A sensitive assay reveals structural requirements for α-synuclein fibril growth

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The accumulation of α-synuclein (α-syn) fibrils in neuronal inclusions is the defining pathological process in Parkinson’s disease (PD). A pathogenic role for α-syn fibril accumulation is supported by the identification of dominantly inherited α-syn (SNCA) gene mutations in rare cases of familial PD. Fibril formation involves a spontaneous nucleation event in which soluble α-syn monomers associate to form seeds, followed by fibril growth during which monomeric α-syn molecules sequentially associate with existing seeds. To better investigate this process, we developed sensitive assays that use the fluorescein arsenical dye FlAsH (fluorescein arsenical hairpin binder) to detect soluble oligomers and mature fibrils formed from recombinant α-syn protein containing an N-terminal bicysteine tag (C2-α-syn). Using seed growth by monomer association (SeGMA) assays to measure fibril growth over 3 h in the presence of C2-α-syn monomer, we observed that some familial PD-associated α-syn mutations (i.e. H50Q and A53T) greatly increased growth rates, whereas others (E46K, A30P, and G51D) decreased growth rates. Experiments with wild-type seeds extended by mutant monomer and vice versa revealed that single-amino acid differences between seed and monomer proteins consistently decreased growth rates. These results demonstrate that α-syn monomer association during fibril growth is a highly ordered process that can be disrupted by misalignment of individual amino acids and that only a subset of familial-PD mutations cause fibril accumulation through increased fibril growth rates. The SeGMA assays reported herein can be utilized to further elucidate structural requirements of α-syn fibril growth and to identify growth inhibitors as a potential therapeutic approach in PD.

Parkinson’s disease (PD) is a debilitating, progressive neurodegenerative disorder characterized by impaired movement.
faster aggregation than WT α-syn, whereas one study reported a longer lag time relative to WT (31–34). Several studies have investigated interactions between mutant and WT α-syn proteins during fibril formation, with mixed results as to how they influence α-syn fibril accumulation in individuals with dominantly inherited PD (21, 34–36).

To further characterize structural determinants and mechanisms underlying fibril formation, we developed assays that use the biarsenical dye fluorescein arsenical hairpin binder (FlAsH) to detect the association of two or more α-syn monomers containing bicysteine tags. FlAsH is a profluorescent biarsenical dye that binds to short peptides containing four cysteine residues (37, 38). The intermolecular association of two peptides can also be monitored by biarsenical dyes when each of the two peptides contains a peptide tag with two cysteine residues (39–42). In this bipartite tetracysteine approach, close association (~10 Å) of the bicysteine tags in assembled complexes is required to bind FlAsH and induce fluorescence (40).

We found that FlAsH has high sensitivity and specificity for the detection of both soluble oligomeric species and fibrils formed by bicysteine-tagged α-syn (C2-α-syn). Additionally, the high sensitivity of FlAsH for oligomeric and fibrillar α-syn can be utilized to establish novel seed growth by monomer association (SeGMA) assays, which measure the rate at which monomer is consecutively added to preformed seeds consisting of either soluble oligomers or fibrils. We examined the effects of α-syn mutations on seed growth rates and found that some PD-associated mutations significantly increase the rate, whereas others significantly decrease the rate. Furthermore, in SeGMA assays in which the sequence of monomer protein differs from the sequence of seeds, we observed that single-amino acid mismatches between seeds and monomer frequently decrease the rate of fibril growth.

Results

**Bipartite tetracysteine tags enable detection of α-syn fibril formation**

To determine whether the formation of α-syn fibrils can be detected with a bipartite tetracysteine reporter, we produced recombinant human C2-α-syn protein.

We incubated native α-syn and C2-α-syn protein at 37 °C with shaking to form fibrils. The temporal profiles of fibril formation using native α-syn versus C2-α-syn, measured by ThioT fluorescence, were very similar (Fig. 1A), indicating that the bicysteine tag does not alter the rate of fibril formation. We next examined the ability of the FlAsH dye to detect fibril formation and observed a robust increase in fluorescence upon formation of C2-α-syn amyloid fibrils, which paralleled the increase in ThioT fluorescence (Fig. 1B). In contrast, FlAsH fluorescence did not increase in the presence of native WT α-syn fibrils. We further characterized FlAsH detection of C2-α-syn fibrils by combining 25 nM FlAsH with increasing concentrations of C2-α-syn fibrils and measuring fluorescence. Analysis of the data with a saturation binding model indicated that the average apparent affinity constant \( K_{\text{app}} \) for association of FlAsH with C2-α-syn fibrils is 27 μg/ml (Fig. 1C). Atomic force microscopy (AFM) verified that native α-syn and C2-α-syn fibrils have similar morphology (Fig. 1, D–G), providing further evidence that the C2 tag does not significantly alter the α-syn fibril formation process. Fibril formation reactions contained freshly added dithiothreitol (DTT) to reduce disulfides formed by cysteine residues. In our early fibril formation experiments performed in the absence of DTT, C2-α-syn formed fibrils at a slightly faster rate compared with native α-syn (data not shown). When DTT was added in subsequent experiments, rates were more similar, as shown in Fig. 1A.

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**Figure 1. Bipartite tetracysteine detection of C2-α-syn fibril formation using FlAsH dye.**

A, similar kinetic profiles are observed when ThioT is used to monitor fibril formation by WT α-syn and WT C2-α-syn. B, formation of fibrils by WT C2-α-syn can be measured by FlAsH fluorescence and displays a profile similar to that observed with ThioT. C, a saturation binding assay using increasing concentrations of C2-α-syn fibrils incubated with 25 nM FlAsH indicates a \( K_{\text{app}} \) of 23 μg/ml of FlAsH to C2-α-syn fibrils. D–G, AFM analysis shows similar morphology of WT α-syn fibrils (D and E) and WT C2-α-syn fibrils (F and G) formed following incubation for 72 h. Similar results were observed in three independent experiments (mean ± S.E. (error bars), n = 3).
FlAsH detects soluble α-syn oligomers

To characterize soluble oligomeric α-syn species, we incubated native α-syn and C2-α-syn monomer at 37 °C for 32 h, centrifuged the samples at 100,000 × g, and then fractionated the supernatants using SEC. Western blot (WB) analysis demonstrated that α-syn elutes from the column in two distinct peaks: a high-\(M_r\) oligomer peak eluting near the void volume (relative \(M_r = 1,100,000\)) and a lower-\(M_r\) peak eluting at a relative \(M_r\) of 59,000 (Fig. 2, A–C). SEC analysis of recombinant α-syn that was not previously incubated at 37 °C demonstrated only the low-\(M_r\) peak. WB analysis indicated that native α-syn and C2-α-syn produced similar levels of high-\(M_r\) oligomer species when incubated at 37 °C for 32 h.

We analyzed the C2-α-syn SEC fractions in the FlAsH fluorescence assay. We observed increased fluorescence exclusively in high-\(M_r\) fractions, corresponding to the α-syn identified by WB in these fractions. Despite the presence of substantially higher levels of α-syn in WB analysis of the low-\(M_r\) fractions, there was no increase in FlAsH fluorescence above baseline, confirming both the specificity of the FlAsH assay for high-\(M_r\) oligomers and the absence of high-\(M_r\) oligomers in the low-\(M_r\) fractions.

