1.1 \pm 0.3 \mu\text{g}/\text{ml}$). As resistance to proteolytic, and specifically proteinase K, digestion has been found generally to correlate with the degree of persistent structure and β sheet content of aggregated species (Kocisko et al., 1995; Miake et al., 2002; Nordstedt et al., 1994), our results strongly suggest that type-A oligomers lack persistent structure, although their structure cannot be random, because they give rise to a distinct FRET peak. In contrast, type-B oligomers are likely to contain a significant degree of β sheet structure. This conclusion is in agreement with previous studies of α S aggregation, where a progressive increase in β sheet structure was observed during α S aggregation before the formation of mature amyloid fibrils (Apetri et al., 2006).

Disaggregation of α S Fibrils Leads to the Release of Oligomeric Species

A number of studies have shown directly or indirectly that amyloid fibrils are able to disaggregate, for example, as a result of changes in pH (Picotti et al., 2007) or addition of chemical denaturants (Calamai et al., 2005; MacPhee and Dobson, 2000). We therefore set out to probe the disaggregation of α S fibrils under near-physiological conditions by incubating preformed labeled α S fibrils at 37°C in monomer-free buffer (see Experimental Procedures) and carrying out a series of smFRET experiments over a period of several weeks (Figure 4). There was greater variability in the data from these experiments than from the aggregation experiments, an observation attributable to a significant variability in the degree of maturation of the fibrils in the different samples. We observed in all cases that, however, disaggregation results in species with a wide variation in size (Figure 4), indicating that monomeric, as well as oligomeric, species detach from the fibrils; after longer incubation times, however, these oligomers were found to have dissociated into monomers (see Figures 4, S4A, and S4B). Interestingly, a high fraction of oligomers formed at the beginning of the disaggregation experiment possesses FRET efficiencies similar to the type-B oligomers that are formed in the late stages of aggregation (see Table S1).

Because standard confocal smFRET techniques applied in solution have limitations in the detection of large species, we used single-molecule total internal reflection fluorescence (smTIRF) experiments to probe further the nature of the aggregates generated by fibril disaggregation and in the late stages of the aggregation process (see Extended Experimental Procedures and Figures S4C–S4F). Under the latter conditions, two FRET distributions were observed, corresponding to the two distributions detected in solution by smFRET experiments (type A and B), whereas in the fibril disaggregation sample, at early incubation times, the data show essentially a single distribution at high-FRET efficiencies, corresponding to type-B oligomers. Thus, the TIRF experiments confirm that fibrils initially disaggregate largely into type-B oligomers, a finding

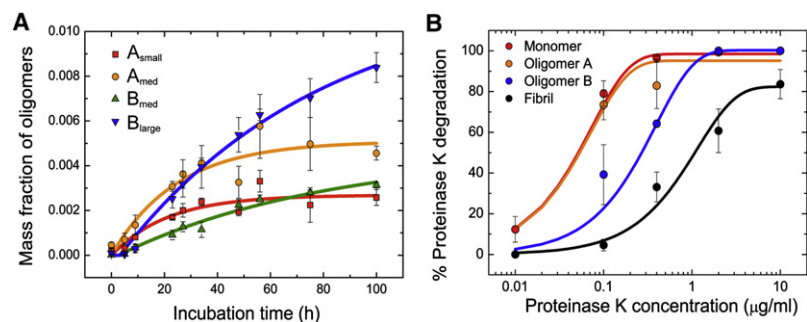


Figure 3. Characterization of the Different Oligomeric Species Formed during α S Aggregation and Fibril Formation

(A) The time dependence of the mass fraction of the four oligomeric distributions A_{small} (red squares), A_{medium} (orange circles), B_{medium} (green triangles), and B_{large} (blue triangles). The data shown correspond to the average and standard error of five different experiments.

