Human β-Synuclein Rendered Fibrillogenic by Designed Mutations

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Filamentous inclusions made of α-synuclein are found in nerve cells and glial cells in a number of human neurodegenerative diseases, including Parkinson disease, dementia with Lewy bodies, and multiple system atrophy. The assembly and spreading of these inclusions are likely to play an important role in the etiology of common dementias and movement disorders. Both α-synuclein and the homologous β-synuclein are abundantly expressed in the central nervous system; however, β-synuclein is not present in the pathological inclusions. Previously, we observed a poor correlation between filament formation and the presence of residues 73–83 of human α-synuclein, which are absent in β-synuclein. Instead, filament formation correlated with the mean β-sheet propensity, charge, and hydrophilicity of the protein (global physicochemical properties) and β-strand contiguity calculated by a simple algorithm of sliding averages (local physicochemical property). In the present study, we rendered β-synuclein fibrillogenic via one set of point mutations engineered to enhance global properties and a second set engineered to enhance predominantly β-strand contiguity. Our findings show that the intrinsic physicochemical properties of synucleins influence their fibrillogenic propensity via two distinct but overlapping modalities. The implications for filament formation and the pathogenesis of neurodegenerative diseases are discussed.

α-Synuclein (α-syn) filaments are found mainly inside nerve cells in Parkinson disease and dementia with Lewy bodies and in glial cells in multiple system atrophy (1–7). The polymerization process by which filaments form is likely to be directly involved in neurodegeneration, such that preventing it from happening may also prevent disease from occurring (8). The heterozygous expression of α-syn missense mutants results in inherited forms of Parkinson disease and dementia with Lewy bodies (9–11). Overexpression of wild-type α-syn also causes Parkinson disease and dementia with Lewy bodies (12–15). Recombinant α-syn assembles into amyloid-like filaments in vitro (16–19); however, β-synuclein (β-syn), which shares ~60% sequence identity with α-syn (20), does not assemble into filaments (19, 21–24) and is not present in the pathological inclusions (2, 3, 25).

The correlation between the amino acid sequence of α-syn and its propensity to assemble in vitro has been studied using deletion mutants (22, 26) and carboxy-terminally truncated fragments (16, 19, 26–28) as well as peptides corresponding to a hydrophobic region spanning residues 61–95 of α-syn (29–32). As a result, it is known that the hydrophobic central region of α-syn is favorable for assembly, whereas the negatively charged carboxyl-terminal region is inhibitory. Subsequently, we performed a systematic analysis of sequence determinants for α-syn assembly using deletion mutants, amino-terminally truncated fragments, α-syn/β-syn chimeras, and point mutants (33). This revealed that the fibrillogenic propensity of α-syn is determined by both amino acid composition (a “global” property) and β-strand contiguity (a “local” property) (33, 34).

In the present study, we have designed point mutants of human β-syn to primarily target either global properties or local properties, to turn β-syn into a fibrillogenic protein. One set of mutations modified global physicochemical properties such as mean β-sheet propensity and mean charge, whereas the other set incorporated changes that enhanced β-strand contiguity (β-SC), a local property. Each approach was successful in producing mutants of β-syn that readily assembled into filaments. The results were also compared with those for β-syns from mouse and chicken, predicted to have higher fibrillogenic propensities than human β-syn.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-type and Mutant Synucleins—The expression constructs of human α-syn and β-syn in pRK172 have been described (20). Point mutations in α-syn and β-syn were introduced using site-directed mutagenesis with QuikChange (Stratagene) and checked by DNA sequencing. Chicken β-syn (35) was subcloned into pRK172 from pBluescript (kindly donated by Dr. V. Buchman). Mouse β-syn (36) was amplified by PCR from mouse brain QUICK-Clone cDNA (Clontech Laboratories Inc.) and subcloned into pRK172. Bacterial expression and purification were as described (20, 24). Protein concentrations were determined by quantitative amino acid analysis.
Filament Assembly—For assembly, synuclein proteins were used at 400 μM in 30 mM MOPS, pH 7.2, containing 0.02% sodium azide and 20 μM thioflavin T (THI; Sigma-Aldrich), placed in a shaking incubator at 37 °C for up to 96 h and quantitatively assayed by THI fluorescence, as described (33). After 96 h, degradation of some proteins was variably present. No synucleins were observed to begin to assemble after 96 h; we therefore decided that a 96 h cut-off would provide appropriate data to assay for filament formation while avoiding any undue influence of protein degradation. For a semi-quantitative assessment of filament assembly, transmission electron microscopy was used, as described (33). Briefly, aliquots of assembly mixtures were placed on carbon-coated 400-mesh grids and stained with 1% potassium phosphotungstate, and micrographs recorded at a nominal magnification of ×20,000 on a Philips model EM208S microscope.