FlAsH detects soluble α-syn oligomers

Samples of WT α-syn and WT C2-α-syn monomer incubated for 32 h were centrifuged at 100,000 × g, and supernatants were fractionated by SEC. Western blot analysis for α-syn in SEC fractions revealed α-syn oligomers in early, high-\(M_r\) fractions, in addition to α-syn monomer in late, low-\(M_r\) fractions (not shown). Similar amounts of oligomeric α-syn are present in high-\(M_r\) fractions from WT α-syn (A) and WT C2-α-syn (B) reactions. Oligomeric α-syn species are detected by FlAsH fluorescence in high-\(M_r\) fractions. The specificity of FlAsH for oligomeric species is demonstrated by the absence of FlAsH fluorescence in low-\(M_r\) fractions containing high levels of α-syn monomer detected by WB. D, FlAsH detects oligomeric species only after incubation of purified α-syn monomer, and ThioT has poor sensitivity for detecting soluble oligomers present in SEC fractions. E, FlAsH analysis of SEC fractions demonstrates progressive accumulation of oligomeric species with increasing incubation time. F, calibration curve obtained by fractionating \(M_r\) standards on the SEC column. G–J, AFM analysis of WT α-syn (G and H) and WT C2-α-syn (I and J) oligomers show similar morphology. Similar results were observed in two independent experiments.

Figure 2. FlAsH detects soluble α-syn oligomers. Samples of WT α-syn and WT C2-α-syn monomer incubated for 32 h were centrifuged at 100,000 × g, and supernatants were fractionated by SEC. A and B, Western blot analysis for α-syn in SEC fractions reveals α-syn oligomers in early, high-\(M_r\) fractions, in addition to α-syn monomer in late, low-\(M_r\) fractions (not shown). Similar amounts of oligomeric α-syn are present in high-\(M_r\) fractions from WT α-syn (A) and WT C2-α-syn (B) reactions. C, oligomeric α-syn species are detected by FlAsH fluorescence in high-\(M_r\) fractions. The specificity of FlAsH for oligomeric species is demonstrated by the absence of FlAsH fluorescence in low-\(M_r\) fractions containing high levels of α-syn monomer detected by WB. D, FlAsH detects oligomeric species only after incubation of purified α-syn monomer, and ThioT has poor sensitivity for detecting soluble oligomers present in SEC fractions. E, FlAsH analysis of SEC fractions demonstrates progressive accumulation of oligomeric species with increasing incubation time. F, calibration curve obtained by fractionating \(M_r\) standards on the SEC column. G–J, AFM analysis of WT α-syn (G and H) and WT C2-α-syn (I and J) oligomers show similar morphology. Similar results were observed in two independent experiments.
These results demonstrate that FlAsH detects soluble high-

C2-ellipsoid species. Native FlAsH. AFM images revealed a combination of spherical and oligomer species and the absence of oligomeric species in the lower-M_r peak (Fig. 2C).

In a separate experiment, we compared the abilities of ThioT and FlAsH to detect soluble oligomers present in SEC fractions from C2-α-syn incubated for 32 h. FlAsH produced a robust increase in fluorescence for high-M_r SEC fractions of incubated C2-α-syn, compared with non-incubated C2-α-syn. In contrast, we did not detect a change in ThioT fluorescence for high-M_r SEC fractions (Fig. 2D) regardless of incubation time. These results demonstrate that FlAsH detects soluble high M_r oligomers that do not bind ThioT.

To examine the temporal evolution of oligomeric species, we used FlAsH to analyze SEC fractions obtained after C2-α-syn incubated for varying lengths of time. Oligomer levels progressively increased with longer incubation times (Fig. 2E). The distribution of FlAsH-positive oligomers was similar among several time points, with significant FlAsH fluorescence present in fractions corresponding to relative M_r values of 440,000 and higher, based on comparison with a M_r standard calibration curve (Fig. 2F).

**AFM characterization of α-syn oligomers**

We used AFM to further characterize α-syn oligomers detected by FlAsH. AFM images revealed a combination of spherical and ellipsoid species. Native α-syn (Fig. 2, G and H) and C2-α-syn (Fig. 2, I and J) oligomers displayed similar morphologies.

**SeGMA assay with sonicated fibrils**

In the fibril formation experiments, we estimated an oligomer/fibril ratio of < 1% after incubation of purified C2-α-syn monomer for 72 h, indicating that kinetics favor conversion to fibrils. To further characterize seed extension by sequential association of monomer using fibrils as seeds, we used a water bath sonicator to fragment and disperse fibrils obtained after a 72-h incubation of C2-α-syn. Characterization of these seeds by dynamic light scattering (DLS) indicated consistent size distribution and concentration across independently generated batches (Table 1). Incubation of 30 μg/ml sonicated fibrils with 500 μg/ml C2-α-syn monomer in SeGMA assays produced a linear increase in FlAsH signal over 4.5 h. In control reactions with no added fibril seeds, FlAsH signal did not increase, reflecting the absence of spontaneous nucleation over the 4.5-h incubation period (Fig. 3C).

**FlAsH is more sensitive than ThioT for measuring fibril growth rates**

To compare the sensitivity of FlAsH and ThioT in SeGMA assays, we incubated different amounts of sonicated C2-α-syn fibril seeds with 500 μg/ml C2-α-syn monomer for 3 h and quantified seed extension with either FlAsH (Fig. 4A) or ThioT (Fig. 4B). We observed a linear increase for both FlAsH and ThioT fluorescence with respect to seed concentration. However, the signal/background ratio was substantially higher for

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**Figure 3. FlAsH detects extension of oligomer and fibril seeds by association of WT C2-α-syn monomer.** A, diagram of the conceptual basis for the SeGMA assay. When SEC-isolated oligomers or sonicated fibril seeds are incubated with C2-α-syn monomer at 37 °C, C2-α-syn monomer sequentially associates with seeds, which can then be detected by binding of FlAsH dye to closely associated C2-α-syn monomers. B and C, results of SeGMA assays in which SEC-isolated oligomers (B) or sonicated fibril seeds (30 μg/ml) (C) were incubated with WT C2-α-syn monomer (500 μg/ml) for different lengths of time. A linear increase in FlAsH fluorescence indicates that FlAsH detects sequential association of monomer with seeds. No change in FlAsH fluorescence is detected when WT C2-α-syn is incubated with control SEC fractions or fibril buffer (“no seeds”). Similar results were observed in three independent experiments (mean ± S.D. (error bars), n=3).
FlAsH fluorescence compared with ThioT fluorescence for the lower levels of fibril formation in SeGMA assays. We calculated the $Z$-factor as an estimate of the dynamic range and separation band for assays measuring seed extension by FlAsH and ThioT (Table 2). Assays with FlAsH produced a $Z$-factor between 0.62 and 0.96 for all fibril seed concentrations tested in the SeGMA assay, indicating robust assay performance and suitability as a screening assay. $Z$-Factors for ThioT assays were in the range of 0.00–0.26, indicating smaller separation bands and marginal assay performance. We used the same conditions, 11.5 $\mu$g/ml C2-$\alpha$-syn fibril seeds, and 500 $\mu$g/ml C2-$\alpha$-syn monomer to compare the signal/background ratio for FlAsH (Fig. 4C) and ThioT (Fig. 4D) over 96 h of fibril growth and also saw a higher signal/background ratio for FlAsH compared with ThioT during the longer incubation time. At 96 h, the signal/background ratio was 26.2 for FlAsH and 2.1 for ThioT. The advantage of FlAsH over ThioT was less pronounced for the measurement of fibril formation when $\alpha$-syn monomer was incubated at higher concentrations with shaking (Fig. 1), where the amount of fibril formation was 20-fold higher, based on measurement of fibril concentration by a BCA sedimentation assay.