(B) Proteinase K degradation curves of the different protein species (monomer in red, type A oligomer in orange, type B oligomer in blue, and fibrils in black). The data shown here correspond to the average and standard error of three different experiments. See also Figure S3.

that strongly supports the view that the latter possess a β sheet structure that could resemble that found in the amyloid state. Moreover, the fact that both types of oligomers are detected in fibril disaggregation experiments provides further evidence for a structural conversion between the two types of oligomers and that such a conversion is likely to be a key step in fibril formation.

Kinetic Analysis of the Early Stages of Amyloid Fibril Formation

We then set out to determine the rate constants for the reactions associated with the early stages of aggregation using our smFRET experiments. For this purpose, we needed a kinetic model that could explain the following experimental observations: (1) a lag phase in the formation of fibrils; (2) the presence of two different structural types of oligomers; and (3) a lag phase in the appearance of type-B, but not type-A, oligomers.

A general nucleation and growth model can explain the lag phase observed in bulk α S fibril formation experiments. This model then needs to include a conformational conversion step to take account of the two types of oligomeric states observed during aggregation and of the lag phase for type-B oligomers. The simplest model that is consistent with these observations is a nucleation and growth model with a conformational conversion step, analogous to that used to describe the conformational conversion mechanism suggested for prions (Serio et al., 2000). We therefore extended the analytical solution that we have generated to interpret the kinetic rate equations for a nucleation and growth model (Knowles et al., 2009) by the addition of a conversion step. As shown in Figure 5A, this model assumes that primary nucleation results in the creation of oligomers of type A from monomeric protein molecules. Type-A oligomers can grow through monomer addition, but they can also convert into type-B oligomers, indicating that multiple parallel pathways could result in the formation of type-B oligomers of a given size; details of the early-time analytical and exact numerical solutions of the model are presented in the Supplemental Information.

This model was then used to fit the experimental kinetic data describing the formation of the two types of oligomers over the first 30 hr of incubation, that is, during the lag phase observed in the kinetics of formation of fibrils under bulk conditions; as shown in Figure 5B, the model fits the data well. At later times, additional events, such as the formation of large fibrillar species, the contribution of the reverse processes involving conversion of type-B oligomers into type-A oligomers, and the dissociation of type-A oligomers, become significant and result in underfitting

(see Extended Experimental Procedures). Overall, therefore, this kinetic description in terms of the dominant events associated with both fibril formation and disaggregation enables the data obtained from single-molecule experiments to be understood on the basis of a small number of well-defined microscopic parameters, the values of which can be determined directly from the experimental data. Thus, the primary nucleation rate constant for oligomers of type A can be defined as $\sim 4 \times 10^{-8} \text{ s}^{-1}$ and the conversion rate constant from type-A to type-B oligomers as $\sim 5 \times 10^{-6} \text{ s}^{-1}$. Note that, because the number of monomeric species able to participate in primary nucleation greatly exceeds the number of oligomers that are involved in the structural conversion, the overall primary nucleation rate is larger than the conversion rate, a situation that results in the observed build-up of the type-A oligomers during the aggregation process.

A particularly interesting result is that the rate of structural conversion between the two types of α S oligomers determined from the analysis corresponds to a half-time of ca. 35 hr, a time which is very long compared, for example, with the time required for the folding of small single-domain globular proteins, the characteristic times for which range from microseconds to seconds (Jackson, 1998). The slow rate indicates that the energy barrier for the conversion from a relatively unstructured oligomer to a β sheet rich oligomer is very high, an observation that can be attributed in part to the multiple permutations of interstrand hydrogen bonding interactions and in part to the fact that the β sheet rich species are likely to have a significant fraction of their hydrophobic side chains exposed to the solvent as found in amyloid fibrils (Heise et al., 2005; Vilar et al., 2008). Furthermore, previous studies of the folding kinetics of pertactin, a protein that has an elongated parallel β -helix shape resembling that of an amyloid fibril, found a half-time for refolding of 3 hr (Junker and Clark, 2010), only an order of magnitude faster than the rate of reorganization of oligomeric α S into intermolecular β sheet structures, suggesting that the intrinsic formation of an amyloid-like structure proceeds through an energy landscape with significant barriers between different regions of conformational space, in contrast to the smooth funnel-like landscape found for the majority of small naturally evolved proteins (Onuchic et al., 1997; Dinner et al., 2000).