As in our previous study (33), THI was included in the assay solution ab initio. As a result, we found fluorescence values to be more stable and more sensitive to small increases in the early stages of filament formation. The correlation of THI fluorescence with filament numbers as observed by time point electron microscopy was clearer for early stages of growth than for late stages of growth.

Averaged Thioflavin T Fluorescence Growth Curves—The THI fluorescence curves shown were plotted from the averages of all available fluorescence data, including experiments in which fluorescence had not increased from the zero time point, indicating that filament assembly had not begun (the emission readings at 480 nm were often marginally above the value of the buffer alone, but either they did not increase over time, or their increase was very small). This was done so that each THI fluorescence plot would more accurately represent the fibrillogenic propensity of a given synuclein, such that where filaments were observed to form in only a proportion of the assays, the average plot should be lower than a plot that excluded these data.

We preferred to remain as close as possible to the raw data and therefore fitted growth curves to the data points with a simple scatter plot using Excel (Microsoft). Filament assembly growth curves have previously been fitted to a variety of curve equations (reviewed by Morris et al. (40)). However, we did not find that fitting the data to a logistic curve, for example, improved our results, nor did we want to risk biasing lag time estimations or assuming any particular kinetic model(s).

Estimating the Fibrillogenic Propensity from Thioflavin T Fluorescence Growth Curves—The fibrillogenic propensity of synucleins, in terms of the “lag time” of assembly, was estimated as before (33). The lag time measured in this way correlated well with the earliest observed filaments at the start of assembly and therefore serves as a description of the time taken for filaments to start forming. Lag times were calculated for each experiment in which filament had started to form, and the averages and S.E. were calculated from these and not from the averaged growth curves. This means that the apparent lag time of the average plot will usually not be the same as the quoted averaged lag times from the various separate experiments.

Other studies have calculated the lag time by extrapolation of the midpoint slope of the growth curve to intersect the time axis (37–39). We avoided this method, because it assumes that the growth rate is coupled to the start of assembly, such that the faster the rate of filament growth at the midpoint, the longer the preceding lag time.

Calculation of Fibrillogenic Properties—The MBP, MHC, MNC, and MTC were calculated in the same way as in our previous papers (33, 34), using data from (Refs. 41 and 42). Trendlines in Figs. 2b, 4b, and 7b were fitted using Excel (Microsoft).

SALSA β-SC plots were produced as described (34). The integrals of the SALSA β-SC (β-SC) plots measure the area covered by all peaks from the whole sequence. Trendlines in these plots were fitted using Excel (Microsoft).

The ordering of constructs in Fig. 2e is based on calculated fibrillogenic propensities using a linear combination of the fibrillogenic propensity of human α-synuclein and β-synuclein (34, 35, 36). For each separate calculated property, the observed lag times for the different constructs can be fitted approximately by an exponential curve. A better fitting to the measured lag times can be produced by an exponential fit using a linear combination of the five calculated properties, as in the following equations: mprops = α(nMBP) − β(nMHC) − c(nMNC) − d(nMTC) and combined = emprops + f(mβ-SC), where n indicates a normalized value. Based on the observed lag times for 32 synuclein constructs, the fitted values of the coefficients are α = 0.074, b = 0.066, c = 0.063, d = 0.060, e = 0.403, and f = 0.209 (R² = 0.71). For any given construct, using this model, the combined calculated properties thus give a prediction of relative fibrillogenic propensity.