Familial-PD mutations alter fibril growth rates

To determine the effects of familial PD-associated $\alpha$-syn mutations on the kinetics of fibril formation, we measured seed growth rates for C2-WT and mutant C2-$\alpha$-syn seeds combined with corresponding C2-WT and mutant C2-$\alpha$-syn monomer proteins (homologous SeGMA assay). We incubated 11.5 $\mu$g/ml seeds with 500 $\mu$g/ml C2-$\alpha$-syn monomer for various lengths of time (0–5 h) and measured growth rates in the FlAsH assay (Fig. 5, A and B). All five missense amino acid mutations altered the rate of seed extension relative to WT. The A53T and H50Q mutations increased the rate of seed extension compared with WT. The A53T and H50Q mutations increased the rate of seed extension relative to WT.

Table 1

<table>
<thead>
<tr>
<th>C2-$\alpha$-syn fibril seeds</th>
<th>Z-average (diameter ± S.D.)</th>
<th>PDI (average ± S.D.)</th>
<th>Independent measurements (n)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>57.0 ± 2.9</td>
<td>0.245 ± 0.02</td>
<td>10</td>
</tr>
<tr>
<td>A30P</td>
<td>57.0</td>
<td>0.263</td>
<td>2</td>
</tr>
<tr>
<td>A53T</td>
<td>72.2</td>
<td>0.276</td>
<td>2</td>
</tr>
<tr>
<td>E46K</td>
<td>80.8 ± 7.0</td>
<td>0.187 ± 0.16</td>
<td>6</td>
</tr>
<tr>
<td>G51D</td>
<td>67.9 ± 6.0</td>
<td>0.244 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>H50Q</td>
<td>53.5 ± 3.8</td>
<td>0.306 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>Fibril buffer</td>
<td>8.9 ± 0.2</td>
<td>0.260 ± 0.02</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 4. FlAsH has greater sensitivity compared with ThioT for detection of fibril growth. Increasing concentrations of sonicated fibril seeds (0–14.5 $\mu$g/ml) were incubated with C2-$\alpha$-syn monomer (500 $\mu$g/ml) for 3 h, and the reactions were analyzed with either FlAsH (A) or ThioT (B) fluorescence assays (mean ± S.D. (error bars), $n = 3$, independent experiments = 2). The signal/background (3 h/0 h) ratio for FlAsH fluorescence is significantly greater than ThioT fluorescence at all concentrations of seeds in the SeGMA assay. Comparison of $Z$-factors for each measurement demonstrates more robust assay performance for FlAsH (shown in Table 2). To compare signal/background ratios over longer time periods, 11.5 $\mu$g/ml C2-$\alpha$-syn seeds and 500 $\mu$g/ml C2-$\alpha$-syn monomer were incubated at 37 °C without agitation for varying lengths of time up to 96 h. Fibril growth at each time point was determined by FlAsH (C) or ThioT (D) fluorescence. Although signal/background ratios for ThioT improved with increasing incubation time, they were significantly lower compared with FlAsH.
and 283% of the rate of WT, respectively. In contrast, the E46K, G51D and A30P mutations decreased the seed extension rate to 60, 42, and 30% of WT, respectively (Fig. 5C). Similar results were observed in multiple independent experiments.

Fibrils used in SeGMA assays were produced by incubating 2000 μg/ml C2-α-syn monomer at 37 °C with shaking for 72 h. We used a BCA assay to measure the amount of fibrils produced by WT and mutant C2-α-syn under these conditions, in which higher monomer concentrations and shaking are used to promote nucleation and fibril growth (Table 3). Comparison of fibril production rates for WT and mutant C2-α-syn revealed differences that were similar to those observed for WT and mutant C2-α-syn in SeGMA assays. A53T and H50Q produced higher levels of fibrils at 72 h, whereas A30P, E46K, and G51D produced lower levels of fibrils.

To evaluate whether the sensitivity of FlAsH for measuring fibrils varies among different fibril species, we measured FlAsH fluorescence for equal concentrations of WT and mutant C2-α-syn fibrils (Table 4). Most mutant fibril species produced FlAsH fluorescence values that were similar to WT. FlAsH sensitivity was modestly lower for G51D fibrils, suggesting that the results in Fig. 5C may overestimate the degree to which G51D seed extension is slower than WT. We used DLS to measure the size of WT α-syn fibrils produced by sonication. The hydrodynamic radius (z-average) measured for WT, A30P, and A53T C2-α-syn fibrils (Fig. 5) was modestly lower for G51D fibrils, suggesting that the results with that of WT α-syn (mean ± 95% confidence interval (error bars), n = 3, independent experiments = 2).

### Table 2

<table>
<thead>
<tr>
<th>Seed concentration μg/ml</th>
<th>FlAsH fluorescence (afr ± S.D.)</th>
<th>Z' Factor</th>
<th>ThioT fluorescence (afr ± S.D.)</th>
<th>Z' Factor</th>
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<tbody>
<tr>
<td>3.6</td>
<td>12,815 ± 401</td>
<td>0.62</td>
<td>9933.3 ± 131</td>
<td>0.10</td>
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<tr>
<td>7.2</td>
<td>24,278 ± 88.5</td>
<td>0.96</td>
<td>11,147.6 ± 87</td>
<td>0.26</td>
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<tr>
<td>14.5</td>
<td>48,310 ± 774.5</td>
<td>0.83</td>
<td>13,807 ± 309</td>
<td>0.00</td>
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</table>

**Figure 5. Comparison of homologous fibril growth rates for WT and mutant C2-α-syn.** The rate of fibril growth was measured by FlAsH fluorescence after incubating homologous combinations of 500 μg/ml C2-α-syn monomer with 11.5 μg/ml matched preformed fibril seeds for different lengths of time. A, comparison of WT, A30P, and A53T C2-α-syn; B, comparison of WT, E46K, G51D, and H50Q C2-α-syn; C, bar graph shows the average rates of mutant α-syn fibril growth (A30P, A53T, E46K, G51D, and H50Q) as a percentage of the rate of WT α-syn (mean ± 95% confidence interval (error bars), n = 3, independent experiments = 2).