PK-Resistant Oligomers Induce Higher Aberrant Levels of ROS in Cells Than Do PK-Sensitive Oligomers

Given the long-standing pathophysiological link between increased levels of oxidative stress and PD (Jenner and Olanow,

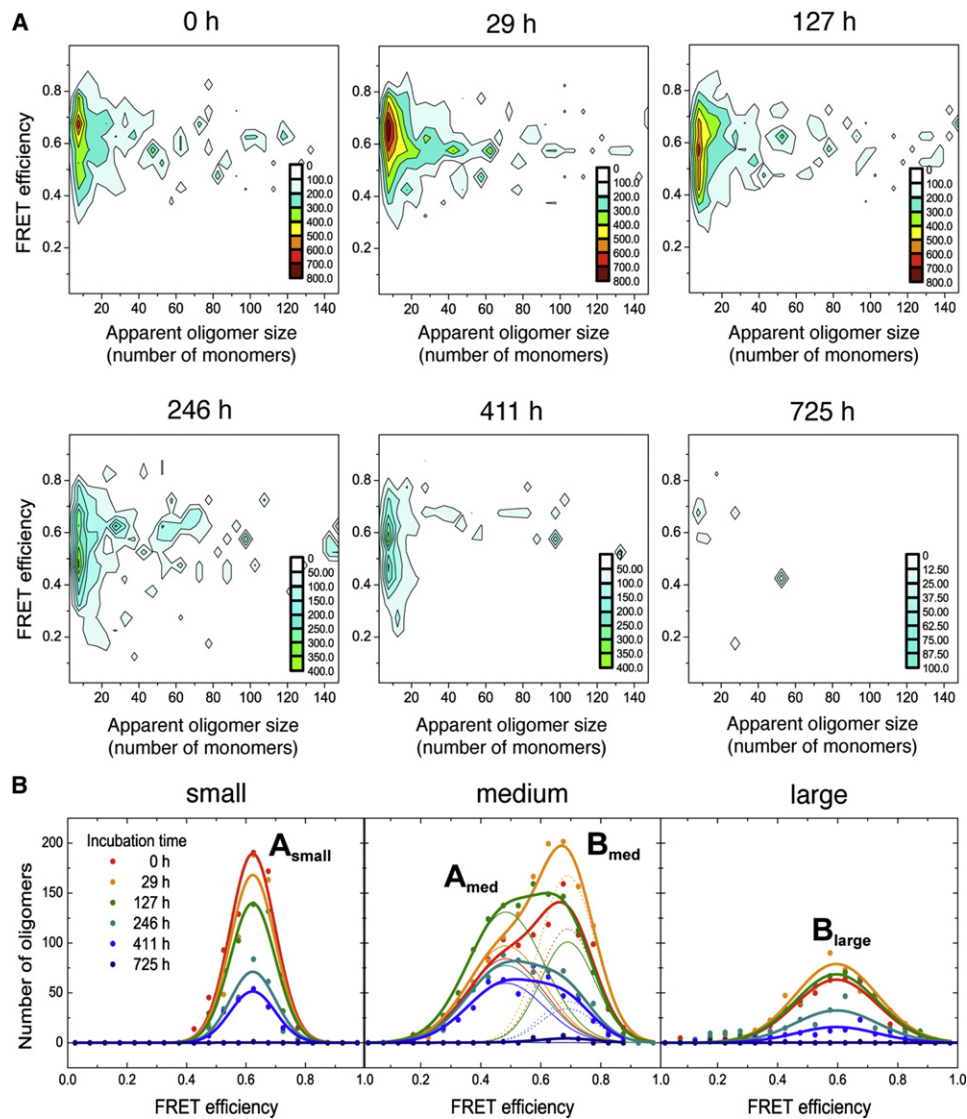


Figure 4. α S Fibril Disaggregation

(A) 2D plots of the relative mass distribution of oligomers according to apparent size and FRET efficiency, as explained in Figure 2A.