RESULTS

Enhancing the Global Fibrillogenic Propensity of Human β-Synuclein—The amino acid sequences of human α-syn and β-syn are aligned in Fig. 1. To enhance the fibrillogenic propensity of human β-syn by global changes, five alanines were substituted with five valines (β-syn(5v)), thereby increasing the
MβP. The positions of the mutations were spread out and located (Table 1) in a way that would minimize any effects on local β-strand propensity, measured by SALSA β-SC. Besides these five substitutions, we engineered a further four mutants called β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), and β-syn(5V/8Q), with two, four, six, and eight glutamate-to-glutamine substitutions (Table 1), such that in combination with an increased MβP, there was also a reduction in the absolute value of MNC and MTC. We made an additional mutant that lacked the alanine-to-valine substitutions (β-syn(8Q)), so that although the MNC and MTC were equivalent to those of β-syn(5V/8Q), the MβP was nearly identical to that of β-syn. The positions of the mutations are listed in Table 1 and illustrated in Fig. 2a.

The effect on the global physicochemical properties (mprops) is compared for the seven proteins in Fig. 2b, arranged in order of their fibrillogenic propensities according to their lag times. α-syn is included alongside the β-syn for comparison. Overlapping SALSA β-SC plots of β-syn and the six β-syn mutant proteins are shown in Fig. 2c, split into two plots for improved clarity. The β-SC plot of α-syn is included for comparison. Below this, the integrals of SALSA β-SC plots are shown, arranged in order of their fibrillogenic propensities, according to their lag times. The integral of the β-SC of α-syn is included alongside for comparison. Filament assembly of α-syn was compared with that of β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q) (Fig. 2d). The lag time of each experiment is illustrated for each protein, arranged in order of fibrillogenic properties, according to combined mprops and β-SC (Fig. 2e). α-syn is included alongside the β-syns for comparison. This figure clearly illustrates the overall trend, which correlates with the combined mean physicochemical properties of the seven β-syn proteins. This is despite the significant variation in lag times observed between different assays of a particular protein.

The assembly properties of α-syn, β-syn, the β-syn(5VnQ) mutants, and β-syn(8Q) are listed in Table 2, showing the proportion of assays in which filaments had started to form by 96 h. Thus, of a total of 44 α-syn preparations run concurrently with the assembly assays, all assembled into filaments within 96 h. The fluorescence readings of the mutants as a percentage of α-syn at 96 h were calculated from these plots and are listed in the fourth column of Table 2.

The average lag time for all α-syn assemblies run concurrently with the β-syn(5VnQ) mutants was 11.0 (± 1.1) h (n = 28). β-syn had not begun to assemble by 96 h (n = 19). Although there were no significant changes in the ThT fluorescence readings for β-syn, the background value was not subtracted, so that at 96 h the average fluorescence reading (essentially unchanged from 0 h) was 1% of that of α-syn. Occasionally, assembly into short filaments was observed for β-syn (data not shown), but in this case, assembly always coincides with premature degradation of the protein. β-syn(5V) assembled into filaments in five of ten assays with an average lag time of 69 (± 5.3) h. Filament formation was not detected in the other five assays. The average ThT growth curve had not plateaued by 96 h, when it had reached 10% of the average plot of the concurrently run α-syn. β-syn(5V/2Q) assembled into filaments in two of seven assays, with lag times of 19 and 36 h. The average ThT growth curve had not plateaued by 96 h, when its value had reached 3.8% of that of α-syn. β-syn(5V/4Q) assembled into filaments in six of eight assays with an average lag time of 33 (± 14) h. The average ThT growth curve had begun to plateau from ~48 h, and its value had reached 28% of that of α-syn by 96 h. β-syn(5V/6Q) assembled into filaments in all assays with an average lag time of 30 (± 7.9) h (n = 9). The average ThT growth curve had not plateaued by 96 h, when its value had reached 155% of that of α-syn. β-syn(5V/8Q) assembled into filaments in 12 of 13 assays with an average lag time of 21 (± 4.9) h. The average ThT growth curve had not plateaued by 96 h, when its value had reached 128% of that of α-syn. β-syn(8Q) assembled into filaments in one of four assays with a lag time of 71 h. The average ThT growth curve suggested that assembly had not plateaued by 96 h, when its value had reached 3.3% of that of α-syn.

By electron microscopy the number of filaments matched well with the ThT growth curves (Fig. 3). α-syn produced several different filament morphologies. A number of straight and variably twisted ribbons could be observed, even within individual samples. The β-syn(5VnQ) mutants produced a range of filament morphologies, apparently made up of two protofilaments twisting around one another. These could be distinguished by the cross-over spacings, as well as by their general appearance, not unlike the straight and paired helical tau filaments of Alzheimer disease (44). The five mutants (β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), and β-syn(5V/8Q)) appeared to produce similar filament morphologies but
with different proportions of the various classes. Examples from three of the five mutants are shown in Fig. 3.