### Table 3

*De novo* fibril formation at 72 h assessed by BCA assay

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A30P</th>
<th>A53T</th>
<th>E46K</th>
<th>G51D</th>
<th>H50Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>912</td>
<td>543</td>
<td>1304</td>
<td>604</td>
<td>423</td>
<td>1320</td>
</tr>
<tr>
<td>0 h</td>
<td>912</td>
<td>543</td>
<td>1304</td>
<td>604</td>
<td>423</td>
<td>1320</td>
</tr>
</tbody>
</table>

* STRUCTURAL DETERMINANTS OF α-SYNUCLEIN FIBRIL GROWTH*

In individuals with dominantly inherited PD who have one mutant SNCA allele and one WT SNCA allele, α-syn fibril accumulation can also be influenced by interactions between mutant and WT α-syn protein. We determined the effects of α-syn mutations in heterologous SeGMA assays, in which either C2-α-syn WT seeds were extended by association of mutant C2-α-syn monomer or C2-α-syn mutant seeds were extended by association of WT monomer. We measured fibril growth rates for each combination of seed and monomer and then determined average rates based on measurements at multiple seed concentrations (Fig. 6). We then compared rates of heterologous seed extension (Fig. 7, B and C) with that of ho-
mologous seed extension (Fig. 7A), in which seeds and monomer were matched. Relative rates of homologous seed extension in this paradigm were similar to those observed in the homologous SeGMA time course experiments (Fig. 5). Mismatches between seeds and monomer sequences significantly decreased rates of fibril growth. When WT seeds were incubated with mutant monomers, we observed very low fibril growth rates, with the exception of WT seeds extended by H50Q monomer (Fig. 7B). In the case of H50Q monomer association with WT seeds, the rate was similar to the rate of WT monomer association with WT seeds, but this rate was only 33% of the rate of H50Q monomer association with H50Q seeds. Furthermore, when mutant seeds were incubated with WT monomer (Fig. 7C), we observed consistently low rates ranging from 7.6 to 30% of the rate of extension of WT seeds. The homologous and heterologous fibril growth rates are summarized in Table 5. These results demonstrate that single-amino acid mismatches in the N-terminal region of α-syn significantly alter the rate of fibril growth.

Because individuals with dominantly inherited α-syn gene mutations have a combination of WT and mutant α-syn protein production, we measured fibril growth rates for each seed species in the presence of 50% WT and 50% mutant α-syn monomer (Fig. 8). Overall, the rates produced by WT and mutant mixtures were very close to the average of the individual rates for WT and each mutant monomer (Fig. 6), indicating that rates produced by monomer mixtures are equal to the sum of the individual monomer rates, with no evidence of synergistic interactions between WT and mutant proteins.

We also examined homologous and heterologous seeded fibril growth over 96 h using the same concentrations of seeds (11.5 μg/ml) and monomer (500 μg/ml) used for 3-h rate measurements (Fig. 9 and Table 6). The relative rates measured over longer time periods were consistent with 3-h rate measurements for homologous seed-monomer combinations. Most seed-monomer combinations eventually reached equilibrium states, in which the amount of fibrils measured by FlAsH remained constant. The faster rates of A53T and H50Q translated into higher levels of fibrils at equilibrium relative to WT, whereas the slower rates of A30P, E46K, and G51D translated to lower levels of fibrils at 96 h.

The rates of heterologous seeded fibril growth were also consistent between 3- and 96-h experiments. For example, the slow
Structural determinants of α-synuclein fibril growth

Figure 7. Reduced rates of heterologous fibril growth for both WT seeds extended by mutant monomer and mutant seeds extended by WT monomer. Rates of homologous and heterologous fibril growth were compared for different concentrations of seeds incubated with a fixed concentration of monomer for 3 h. All data were normalized to the rate of WT seeds extended by WT monomer. A, normalized data show the rate of fibril growth when the same species of preformed fibril seeds and C2-α-syn monomer protein were used in each reaction. B, normalized data show the rate at which WT C2-α-syn seeds were extended by either WT or mutant (A30P, A3T, E46K, G51D, and H50Q) C2-α-syn monomer. C, normalized data show the rate of extension of WT or mutant (A30P, A3T, E46K, G51D, and H50Q) C2-α-syn seeds with WT monomer (mean ± 95% confidence interval (error bars), n = 3, independent experiments = 3). The average rate of fibril growth was determined using four different concentrations of seeds during a 3-h incubation period (shown in Fig. 6) and is expressed as a percentage of the rate for WT seeds extended by WT monomer. Because all rates were normalized to the rate of WT seeds extended by WT monomer, rates of homologous and heterologous fibril growth can be compared across the three graphs.

growth of WT seeds in the presence of A53T monomer was maintained over 96 h, which indicates that the structural feature of WT fibrils dictating slow A53T monomer association remains stable despite the sequential addition of A53T monomer. Stable maintenance of fibril structure, as reflected by monomer association rates, was observed for all of the heterologous seed–monomer combinations.

We compared FLAsH measurements with two other measurements of fibril formation after 96 h of seeded fibril growth (Fig. 10). In addition to ThioT measurements, we used a BCA sedimentation assay, which measures the concentration of fibrils by determining the change in monomer concentration in the supernatant between 0 and 96 h following centrifugation at 100,000 × g for 20 min. We focused on measuring fibril formation after 96 h of seeded fibril growth, because the sensitivities of ThioT and BCA assays were not optimal for quantifying fibril growth during shorter time periods. The amount of fibril formation determined by BCA sedimentation assays strongly correlated with the amount of fibril formation determined by FLAsH for the same reactions. Relative rates of fibril formation determined by ThioT measurements also generally agreed with FLAsH measurements, although ThioT generally showed that heterologous growth rates were less reduced relative to the homologous WT rate.

We assessed whether the presence of the N-terminal bicysteine (C2) tag influenced the measurement of heterologous fibril growth rates. We prepared fibril seeds from native WT α-syn and combined them with native WT and mutant α-syn monomer proteins (without a C2 tag) in 96-h heterologous fibril growth experiments matching the experimental design of Fig. 10, D–F. We measured fibril growth by a BCA sedimentation assay (Fig. 11A) and ThioT fluorescence (Fig. 11B). The relative differences in growth rates for untagged WT and mutant α-syn monomer species were similar to those observed with C2-α-syn monomer species (Fig. 10, E and F). Sensitivity limits do not allow comparison of fibril growth rates in 3-h experiments, and we have not prepared fibrils from untagged mutant α-syn to further characterize homologous and heterologous growth rates for the native mutant α-syn seeds. However, the results with native WT α-syn seeds and mutant α-syn monomer indicate that measurements of relative growth rates with C2-tagged α-syn monomer species reflect the growth rates for native α-syn monomer species.

Discussion

In this study, we developed a novel assay that utilizes bipartite tetracysteine detection to analyze the effects of single-amino acid sequence changes on fibril growth, a critical step in α-syn fibril accumulation. To that effect, we produced recombinant α-syn protein containing a bicysteine tag. We demonstrated that FLAsH is capable of detecting a wide range of soluble oligomeric and fibrillar species produced by incubation of the monomeric C2-α-syn protein. This property enabled the development of a highly sensitive in vitro SeGMA assay that measures the rate of monomeric α-syn association with either preformed oligomer or fibril seeds. We observed that two mutations associated with familial PD (H50Q and A53T) dramatically increase the rate of seed extension, whereas three mutations (A30P, E46K, and G51D) significantly decrease the rate of seed extension. Our heterologous seed extension experiments demonstrated that WT seeds are extended inefficiently
growth is a highly ordered process. Although fibril growth was measured by FlAsH fluorescence after combining 11.5 monomer.

**Table 5**
Rates of homologous and heterologous fibril growth in 3-h SeGMA assays

Rates of seed growth for homologous and heterologous seed plus monomer combinations were measured by FlAsH fluorescence for three different seed concentrations (2.8–11.5 μg/ml) and a constant monomer concentration of 500 μg/ml. Average rates were expressed as a percentage of the rate obtained for WT seeds extended by WT monomer.