(B) Representative global fits of the size-derived FRET oligomer distributions as a function of the incubation time. See also Figure S4 and Table S1.

1998; Jenner, 2003) and the reported specific generation of intracellular reactive oxygen species (ROS) by aggregating proteins implicated in neurodegenerative diseases (Tabner et al., 2001; Parihar et al., 2009), we examined if the production of ROS changed on exposure of rat primary midbrain neurons to samples containing different α S species, using samples from identical aggregation experiments to those described above. Two samples containing a mixture of monomeric and oligomeric species with different concentrations of type-A and type-B oligomers were obtained by taking aliquots of an aggregating sample at two different incubation times. The first sample was enriched in type-A oligomers (denoted as the “oligomer A sample”) and was obtained after 20–25 hr of incubation; it contained ca. 200 nM of type-A oligomers and ca. 50 nM of type-B oligomers (concentrations are in number of species) in

the presence of 70 μ M of monomers. The second sample (denoted the “oligomer B sample”), taken after 70 hr of incubation, contained ca. 200 nM of type-A oligomers and ca. 150 nM of type-B oligomers in the presence of 50 μ M of monomers. In both cases, fibrillar species were removed from the sample by centrifugation. In addition, we took an aliquot from the sample before initiating the aggregation as a monomeric control sample (at a concentration of 70 μ M) and another aliquot at the end of the aggregation as a fibrillar control sample. The latter was centrifuged and the fibrillar material resuspended in the same volume as that of the initial sample, which contained ca. 200 nM of fibrils (in number concentration).

We first assessed the ability of cells to take up the different forms of α S using confocal microscopy; the rate of uptake of the differently labeled α S species was calculated by measuring

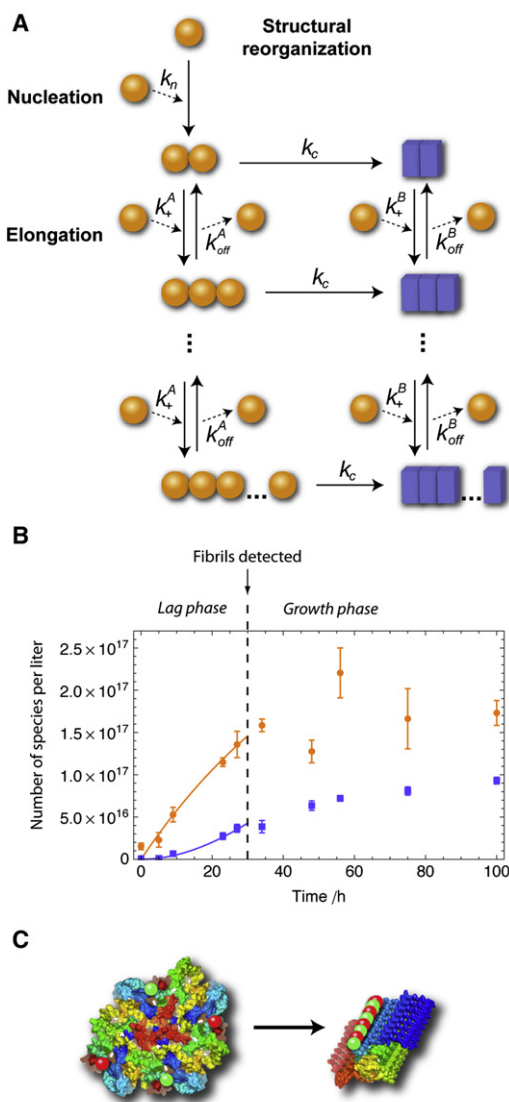


Figure 5. Kinetic Analysis of α S Oligomerization

(A) Scheme for the minimalistic kinetic model used to fit the early stages of α S aggregation.