**Enhancing Local Fibrillogenic Propensities of \( \beta \)-Synuclein—**

To enhance the fibrillogenic propensity of human \( \beta \)-syn by local changes, we substituted amino acids that would produce the largest increase in \( \beta \)-SC with the least number of substitutions, while minimizing changes to global properties. To achieve this, a single or double substitution was located to the midpoint of peaks I and II in the \( \beta \)-SC plot, bridging them into a single larger peak. Thus, an arginine was substituted with a valine at position 45, producing \( \beta \)-syn(R45V). A glutamate was substituted with a valine at position 46, producing \( \beta \)-syn(E46V). A double mutant \( \beta \)-syn(45VV46) was also made. The same changes were made in \( \alpha \)-syn, which has a lysine instead of an arginine at position 45. The positions of the mutations are illustrated in Fig. 4a. In Fig. 4b, the effect on the global mprops is compared for each of the two sets of four proteins, arranged in order of their fibrillogenic propensities according to their lag times. In Fig. 4c, the overlapping SALSA \( \beta \)-SC plots are shown above their integrals, arranged in order of their fibrillogenic propensities according to their lag times. Filament assembly of \( \alpha \)-syn was compared with that of \( \alpha \)-syn(K45V), \( \alpha \)-syn(E46V), and \( \alpha \)-syn(45VV46) (Fig. 4d). Filament assembly of \( \alpha \)-syn was also compared with that of \( \beta \)-syn, \( \beta \)-syn(R45V), \( \beta \)-syn(E46V), and \( \beta \)-syn(45VV46) (Fig. 4e). The average lag time for \( \alpha \)-syn assemblies run concurrently with the \( \alpha \)-syn and \( \beta \)-syn mutants was 9.6 (± 1.3) h \((n = 15)\). \( \beta \)-syn had not begun to assemble by 96 h \((n = 6)\). As above, the background value was not subtracted, so that the average fluorescence reading of \( \beta \)-syn was 0.6% of that of \( \alpha \)-syn. \( \alpha \)-syn(K45V) assembled into filaments with an average lag time of 5 (± 1.2) h \((n = 4)\). The average ThT growth curve had plateaued by 24 h, and by 96 h its value had reached 297% of that of \( \alpha \)-syn. \( \alpha \)-syn(K45V) assembled into filaments with an average lag time of 2 (± 0.3) h \((n = 3)\). The average ThT growth curve had plateaued by 24 h, and its value had reached 148% of that of \( \beta \)-syn by 96 h. \( \alpha \)-syn(45VV46) assembled into filaments with an average lag time of 1 (± 0.5) h \((n = 8)\). The average ThT growth curve had plateaued by 24 h, and by 96 h its value had reached 160% of that of \( \alpha \)-syn. \( \beta \)-syn(R45V) assembled into filaments in one of four assays with a lag time of 70 h. It is unclear whether the ThT growth curve averaged from these four assays had plateaued, but its value at 96 h was 5% of that of \( \alpha \)-syn. \( \beta \)-syn(E46V) assembled into filaments with an average lag time of 2 (± 0.3) h \((n = 3)\). The average ThT growth curve had plateaued by 24 h, and its value had reached 148% of
Fibrillogenic Propensity of Mouse and Chicken β-Synucleins—

Based on available sequences, the mean physicochemical properties and β-SCs of fish β-syns predict that they are less fibrillogenic than human β-syn. The properties of amphibian and mammalian β-syns, other than mouse, are virtually the same as those of human β-syn. Mouse β-syn has slightly less MHL, more MβP, less MTC, and more β-SC than human β-syn. It therefore has a higher predicted fibrillogenic propensity than human β-syn. Human and mouse β-syn sequences differ only at four positions (Fig. 6), with mouse β-syn having a lysine in place of an arginine at position 86 and a serine in place of a proline at position 122, with the latter inducing a small increase in M

The positions of the sequence differences are illustrated in Fig. 7a. The four mprops are compared for all four β-syns in Fig. 7b, arranged in order of their fibrillogenic propensities according to their lag times. Human α-syn is included alongside the β-syns for comparison. Overlapping SALSA β-SC plots of all four proteins are shown in Fig. 7c with the β-SC plot of human α-syn included for comparison. Below this, the integrals of SALSA β-SC plots are shown, arranged in order of their fibrillogenic propensities according to their lag times. The integral of the β-SC of human α-syn is included alongside for comparison. Filament assembly of human α-syn was compared with that of human β-syn, mouse β-syn, chicken β-syn, and human β-syn(45VV46) (Fig. 7d).