<table>
<thead>
<tr>
<th>Rate of seed extension (%WT) at 3 h, mean (95% CI)</th>
<th>WT</th>
<th>A30P</th>
<th>A53T</th>
<th>E46K</th>
<th>G51D</th>
<th>H50Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homologous: matched seeds + monomer</td>
<td>100 (91.7–108.3)</td>
<td>23 (18.6–27.4)</td>
<td>552.6 (481.6–623.6)</td>
<td>48 (42.5–53.5)</td>
<td>21 (17.8–24.2)</td>
<td>323 (302–344)</td>
</tr>
<tr>
<td>Heterologous: WT seeds + mutant monomer</td>
<td>4.7 (0–9.6)</td>
<td>3.7 (0–8.7)</td>
<td>0.3 (0–1.1)</td>
<td>2.9 (1.7–4.1)</td>
<td>97 (91.4–102.6)</td>
<td></td>
</tr>
<tr>
<td>Heterologous: mutant seeds + WT monomer</td>
<td>30 (26.4–33.6)</td>
<td>17.7 (6.2–29.2)</td>
<td>16 (13–19)</td>
<td>22 (19–25)</td>
<td>7.6 (2.4–12.8)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8. Rates of fibril growth for WT and mutant seeds in the presence of 50% WT and 50% mutant C2-α-syn monomer. The rate of fibril growth was measured by FlAsH fluorescence after combining 11.5 μg/ml seeds with different combinations of WT and mutant monomer at a 1:1 ratio and a total concentration of 500 μg/ml. A, fibril growth rates for WT seeds combined with 50% WT and 50% of each different mutant monomer. B, fibril growth rates for each mutant seed species when combined with 50% WT monomer and 50% matched mutant monomer. Rates are compared with the rate observed for WT seeds extended by 100% WT mutant monomer. The rates for the monomer mixtures containing 50% each of WT and mutant monomer were approximately the average of the two rates produced by 100% WT monomer and 100% mutant monomer, with no evidence of synergistic interactions between different monomer species (mean ± S.D. (error bars), n = 3, independent experiments = 1).

by mutant monomer proteins and that mutant seeds are also extended inefficiently by WT monomer. These results provide important insight into a critical step regulating the accumulation of α-syn fibrils. They demonstrate that fibril growth is a highly ordered process. Although fibril growth probably depends on monomer-monomer interactions comprising the highly structured β-sheet region of α-syn fibrils, it is very sensitive to single-amino acid sequence changes in the N-terminal region.

The bicysteine tag has several advantages for monitoring the kinetics of fibril formation. It is smaller than other fluorescent tags, such as fluorescent proteins, and therefore has less potential to influence fibril structure and the kinetics of fibril formation. Commonly used amyloid-binding dyes, such as ThioT or thioflavin S, depend on specific binding sites present on the cross-β-sheet conformation of the aggregated protein. Thus, it is difficult to obtain accurate kinetic measurement for smaller oligomeric species, which may lack ThioT-binding sites. FlAsH is able to detect soluble oligomeric species that are not detected by ThioT, indicating a greater sensitivity to monitor the formation of early and intermediate species in fibril formation. The most important property of bipartite tetracysteine detection is the ability to sensitively detect growth of either oligomer or fibril seeds by association of C2-α-syn monomer. This property enables linear rates of seed extension to be measured during very short incubation times (1–5 h) under quiescent conditions, providing novel information about homologous and heterologous fibril growth rates for α-syn. BCA sedimentation assays support the reliability of FlAsH measurements for both WT and mutant fibril species, which is probably explained by the fact that the bicysteine tag is located at least 30 amino acids away in the unstructured N-terminal end of α-syn fibrils. Additional experiments comparing relative growth rates of native WT α-syn seeds in the presenting of WT and mutant native α-syn monomer species also support the reliability of SeGMA assays for investigating structural requirements of fibril growth.

Previous studies have examined the effect of familial-PD mutations on α-syn aggregation in paradigms involving the rate of spontaneous conversion of monomeric protein to fibrils. Our mutant seed extension data agree with previously published studies with A30P, A53T, G51D, and H50Q, in which rates of spontaneous fibrillization were measured in vitro. In our seed growth assay, E46K demonstrated a slow rate of growth compared with WT, which is in contrast to other studies examining rates of spontaneous fibril formation. It should be noted that the rates measured in these previous studies represented a combination of nucleation plus fibril growth rates, where parameters such as the 50% conversion time (t1/2, hours or days) or the lag time (hours or days) can be compared. However, we also observed slower rates of spontaneous fibril formation by E46K α-syn under shaking conditions, suggesting that differ-
ences in fibril formation kinetics for this protein may be attributable to different conditions utilized for fibril formation studies. Different fibril formation conditions can lead to differences in WT/H9251-syn fibril structure as assessed by solid-state NMR (44, 46). Furthermore, variations in fibril structure could arise from conditions driving WT/H9251-syn fibril formation in vivo, not only for mutant α-syn fibrils in familial PD, but also the occurrence of α-syn fibril accumulation in other synucleinopathies, such as multiple system atrophy. The SeGMA assays reported herein can be utilized to further investigate and compare structural requirements for fibril growth of different α-syn fibril conformers.

The high degree of sequence specificity observed in our heterologous seed extension experiments, where rates are measured over 3 h, has not been reported previously. The basis for this specificity is probably related to the rigid β-sheet structure observed in previous solid-state nuclear magnetic resonance studies of α-syn fibrils (43, 44). Residues 30–97 comprise the core fibrillar region, with multiple rigid β-strands and loops. Fibril growth thus requires newly associating monomer pro-

Figure 9. Time course of homologous and heterologous seeded fibril growth over 96 h. Homologous and heterologous mixtures of 11.5 μg/ml C2-α-syn seeds and 500 μg/ml C2-α-syn monomer were incubated at 37 °C without agitation for varying lengths of time up to 96 h. Fibril growth at each time point was determined by FlAsH fluorescence. The rate for each seed-monomer combination was compared with the rate of fibril growth for WT seeds incubated with WT monomer. A–E, time course of fibril growth for homologous seed-monomer combinations demonstrated that the rates over longer incubation periods were very similar to the rates observed in 3-h experiments (Fig. 5). Furthermore, faster and slower rates of fibril growth corresponded to higher and lower levels of fibril accumulation as rates plateaued by 96 h. F–J, time course of fibril growth for WT seeds incubated with each of the different mutant monomer species. K–O, time course of fibril growth for each of the different mutant seeds incubated with WT monomer. The time course of heterologous fibril growth over longer time periods was also similar to the rates observed in 3-h experiments (Fig. 6). P–T, time course of fibril growth when each monomer species was incubated in the absence of seeds (mean ± S.E. (error bars), n = 3, independent experiments = 2).