(B) Results of the global fitting (continuous lines) of the kinetics of formation of the two types of oligomeric species estimated under bulk conditions from smFRET experiments. Data for type A oligomers and type B oligomers are shown as orange circles and blue squares, respectively (average and standard error of five different experiments). The vertical dashed line is at 30 hr, corresponding to the lag phase of fibril formation estimated under bulk conditions, up to which time our model accounts well for the different microscopic processes governing the aggregation reaction.

(C) Cartoon showing the conversion of an 8-mer of α S from a collapsed to an ordered proteinase-K-resistant form. Residues of each monomer are colored according to their location in the amino acid sequence. The average distance between fluorophores, represented as green and red spheres, is different for each type of oligomer and hence gives rise to different average FRET efficiencies.

the increase in the fluorescent intensity signal within the cell body (Figures 6A and S5). We observed a rapid (between 6–8 min) and efficient uptake of all α S species into both neurons

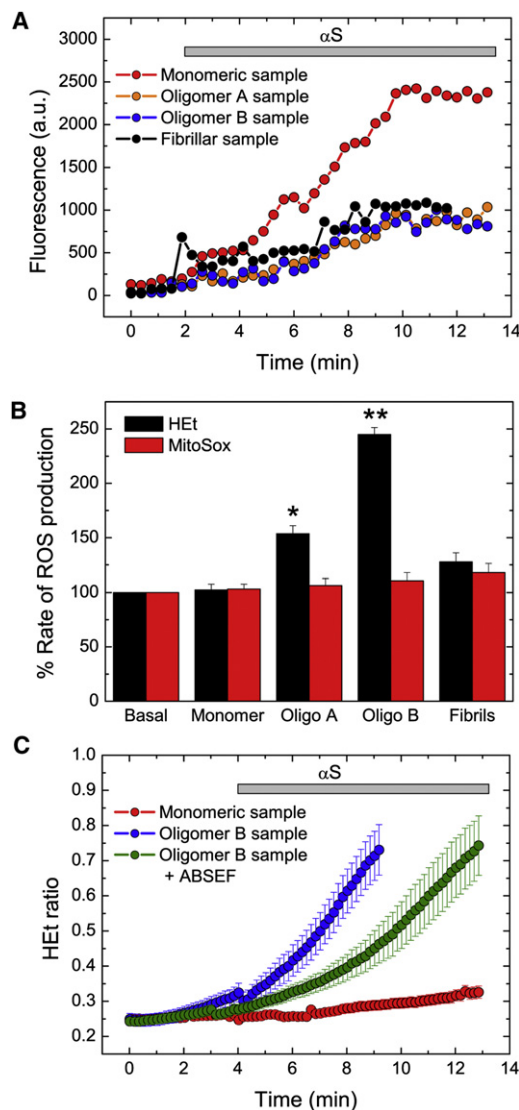


Figure 6. Evaluation of the Pathophysiological Effects of the α S Species on Rat Primary Neuronal Cultures

(A) Representative traces showing the rate of uptake of the different types of α S species into the cell body. The fluorescence of AF488-labeled α S was used to track monomeric α S uptake, whereas the fluorescence emission of AF647-labeled α S was used to follow the uptake of oligomeric and fibrillar forms of the protein (see also Figure S5), as only these species are able to produce FRET signals.

(B) Bar chart displaying cytoplasmic (HET) and mitochondrial (MitoSox) ROS production induced in cells after exposure to each α S sample (see “PK-Resistant Oligomers Induce Higher Aberrant Levels of ROS in Cells than Do PK-Sensitive Oligomers” section in the main text for a detailed explanation of the composition of each sample). *p < 0.05; **p < 0.005.