The average lag time for the human α-syn assemblies run concurrently was 11 (± 2) h (n = 11). Human β-syn had not begun to assemble by 96 h (n = 5). The average ThT fluorescence value had reached 1.2% of that of human α-syn (which is only marginally above background). Mouse β-syn assembled into filaments in two of six assays, with lag times of 49 and 88 h. In the remaining four assays, small increases in fluorescence were detected at 96 h. The average ThT growth curve had not

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**TABLE 2**

| Name of protein | Assembled | Average lag time | αWT |
|----------------|-----------|------------------|-----|
| α-syn          |           | 11 ± 0.9         | 100 |
| β-syn          |           | 0 ± 0.2          | 1   |
| β-syn(5V)      | 5/10      | 69 ± 5.3         | 10  |
| β-syn(5V/2Q)   | 2/7       | 19 and 36        | 3.8 |
| β-syn(5V/4Q)   | 6/8       | 35 ± 14          | 28  |
| β-syn(5V/6Q)   | 9/9       | 30 ± 7.9         | 155 |
| β-syn(5V/8Q)   | 12/13     | 21 ± 4.9         | 128 |
| β-syn(8Q)      | 1/4       | 71               | 3.3 |
| β-syn(αK45)    | 4/4       | 51 ± 1.2         | 297 |
| β-syn(E46V)    | 3/3       | 20 ± 1.3         | 148 |
| β-syn(45VV46)  | 8/8       | 1.3 ± 0.5        | 160 |
| β-syn(45V)     | 1/4       | 70               | 5.1 |
| β-syn(E46V)    | 5/5       | 15 ± 6.1         | 36.5|
| β-syn(45VV46)  | 5/5       | 23 ± 10          | 58  |
| Mouse β-syn    | 2/6       | 49 and 88        | 7.8 |
| Chicken β-syn  | 0/5       | NA               | 1.7 |

* Calculated for each individual experiment with filament formation and then averaged (not calculated from the averaged ThT growth curves).
* NA, not applicable.

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**FIGURE 2.** a, schematic representation of human β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q). The locations of amino acid substitutions are indicated by black bars. Substitutions are given in detail in Table 2. b, MβP (orange), MHL (blue), MTC (red), and MTC (green) for β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q), placed in order of their lag times. They are separated into three groups, from the left, those that assembled within 96 h in: ≥50% of assays, <50% of assays, and 0% of assays. Trendlines are shown. α-syn mprops are included for comparison (white diagonal stripes). c, overlapping SALSA β-SC plots for β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q), placed in order of their lag times. They are separated into three groups, from the left, those that assembled within 96 h in: ≥50% of assays, <50% of assays, and 0% of assays. Trendlines are shown. The β-SC of α-syn is included for comparison (white diagonal stripes). d, growth curves of filament assembly for α-syn, β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q). Assembly was monitored by the enhancement of ThT fluorescence over time. The results are presented as normalized fluorescence (with the value for α-syn at 96 h taken as 100) and expressed as the means ± S.E. of the measurements obtained from at least three separate protein preparations. e, the trend of assembly lag times with fibrillogenic propensities, calculated by mprops. Lag times of α-syn (n = 28), β-syn (n = 19), β-syn(5V) (n = 10), β-syn(5V/2Q) (n = 7), β-syn(5V/4Q) (n = 8), β-syn(5V/6Q) (n = 9), β-syn(5V/8Q) (n = 13), and β-syn(8Q) (n = 4) are placed in order of their fibrillogenic propensities, estimated by mprops. Each assembly assay is represented by a white circle.