Table 6
Fibril accumulation in the homologous and heterologous seed plus monomer combinations after incubation for 96 h
Fibril accumulation in the homologous and heterologous seed plus monomer combinations were measured by FlAsH fluorescence after incubation of 11.5 μg/ml seeds with 500 μg/ml monomer without agitation for 96 h. Average rates were expressed as a percentage of the rate obtained for WT seeds extended by WT monomer (mean ± 95% CI, n = 9, independent experiments = 3).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A30P</th>
<th>A53T</th>
<th>E46K</th>
<th>G51D</th>
<th>H50Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homologous: matched seeds + monomer</td>
<td>100 (87–113)</td>
<td>24.6 (15.9–33.4)</td>
<td>240.8 (276.9–339.8)</td>
<td>22.3 (18.3–26.4)</td>
<td>11.5 (10.6–12.4)</td>
<td>203.8 (186.8–220.8)</td>
</tr>
<tr>
<td>Homologous: WT seeds + mutant monomer</td>
<td>18.6 (13.1–24.1)</td>
<td>40.0 (21.8–58.3)</td>
<td>1.3 (0.2–2.5)</td>
<td>10.8 (7.8–13.8)</td>
<td>100.2 (83.1–117.3)</td>
<td></td>
</tr>
<tr>
<td>Homologous: mutant seeds + WT monomer</td>
<td>28.3 (20.3–36.2)</td>
<td>52.8 (40.9–64.8)</td>
<td>18.4 (8.9–27.9)</td>
<td>7.3 (6.5–8.0)</td>
<td>13.0 (9.4–16.7)</td>
<td></td>
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</table>
proteins to adopt a conformation that matches the β-strand structure of the fibril. Constraints on the ability of associating monomer to match the conformation of the fibril seed are likely to result in slower association of α-syn monomers and faster monomer dissociation.

The effects of single-amino acid mismatches on fibril growth were consistently maintained in 96-h experiments, indicating that the structures of fibril seeds are maintained during growth by the addition of heterologous monomer species. Interpretation of these 96-h experiments is aided by the lack of spontaneous nucleation under the conditions we used, which could potentially interfere with measurement of seeded fibril growth rates. The observation that rates are consistently maintained over longer time periods supports the conclusion that fibril structure is templated during heterologous monomer addition, with no evidence of conversion to the structures and rates produced under spontaneous nucleation conditions.

Our findings are consistent with the observation that A53T fibrils seed fibril formation by WT monomer more slowly than WT fibrils seed WT monomer, as measured by sedimentation assays over 1–10 days (21). Furthermore, Sidhu et al. (35) observed inefficient heterologous cross-seeding for WT, A30P, and A53T α-syn, relative to homologous rates. However, two previous studies did not find reduced rates of heterologous growth for at least some species. Ono et al. (34) observed that heterologous mixtures of mutant fibrils and WT monomer formed ThioT-binding fibrils at approximately the same rate as homologous mixtures of fibrils and monomer, in a system that utilized agitation in 20 mM Tris-HCl, pH 7.4, which is significantly different from our quiescent incubation in 20 mM Tris-

Figure 10. Comparison of FlAsH measurements with two other measurements of C2-α-syn fibril formation following seeded fibril growth for 96 h further validates SeGMA assays. Homologous and heterologous mixtures of 11.5 μg/ml seeds and 500 μg/ml monomer were incubated at 37 °C without agitation for 96 h. Fibril formation for each of the homologous and heterologous seed-monomer combinations was assessed by FlAsH fluorescence (A, D, and G); BCA sedimentation assay, which measures fibril concentration based on the change in the supernatant concentration of C2-α-syn after centrifugation at 100,000 × g (B, E, and H); and ThioT fluorescence (C, F, and I) (mean ± S.D. (error bars), n = 3, independent experiments = 2).

Figure 11. Relative rates of heterologous fibril growth for α-syn monomer species without a C2 tag are similar to the relative rates of C2-α-syn monomer species. 11.5 μg/ml native WT α-syn seeds were combined with 500 μg/ml native WT or mutant α-syn monomer and incubated at 37 °C without agitation for 96 h. Fibril formation was assessed by a BCA sedimentation assay (A) and ThioT fluorescence (B). Relative rates of fibril growth with untagged α-syn monomer species shown here are similar to the rates observed for C2-α-syn monomer species in the heterologous assays shown in Fig. 10, D–F (mean ± S.D. (error bars), n = 3, independent experiments = 2).
HCl, pH 8.0, with 100 mM NaCl. Flagmeier et al. (36) observed heterologous fibril growth rates that were equal to or lower than homologous growth rates, in a system that used higher seed/monomer ratios with quiescent incubation in 20 mM phosphate buffer at pH 6.5. The use of agitation and higher concentrations of monomer may produce more complex effects of seeds on the rates of both nucleation and growth. Furthermore, differences in incubation conditions used for nucleation of fibril formation, such as buffer and NaCl concentration, may produce differences in fibril structures (44, 47–50), which in turn may have different effects on the relative rates of heterologous monomer association.

The accumulation of α-syn fibrils is defining histopathologic feature of not only idiopathic PD, but also familial PD caused by the A53T, A30P, E46K, H50Q, and G51D mutations. The observed decrease in seed extension rates for the A30P, E46K, and G51D mutations indicates that these single-amino acid changes cause fibril accumulation in vivo through mechanisms independent of effects on fibril growth rates. This is further supported by the decreased rates of heterologous seed extension for all mutants, indicating that interactions between mutant and WT α-syn proteins do not significantly contribute to fibril accumulation in dominantly inherited PD. For example, the faster rate of oligomer formation observed for A30P α-syn occurs in the setting of slow fibril growth rates by association of either A30P or WT α-syn monomer. This predicts greater accumulation of oligomers than fibrils in individuals with the A30P mutation. However, the A30P mutation does cause accumulation of α-syn fibrils, which has been demonstrated by electron microscopy analysis and by detection of insoluble α-syn in postmortem human brain tissue (51).

Dominantly inherited SNCA mutations could cause fibril accumulation by multiple mechanisms other than effects on fibril growth rate. For example, rates of both nucleation and seed extension depend on the in vivo concentration of free monomer, which is likely to be influenced by protein turnover rates (52), monomer-lipid interactions (53, 54), formation of tetrameric α-syn complexes (55, 56), and chaperone interactions (57, 58). Similarly, interactions with chaperone proteins and protein clearance pathways may influence the clearance rates of soluble oligomeric species and fibrils (59–62). Finally, alterations in post-translational modifications, particularly phosphorylation (63, 64), may affect fibril accumulation in vivo. Translation of the FLAsH system into a cell culture model expressing C2-α-syn could enable further investigation of the effects of mutations on α-syn fibrillization within a cellular environment, enabling the role of additional in vivo factors to be assessed.

Accumulation of misfolded protein may have a pathogenic role in a wide range of both hereditary and sporadic neurodegenerative disorders, including PD, Alzheimer’s disease, frontotemporal lobar dementias, and hereditary polyglutamine repeat disorders, such as Huntington’s disease. The identification of dominantly inherited mutations in these disorders provides further support for the role of protein aggregation pathways in disease pathogenesis, although mechanisms by which aggregated protein species cause neurodegeneration and the role of soluble oligomers versus insoluble fibrils are still being elucidated. A potential common pathogenic mechanism in these diseases is the impairment of protein homeostasis networks caused by the burden of aggregated protein (52, 65), which further impairs cellular function. Furthermore, recent reviews have elaborated on the potential for prion-like transcellular propagation of fibrillar α-syn (66–68), a possible mechanism underlying the progressive involvement of additional brain regions over time. These observations indicate that approaches to decrease the burden of fibrillar protein accumulation could slow or stop disease progression.