(C) Representative cellular traces showing the slower production of ROS induced by type B α S oligomeric samples after cellular treatment with ABSEF. Error bars in (B) and (C) represent SEM.

and astrocytes. The exposure of the cells to the α S species had no detectable effect on cell viability over 24 hr. For the monomeric protein, the time for maximal uptake reported here

corresponds to the time to reach saturation in the quantity of internalized protein, whereas for oligomeric and fibrillar species, due to the reduced quantity of these species in the corresponding solutions, it represents the time the cells needed to take up the total number of FRET-positive species added to the cellular media (see Figure S5).

For cytosolic ROS assessment, cultures were briefly incubated in the presence of hydroethidium (HET) prior to exposure to α S samples, whereas cultures were preincubated with MitoSOX (the mitochondrially targeted equivalent of HET) for mitochondrial ROS measurements. Exposure of the cultures to 10 μ l monomeric or fibrillar α S samples had a marginal and insignificant effect on cytoplasmic and mitochondrial ROS production (Figure 6B). By contrast, exposure of the neuronal cultures to the type-B oligomeric samples, and to a lesser degree to the type-A oligomeric samples, induced a specific, rapid, and significant increase in cytoplasmic ROS production (Figures 6B and 6C; $p < 0.05$ for type-A oligomeric samples and $p < 0.001$ for type-B oligomeric samples, respectively). The lack of ROS production from the mitochondria, which are the primary source of ROS in cells, suggests that the excessive ROS production observed in neurons and astrocytes is induced directly by the oligomeric α S species. In support of this conclusion, application of 20 μ M ABSEF, a potent NADPH oxidase inhibitor, which is the main producer of ROS in both cytosol and mitochondria, was initially able to delay but not completely block the aberrant ROS production induced through uptake of the type-B oligomeric sample (Figure 6C; $n = 67$ cells).

Overall, therefore, these experiments show that the PK-resistant α S soluble oligomers induce much higher levels of ROS in cells than do the PK-sensitive oligomers, suggesting that the different oligomeric structures have different physiological effects. Moreover, our results are in excellent agreement with a recent study of a model protein that showed that only the addition of oligomers exposing hydrophobic surfaces to the cellular media resulted in an increase in intracellular ROS production (Zampagni et al., 2011) and in agreement with previous theoretical and experimental observations in which the degree of hydrophobic exposure in oligomeric species has been directly linked to cellular dysfunction (Bolognesi et al., 2010; Campioni et al., 2010; Cheon et al., 2007).

Conversion to Proteinase-K-Resistant Oligomers Is the Key Step in Aggregation

Of particular importance in this study has been the identification of a slow conversion from the initially formed and readily degradable oligomers to compact and highly structured oligomers that are likely to contain amyloid-like β sheet structure and have a significant fraction of the hydrophobic side chains exposed to the solvent. These latter oligomers induce much higher levels of ROS in cells than do their precursor oligomers, indicating that they are more damaging to cells as aberrant ROS production has been shown to activate apoptotic cascades that result in the death of dopaminergic neurons (Jenner and Olanow, 1998; Jenner, 2003). At the same time, increased ROS levels are thought to accelerate additional α S oligomer formation (Maries et al., 2003), reflecting the positive feedback between such processes, which can explain the cascade of events causing the development of

disease. These results also suggest that the rate of the structural conversion relative to other biologically relevant processes will be a crucial factor in controlling the accumulation of damaging and degradation resistant oligomeric species. It is interesting to note in this regard that this slow structural conversion is on the same timescale as the half-life for α S turnover in vivo reported previously (Cuervo et al., 2004; Okochi et al., 2000). Together with the fact that the initially formed oligomers are highly degradable, this result indicates that there is a significant period of time for the cellular protein degradation machinery to operate and potentially time for therapeutic intervention, prior to the onset of cellular damage. Our results also indicate that stable oligomers of α S can be formed at physiologically relevant concentrations, and once even a relatively small number of β sheet oligomers are formed and free to react, the aggregation reactions can be accelerated dramatically. These findings highlight the key role that molecular chaperones and the cellular protein degradation machinery must play continually in preventing damage caused by such oligomers and hence the onset of disease (Balch et al., 2008; Morimoto, 2008).