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**FIGURE 3.** a, schematic representation of human β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q). The locations of amino acid substitutions are indicated by black bars. Substitutions are given in detail in Table 2. b, MβP (orange), MHL (blue), MTC (red), and MTC (green) for β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q), placed in order of their lag times. They are separated into three groups, from the left, those that assembled within 96 h in: ≥50% of assays, <50% of assays, and 0% of assays. Trendlines are shown. α-syn mprops are included for comparison (white diagonal stripes). c, overlapping SALSA β-SC plots for β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q), placed in order of their lag times. They are separated into three groups, from the left, those that assembled within 96 h in: ≥50% of assays, <50% of assays, and 0% of assays. Trendlines are shown. The β-SC of α-syn is included for comparison (white diagonal stripes). d, growth curves of filament assembly for α-syn, β-syn, β-syn(5V), β-syn(5V/2Q), β-syn(5V/4Q), β-syn(5V/6Q), β-syn(5V/8Q), and β-syn(8Q). Assembly was monitored by the enhancement of ThT fluorescence over time. The results are presented as normalized fluorescence (with the value for α-syn at 96 h taken as 100) and expressed as the means ± S.E. of the measurements obtained from at least three separate protein preparations. e, the trend of assembly lag times with fibrillogenic propensities, calculated by mprops. Lag times of α-syn (n = 28), β-syn (n = 19), β-syn(5V) (n = 10), β-syn(5V/2Q) (n = 7), β-syn(5V/4Q) (n = 8), β-syn(5V/6Q) (n = 9), β-syn(5V/8Q) (n = 13), and β-syn(8Q) (n = 4) are placed in order of their fibrillogenic propensities, estimated by mprops. Each assembly assay is represented by a white circle.

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plateaued by 96 h, when it reached 7.8% of that of human α-syn. Chicken β-syn produced small increases in fluorescence in three of five assays at 96 h, although these increases were too small to allow us to calculate a lag time (n = 5). A few long filaments were observed at later time points in the absence of detectable protein degradation. The average fluorescence had reached 1.7% of that of human α-syn by 96 h.

The majority of filaments of mouse β-syn (Fig. 7e) had a uniform appearance and a width of ~13 nm, but a small minority appeared as narrow twisted ribbons with variable cross-over spacing. Chicken β-syn filaments showed two distinct morphologies (Fig. 7e). Some appeared as twisted filaments with a maximum width of 15 nm and a cross-over spacing of 130 nm; others appeared as uniform straight filaments with a width of ~18 nm.

**Comparison of mprops and SALSA β-SC with Kyte & Doolittle Hydropathy Plots and Amyloidogenicity Prediction Algorithms**—We compared mprops and SALSA β-SC outputs for the 16 synucleins assayed here with those of Kyte & Doolittle hydropathy plots (46) and 13 amyloidogenicity prediction algorithms (see supplemental materials) (47–64). Many of the algorithms did not detect differences between the β-syn(5VnQ) mutants. None of the algorithms (including mprops and SALSA) predicted β-syn(E46V) to be more amyloidogenic than β-syn(K45V/E46V). Overall, the combined calculation of mprops and SALSA β-SC correlated best with the experimental data.

**DISCUSSION**

We have rendered human β-syn fibrillogenic by rational design of global and local physicochemical properties, using two algorithms developed in earlier work (33, 34).