Fibril growth is thus one of several important steps that can be targeted to prevent the progressive accumulation and spread of fibrillar protein species (69). The observed sensitivity to sequence mismatches indicates that monomer association is dependent on precise alignment of the associating polypeptide chains over the region of amino acid sequence (Ala10–Ala53) included in this study. In the setting of these strict structural requirements, fibril growth could be substantially inhibited by a small molecule that interacts with either free monomeric protein or the seed surface to which a new monomer must bind in order to extend the seed. Effective inhibitors could alter the molecular interaction between associating polypeptide chains or modify their conformation in a way that disrupts the alignment necessary for fibril growth. The bipartite tetracysteine approach enables additional studies to further define structure-function relationships for α-syn association during seed extension and could serve as a platform for the development of fibril growth inhibitors as therapeutics in PD.

**Experimental procedures**

**PCR primers for generating N-terminal C2-α-syn**

To add the 6-amino acid bicysteine tag to the N terminus of WT-α-syn (5′-MGCCGG-α-syn-3′), we used PCR amplification using the pRK172-WT-α-syn plasmid plus the following primers: 5′-AAAAACATATGGGCTGCAGCAGCAGCAGGATGGATATTACATGAAGAGA-3′ (forward primer) and 5′-AAAAAAGCTTTAGGCTTCAGGTTCGTAGTC-3′ (reverse primer). The PCR product was subcloned into the pRK172 vector plasmid, and the DNA was sequenced to verify that the insert had been successfully ligated into pRK172.

**Mutagenesis for untagged α-syn mutants and C2-α-syn mutants (A53T, A30P, E46K, G51D, and H50Q)**

To generate untagged α-syn mutants or C2-tagged α-syn mutants, site-directed mutagenesis using the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA) was performed on the pRK172-WT-α-syn plasmid or the pRK172-C2-WT-α-syn plasmid (see above) with the following primers: G157→A (A53T mutation), 5′-GGTTGCGATGGTTCGACGACAGTGGGCTGAGA-3′ (forward primer) and 5′-GGCTCACTGTCGTCAGCAGCAGG-3′ (reverse primer); G88→C (A30P mutation), 5′-GGGTGTGGGCAGAAGCACCAGG-3′ (forward primer) and 5′-GGGCTCCAAAAAAGGAGTCGATG-3′ (reverse primer); G136→A (E46K mutation), 5′-GGGCTCCAAAAAAGGAGTCGATG-3′ (forward primer) and 5′-
Structural determinants of α-synuclein fibril growth

CATGCACACTCCTCTTGTTTGGAGGCC-3' (reverse primer); G152→A (G51D mutation), 5'-GGAGGTGGTGCATGATGGAACAGTG-3' (forward primer) and 5'-CCAC-TGGTGCCAACATGCGACCTCCC-3' (reverse primer); T150→G (H50Q mutation), 5'-GGAGGGAGGTTGTCAGG-GTTGGCAACAG-3' (forward primer) and 5'-CTGTTGCCACACCTGCACTCCCTCC-3' (reverse primer). All mutations were confirmed by sequencing of the entire α-syn coding region.

Preparation of WT α-syn, untagged mutant α-syn, C2-WT, and C2-mutant-α-syn protein

Recombinant α-syn protein was prepared using methods described previously (70, 71) by transforming BL21(DE3)RIL bacterial cells with the pRK172 plasmid containing the desired α-syn construct (WT, untagged mutant, C2-WT, C2-mutant). The recombinant proteins were purified by heat denaturation and precipitation of bacterial proteins, followed by ion-exchange chromatography. Purified monomeric α-syn was dialyzed and stored at −80 °C before use.

Preparation of α-syn oligomers and α-syn fibrils

α-Syn fibrils were prepared by incubating 2000 μg/ml purified monomer in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl (fibril formation buffer) at 37 °C in an Eppendorf Thermomixer with constant shaking at 1000 rpm for 72–96 h. For the C2-α-syn fibril preparation, 1 mM DTT was added to the fibril formation buffer. For preparation of oligomers, the 2000 μg/ml purified monomer was incubated in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT for 32–36 h. For analysis of the time course of fibril formation, 75 μl of samples were removed at each time point and stored at −80 °C before analysis in the fluorescence assay. To determine the concentration of fibrils, samples were centrifuged at 15,000 × g for 15 min at 4 °C to separate fibrils from monomer. The concentration of α-syn monomer in the supernatant was determined in a Micro BCA protein assay (Thermo Scientific Pierce Micro BCA kit, catalog no. 23235) according to the manufacturer’s instructions, using the manufacturer-supplied bovine serum albumin (BSA) for the standard curve. The measured decrease in α-syn monomer concentration was used to determine the concentration of fibrils in each sample.

Determination of K_{app} of FIAsH to preformed C2-α-syn fibrils

Saturation binding assays were performed to quantify the binding affinity of 4,5-bis(1,3,2-dithiasolan2-yl)fluorescein (FIAsH-EDT₂) dye to preformed C2-α-syn fibrils. Fibrils produced by incubation of C2-α-syn protein for 72 h were centrifuged at 15,000 × g for 15 min at 4 °C. The fibril pellet was resuspended in 100 μl of fibril formation buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) and sonicated in a Qsonica (model Q700) sonicator with a cup horn (5.5-inch) attachment for 1 min at power setting 50. Then increasing concentrations (0–180 μg/ml) of sonicated fibril seeds were combined with a FIAsH assay mixture consisting of 3.5 mM tris(2-carboxyethyl)phosphine, 1 mM EDT, 1 mM EDTA, 25 mM FIAsH-EDT₂, and 200 mM Tris-HCl, pH 8.0, in a final volume of 100 μl. After incubation at room temperature for 1 h in Corning Black 96-well plates (Fisher, catalog no. 07-200-762), FIAsH fluorescence was detected as described below. As a control, FIAsH fluorescence at 0 μg/ml fibril concentration was used to obtain the specific binding fluorescence measurement. The data were fit to the equation, y = B_{max} × x/(x + K_{d}) using non-linear regression analysis with GraphPad Prism version 4.0 to obtain an apparent affinity constant measurement.

FlAsh fluorescence assay

The profluorescent biarsenical dye FIAsH-EDT₂ (Invitrogen TC-FIAsHTM II in-cell tetracysteine tag detection kit, catalog no. T34561) was used in bipartite tetracysteine detection assays to quantify oligomer and fibril formation. Samples (5–25 μl) were combined with a FIAsH assay mixture consisting of 3.5 mM tris(2-carboxyethyl)phosphine, 1 mM EDT, 1 mM EDTA, 25 mM FIAsH-EDT₂, and 200 mM Tris-HCl, pH 8.0, in a final volume of 100 μl. After incubation at room temperature for 1 h in Corning Black 96-well plates (Fisher 07-200-762), FIAsH fluorescence was detected in a BioTek plate reader using a 485/20-nm excitation filter, a 528/20-nm emission filter, top 510-nm optical setting, and gain setting 100.

ThioT fluorescence assay

Samples were incubated in Corning Black 96-well plates (Fisher, catalog no. 07-200-762) with 18 μM ThioT in 30 mM Tris-HCl, pH 7.4. A total volume of 100 μl/well was incubated at room temperature for 1 h, and ThioT fluorescence was measured in a BioTek plate reader using a 440/30-nm excitation filter, a 485/20-nm emission filter, and top 50% optical setting. In the case of the spontaneous fibril formation experiment (Fig. 1), the gain setting was set to 60, whereas, in subsequent experiments, the gain was set to 80. Different gain settings produced different afu values but did not change the signal/background ratio (data not shown).