When fibrils of α S are incubated in the absence of monomeric protein molecules, disaggregation occurs to produce the same type of oligomers as those found in the aggregation reaction, in particular damaging PK-resistant oligomers, which indicates that fibrils could act as a source of soluble species that are potentially pathogenic to neuronal cells and whose concentrations are likely to be high in the vicinity of the fibrils. This conclusion suggests that toxic agents are not generated just in the early stages of the aggregation process but also as a result of the disaggregation of amyloid fibrils, a crucial finding that needs to be taken into account when considering the origin and spread of neurodegenerative diseases and when designing therapeutic strategies, especially those based on the disaggregation of fibrils. Indeed, our results support the hypothesis that fibrils can act either to sequester misfolded potentially toxic species (Lansbury, 1999) or, depending on the conditions, to release them into the local environment, thereby unifying two apparently distinct views drawn from observations of protein aggregation in model organisms (Cohen et al., 2006).

EXPERIMENTAL PROCEDURES

Sample Preparation

The A90C mutant variant of α S was purified as a monomeric fraction from *Escherichia coli* as described previously (Hoyer et al., 2002) and labeled with either maleimide-modified AF488 or AF647 dyes (Invitrogen, Carlsbad, CA, USA) via the cysteine thiol moiety as previously reported (Thirunavukkuarasu et al., 2008). The labeled protein was purified from the excess of free dye by a P10 desalting column with Sephadex G25 matrix (GE Healthcare, Waukesha, WI, USA), concentrated using Amicon Ultra Centrifuges (Millipore, Billerica, MA, USA), divided into aliquots, flash frozen, and stored at -80°C . Each aliquot was thawed immediately and used only once. The degree of monomeric protein in the stock solution was assayed by smFRET as described in the Extended Experimental Procedures.

For the aggregation reactions, equimolar concentrations of the AF488- and AF647-labeled A90C α S in Tris 25 mM (pH 7.4) and 0.1 M NaCl (with 0.01% NaN_3 to prevent bacterial growth during the experiments) were mixed to give a final volume of 300 μ l, bringing the total protein concentration to 1 mg/ml (70 μ M). The solutions were incubated in the dark at 37°C , with constant agitation at 200 rpm for 4–8 days, during which time aliquots were taken. At each

time point, a 2 μl aliquot was diluted 10^5 -fold by serial dilution with 0.022 μM -filtered buffer (Tris 25 mM [pH 7.4] and 0.1 M NaCl) for smFRET analysis at room temperature. Glass slides were treated by incubation for 1 hr with BSA at 1 mg/ml to prevent αS aggregates from adsorbing to the surface (as shown by TIRF, see [Extended Experimental Procedures](#)), and immediately after removal of the BSA solution, 500 μl of diluted sample was placed on the slide for analysis.

For the disaggregation experiments, fibrils formed from a mixture of protein labeled with each fluorophore were washed by two cycles of centrifugation and resuspension in 500 μl of fresh buffer. Then, 300 μl of fresh monomer-free buffer solution was added to the fibrillar pellet, which was then gently resuspended and incubated at 37°C without shaking for 4 weeks. The total protein concentration in the fibril disaggregation samples was estimated to be $\leq 1 \mu\text{M}$.