**Changing Global Properties of β-Synuclein**—Four global physicochemical properties influence the fibrillogenic propensities of α-syn and β-syn. They are the MβP, MHL, MNC, and MTC. Filament formation is favored by a higher MβP, less MHL, less MTC, and a lower absolute value of MNC. In the present study, we mutated β-syn to alter three of the four global properties by substituting alanines with valines to raise the MβP and by substituting glutamates with glutamines to lower both the MTC and the absolute value of MNC. The mutant with only the alanine-to-valine substitutions did not assemble as well as mutants that also included glutamate-to-glutamine substitutions, whereas the mutant that had eight glutamate-to-glutamine substitutions, but no alanine-to-valine substitutions, assembled at a low level with a long lag time. The mutated proteins displayed altered fibrillogenic propensities (as monitored by ThT fluorescence and electron microscopy) that were dependent on the combination of changes. Further
Figure 4. a, schematic representation of human α-syn, α-syn(K45V), α-syn(E46V), α-syn(E46V), β-syn, β-syn(R45V), β-syn(E46V), and β-syn(45VV46). The locations of residues 45 and 46 are indicated by black bars. b, MIP (orange), MHL (blue), MNC (black), and MTC (green) are shown on the left half for α-syn, α-syn(K45V), α-syn(E46V), and α-syn(45VV46) and on the right half for β-syn, β-syn(R45V), β-syn(E46V), and β-syn(45VV46), placed in order of their lag times. The β-syns are separated into three groups, from the left, those that assembled within 96 h in: 50% of assays, <50% of assays, and 0% of assays. Trendlines are shown. c, overlapping SALSA β-SC plots shown for α-syn, α-syn(K45V), α-syn(E46V), and α-syn(45VV46) above those for β-syn, β-syn(R45V), β-syn(E46V), and β-syn(45VV46). Three peaks, spanning residues 32–89, are annotated I, II, and III, as before. d, growth curves of filament assembly for α-syn, α-syn(K45V), α-syn(E46V), and α-syn(45VV46). Assembly was monitored by the enhancement of ThT fluorescence over time. The results are expressed as the means ± S.E. of the measurements obtained from at least three separate protein preparations. e, growth curves of filament assembly for β-syn, β-syn(R45V), β-syn(E46V), and β-syn(45VV46). Assembly was monitored by the enhancement of ThT fluorescence over time. The results are expressed as the means ± S.E. of the measurements obtained from at least three separate protein preparations.
more, there was a continuous correlation between the combined global properties and fibrillogenic propensities, suggesting that the amino acid composition influences the thermodynamics of protein association. The \( \beta \)-syn(5VnQ) mutants displayed increasing fibrillogenic propensities with increasing changes in global properties as a result of increasing numbers of amino acid substitutions. The \( \beta \)-syn(5VnQ) mutants have almost identical \( \beta \)-SC plots. Therefore, their different fibrillogenic propensities are the result of their different global properties. Global properties also largely account for the higher fibrillogenic propensity of the \( \beta \)-syn(5VnQ) mutants compared with wild-type \( \beta \)-syn and \( \beta \)-syn(8Q). However, \( \beta \)-SC may also contribute in a minor way to this difference, because two of the five alanine-to-valine substitutions in \( \beta \)-syn(5VnQ) (at positions 11 and 19) result in a small new \( \beta \)-SC peak (spanning residues 1–27) that is absent from wild-type \( \beta \)-syn and \( \beta \)-syn(8Q).

Changing \( \beta \)-Strand Contiguity (Local Property)—\( \alpha \)-Synuclein filaments are characterized by an amyloid-like cross-\( \beta \) core structure (19, 65, 66). The SALSA \( \beta \)-SC method is based on the supposition that within a natively unfolded protein, the more \( \beta \)-strand favorable residues there are in close proximity to each other, the more likely that region is to form the cross-\( \beta \) core (34). Accordingly, we would expect that filament formation will be favored by more \( \beta \)-SC. We have assayed \( \alpha \)-syn(45VV46) alongside two new mutants, in which Lys45 and Glu46 have been mutated individually to valines. Both mutations bridge \( \beta \)-SC peaks I and II, increasing their size by an approximately equivalent amount but less than that of the double valine mutant. The fibrillogenic propensities of these proteins reflect these changes, so that \( \alpha \)-syn(45VV46) assembled with the shortest lag time, followed by \( \alpha \)-syn(E46V) and \( \alpha \)-syn(K45V), which had shorter lag times than...
wild-type α-syn. The fact that α-syn(E46V) assembled with a shorter lag time than α-syn(K45V) reinforces the influence of mprops in MNCs, which in this case is primarily because of different fibrillogenic changes to the other three mean properties and the β-SC. This would explain why, whereas β-syn(45VV46) had a longer lag time than β-syn(45EV6), α-syn(45VV46) had a shorter lag time than α-syn(45EV6), confounding the fact that their relative changes to global properties are the same.