Isolation and characterization of oligomers using SEC

Samples containing α-syn oligomers were fractionated using a GE Healthcare ÄKTA purifier FPLC system, GE Unicorn version 5.20 software, and a GE Superdex 200 10/300 GL (product 17-5175-01) size exclusion column. The column was equilibrated in at least 50 ml of column buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Triton X-100) before analysis. Samples (400 μl) to be analyzed by SEC were prepared by centrifugation at 100,000 × g for 30 min at 4 °C in a Beckman TLA-55 rotor. The supernatant was collected, Triton X-100 was added to a final concentration of 0.1%, and protease inhibitor mixture (Sigma, catalog no. P2714) was added to a final concentration of 1×. The samples were then injected onto the SEC column using a 200-μl injection loop and fractionated at a flow rate of 0.5 ml/min. Twenty-five fractions (0.5 ml each) were collected beginning at 4.7 ml (0.2 column volumes) after the injection of sample. The GE Superdex 200 10/300 GL column was calibrated by injection of ovalbumin (44,000), aldolase (158,000), and ferritin (440,000) molecular weight standards.

WB analysis of Superdex fractions

SEC fractions were analyzed by WB using 26-well Criterion 4–20% Tris-HCl, SDS-polyacrylamide gels (Bio-Rad) (72).
Electrophoresed proteins were transferred to nitrocellulose membranes and incubated with mouse anti-α-syn antibody syn303 (a gift of Dr. Virginia M.-Y. Lee), followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). Bound secondary antibody was detected by enhanced chemiluminescence (ECL) using Immobilon Western ECL Substrate (Millipore). Blots were imaged with an Eastman Kodak Co. Image Station 4400, and the intensity of individual bands was compared using Kodak 1D analysis software.

**AFM analysis of α-syn fibrils and oligomers**

AFM images were collected using an Asylum Research atomic force microscope with the aid of Asylum Research MFP 3D software. Mica coverslips (Ted Pella Inc., catalog no. 50) were mounted onto Fisher brand Plain Superclean microscope slides with superglue. Samples of SEC-purified oligomers or fibrils were diluted in 20 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X. Then 50 μl of sample was applied to the freshly exposed mica surface, incubated for 30 min at room temperature in a humidified chamber, and then washed three times with 50 μl of water. The coverslips were then dried overnight in a desiccating chamber. Images were obtained by scanning at 1 Hz.

**Preparation of sonicated fibril seeds for SeGMA assays**

Fibrils produced by incubation of α-syn protein for 72 h were centrifuged at 15,000 × g for 15 min at 4 °C. The fibril pellet was resuspended in 100 μl of fibril formation buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) and sonicated in a Qsonica (model Q700) sonicator with a cup horn (5.5-inch) attachment for 5 min at power setting 50. The sonicated fibril seeds were further diluted to a working concentration of 285 μg/ml in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Triton X-100 and stored at 4 °C before use.

**SeGMA assays**

Seed extension rates were measured by combining 5 μl of seed samples (sonicated fibrils or SEC fractions) with 20 μl of purified C2-α-syn monomer in thin-wall PCR tubes and incubating at 37 °C for the indicated times in a Bio-Rad thermal cycler. Control reactions contained the appropriate buffer solution in place of seed samples. Additional control reactions were prepared by combining seed samples and purified C2-α-syn monomer without incubation at 37 °C. SeGMA reaction samples were mixed by pipetting before removing 20 μl for addition to the FlAsH assay mixture in 96-well plates. For homologous SeGMA assays, the seeds and C2-α-syn monomer were matched (e.g. A53T seeds with A53T C2-α-syn monomer), whereas for heterologous SeGMA assays, the seeds and C2-α-syn monomer are different (e.g. A53T seeds with WT C2-α-syn monomer or WT seeds with A53T C2-α-syn monomer).

**Estimating the size of WT and mutant fibril seeds using DLS**

A Zetasizer Nano ZS instrument (Malvern Instruments) was used to measure the hydrodynamic diameter of sonicated WT and mutant C2-α-syn seeds at 25 °C. Seeds were prepared by sonicating WT and mutant C2-α-syn fibrils and were diluted in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Triton-X for measurements. Each measurement was performed on at least two independent preparations of each seed species, and all samples were scanned 3–5 times in the DLS instrument.

**Z’-factor determination**

We used Z’-factor as a screening window coefficient to measure statistical separation between the 0- and 96-h incubated SeGMA samples using FlAsH and ThioT fluorescence measurements. The formula for calculation of the dimensionless Z’-factor (22) is as follows.

\[
Z' = 1 - \frac{3 \times (S.D. \text{sample} + S.D. \text{control})}{\text{Mean} \text{sample} - \text{Mean} \text{control}}
\]  
(Eq. 1)

**Comparison of FlAsH fluorescence intensity in the presence of WT and mutant C2-α-syn fibrils**

Mutant and WT C2-α-syn fibrils were centrifuged at 15,000 × g for 15 min at 4 °C, and the fibril pellets were resuspended in 50 μl of fibril formation buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). The samples were sonicated in a Qsonica (model Q700) sonicator with a cup horn (5.5-inch) attachment for 1 min at power setting 50. The sonicated fibril seeds were then diluted to a concentration of ~14.5 μg/ml and incubated with 25 mM FlAsH dye for 1 h at room temperature, before measurement of FlAsH fluorescence. FlAsH fluorescence measured for each mutant C2 fibril sample was then compared with the fluorescence obtained from WT C2 fibrils.

**Time course of seeded fibril growth reactions**

To assess seeded fibril growth over longer time periods, 20 μl of seeds (11.5 μg/ml) were combined with 80 μl of monomer (500 μg/ml) for each seed-monomer combination in 96-well polystyrene plates (Beckman, catalog no. 10-565-368) and incubated at 37 °C under quiescent conditions. Time points were collected at 0, 3, 6, 9, 18, 42, 54, 72, and 96 h for the time course, and the plates were stored at 4 °C before use in further assays. Similarly, 100-μl reactions were set up in microcentrifuge tubes (Beckman, catalog no. 357448) to assess fibril accumulation at 96 h using FlAsH, BCA sedimentation, and ThioT assays. Each sample was mixed by pipetting before removing 5 μl of sample for the FlAsH assay and using 20 μl for the ThioT assay. The remainder was used for the BCA sedimentation assay. Each seed-monomer combination was set up in triplicate for each time point.

**BCA sedimentation assay**

To assess fibril accumulation in each seed-monomer condition after 96 h, microcentrifuge tubes (0 and 96 h) were spun at 100,000 × g for 20 min at 4 °C in a Beckman TLA-55 rotor. Then 15 μl of supernatant was added to the BCA assay (Micro BCA kit, catalog no. 23235) to determine α-syn monomer concentration using a standard curve produced with manufacturer-supplied BSA. The measured decrease in α-syn monomer concentration was used to determine the concentration of fibrils accumulated in the 96-h fibril reaction mixtures.
References

Structural determinants of α-synuclein fibril growth


A sensitive assay reveals structural requirements for \( \alpha \)-synuclein fibril growth

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