Single-Molecule Data Collection and Analysis

The instrumentation used for TCCD and smFRET experiments has been described in detail previously (Orte et al., 2006). For smFRET, only the 488 nm laser was used to excite the sample (Figure 1B), with a total laser power of 47.5 μW . PC-implemented multichannel scalar cards (MCS-PCS, Ortec) were used to collect photon counts from two avalanche photodiode detectors with 1 ms bin times over 8,000 channels on both MCS cards. Typically, 999 frames of 8,000 ms were collected for a total measurement time of 3 hr per aliquot. During the measurements, the microscope stage was moved at a constant scanning rate of 200 $\mu\text{m/s}$ by two orthogonal DC motors (M-112.IDG, Physik Instrumente) so that the encounter rate does not depend on the size of the observed species. The photon time traces were analyzed by first setting an optimized threshold value for each channel under our conditions of measurement to remove the background noise: a 20 photon/ms bin for the donor channel and a 10 photon/ms bin for the acceptor channel. We analyzed the event data to derive the distributions of oligomer sizes and FRET values. Based on the average intensity from a blue monomer, the approximate number of monomers per oligomer event can be extracted after correcting the blue intensity of the oligomer for the fact that it is reduced by FRET and also taking into account the fact that 50% of the monomers are donors (confirmed by TCCD experiments, see [Extended Experimental Procedures](#)), using the following equation:

$$\text{Apparent oligomer size} = 2 \cdot \left(\frac{I_{DA} + I_A / \gamma}{I_{D_monomer}} \right), \quad (\text{Equation 1})$$

where I_A and I_{DA} correspond to the acceptor fluorescence intensity and to the donor fluorescence intensity in the presence of acceptor, respectively; $I_{D_monomer}$ corresponds to the average intensity of donor monomers (ca. 30 counts), and γ corresponds to a correction factor that accounts for different quantum yields and detection efficiencies of the donor and acceptor ($\gamma = 0.26$). We chose a relatively large oligomer bin size of five (i.e., 2–5 mers, 5–10 mers, etc.), both to present and analyze the data.

The FRET efficiency for each oligomer was calculated from the following expression:

$$E_{\text{FRET}} = \frac{I_A}{\gamma I_{DA} + I_A}. \quad (\text{Equation 2})$$

Because oligomers of a wide distribution of sizes were observed, a significant number of oligomer events occupied more than one time bin (1 ms). For these cases, the maximum brightness recorded per bin (i.e., the brightness recorded during 1 ms when the oligomer crosses the center of the confocal volume) was used to calculate both their size and FRET efficiency. Large species, that is, those corresponding to events occupying more than five bins or calculated to be more than 150 mers, were assumed to be fibrillar species and then were excluded from the analysis.

A full description of the methodology used in this study is presented in the [Extended Experimental Procedures](#).

Live-Imaging Studies

Confocal images were obtained using a Zeiss 710 vis CLSM equipped with a META detection system and a 40 \times oil immersion objective. The 488nm

Argon laser line was used to excite AF488-labeled αS and fluorescence emission collected between 505–550 nm (green channel) and 630–650nm (red channel). FRET measurements (excitation of AF488 and emission of AF647) were used to quantify the uptake of oligomeric and fibrillar αS because monomers are unable to undergo FRET. Illumination intensity was kept to a minimum (at 0.1%–0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of $\sim 2 \mu\text{m}$. We recorded from a single focal plane and specifically selected regions within the cell to measure cellular uptake of labeled αS species. For confirmation, we checked that the recorded αS signal was intracellular at the end of each experiment by z-stack scanning and three-dimensional reconstruction using Zeiss software.

ROS Measurements

Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with a 20 \times fluorite objective. For HET and MitoSOX measurements, a ratio of the oxidized/reduced form was measured: Excitation at 540 nm and emission recorded above 560 nm were used to quantify the oxidized form (ethidium), whereas excitation at 360 nm and emission collected from 405 to 470 was used for the reduced form (hydroethidium). All data reported in this study were obtained from at least five coverslips and 2–3 different cell and sample preparations.

For measurement of mitochondrial ROS production, cells were preincubated with MitoSOX (5 μM ; Molecular Probes, Grand Island, NY, USA) for 10 min at room temperature. For measurement of cytosolic ROS production, dihydroethidium (2 μM) was present in the solution during the experiment. No preincubation (“loading”) was used for dihydroethidium to limit the intracellular accumulation of oxidized products. Animal husbandry and experimental procedures were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at [doi:10.1016/j.cell.2012.03.037](https://doi.org/10.1016/j.cell.2012.03.037).

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