Mouse and Chicken β-Synucleins—Synuclein sequences from 26 different vertebrate species had previously been collated, which included 16 β-syns. We calculated the four global properties and SALSA β-SC for each of the 15 nonhuman-β-syn sequences from this list, as well as for five additional β-syns from Anoplopoma fimbria, Takifugu rubripes, Osmerus mordax, Macaca fascicularis, and Sus scrofa. This included β-syns from fish (n = 12), amphibians (n = 2), mammals (n = 5), and a bird (n = 1). None of them had higher predicted fibrillogenic propensities than human-β-syn, except for mouse and chicken. Both mouse and chicken β-syns displayed more β-SC, because of differences from human β-syn at Arg45 and Glu46, such that peaks I and II were larger. Both sequences are characterized by the absence of arginine at position 45 and the substitution of a negatively charged residue with an uncharged polar residue at position 46 (all other β-syns looked at have a positively charged residue at position 45 and a negatively charged residue at position 46). Although it was expected that the effects of these differences would not be as strong as those of the designed β-syn valine mutants, the mprops and SALSA β-SC plots of mouse and chicken β-syn suggested that they might be capable of some filament assembly, albeit with a longer lag time. Mouse β-syn did begin to assemble within 96 h, whereas chicken β-syn produced fluorescence increases in some assays, although these were below the minimum level required for calculating lag times by our method. By electron microscopy, a small number of long chicken β-syn filaments could be seen at later time points that did not appear to result from protein degradation. The similarities in amino acid sequence between residues 30 and 60, in particular at positions 45 and 46, of mouse and chicken β-syns, give rise to similar β-SC plots. However, differences in the rest of the sequence render chicken β-syn less fibrillogenic than mouse β-syn. Based on a combination of mprops and SALSA β-SC, the order of predicted fibrillogenic propensities was mirrored by observed filament assemblies: human β-syn < chicken β-syn < mouse β-syn << human-β-syn(45VV46).

Filament Morphologies—By negative stain transmission electron microscopy, our assembly assays always produced more
than one type of filament morphology. Although classification criteria would need to be defined, we observed up to six distinct morphologies in any one sample. Different constructs produced distinct populations of morphologies. Some morphologies appeared to be common to more than one construct (including α-syn). As noted previously, α-syn(E46K) produced a highly twisted morphology that was reminiscent of that of the disease-causing mutant, α-syn(K45V) and α-syn(E46V) did not produce E46K-like twisted filaments but did appear to produce filament morphologies different from those of α-syn.

mprops and SALSA β-SC Compared with Kyte & Doolittle Hydropathy Plots and Amyloidogenicity Algorithms—Rationalizing the sequence determinants for protein aggregation was initially qualitative (67, 68) and then increasingly quantitative (69–71). More recently, a number of algorithms have been developed to quantify sequence determinants of protein aggregation (33, 34, 47–64). We compared their outputs with those of mprops and SALSA β-SC, as well as with hydropathy plots produced using the algorithm of Kyte & Doolittle (46). We found that the combination of mprops and SALSA β-SC correlates better than the other 14 algorithms with the fibrillogenic propensities of synucleins. Part of the reason for this may be the fact that mprops was informed by our experimental data with synucleins and would therefore be expected to correlate better with our ThT data. SALSA β-SC was inspired by studies with synuclein filament assembly, but its algorithm was not calibrated by our ThT data (34). Another reason for the better correlation may be that the other algorithms are not designed to measure relative fibrillogenic propensities of whole proteins but rather to identify amyloidogenicity or aggregation-prone regions within the sequence of a protein or otherwise to determine whether a short peptide will assemble. mprops is not designed to identify contiguous regions of hydrophobicity, β-sheet propensity, charge, or amyloidogenicity, and it is the only algorithm that measures physicochemical properties as a mean of the whole protein. Many algorithms failed to detect differences between the β-syn(5VnQ) mutants, but this could be corrected in some cases by taking the total sum
of ordinates (rather than the sum of ordinates above a pre-determined threshold), thereby representing a quasi-global measurement.

We hypothesize that the preference of amino acid side chains for particular three-dimensional structures in the amyloid \( \beta \)-sheet core may have more influence on filament assembly at the level of protofibril associations and filament morphologies than on fibrillogenic propensities and nucleation. As such, a more comprehensive understanding of sequence determinants for filament assembly might benefit from identifying which sequence properties most affect which stages.

**Summary and Molecular Model Encompassing Global and Local Stages in Filament Formation**—Our previous (33) and present findings demonstrate that two separate physicochemical modalities influence the nucleation and early stages of synuclein filament formation. We hypothesize that the way in which the two sets of properties (global and local) are calculated correlates with two distinct stages in filament formation. Furthermore, our data indicate that there may be a temporal relationship between these stages, namely that global properties influence an earlier stage in filament formation than \( \beta \)-SC. The global manner in which the four mean properties are calculated may correlate with an association stage in the generation of amorphous aggregates. The formation of amorphous composites and intermediates, prior to the formation of filaments, has been reported for \( \alpha \beta \), Sup35NM, and the prion protein (59, 61, 63).

**Concluding Remarks**—Our findings are 3-fold: (i) we have, for the first time, rendered the nonfibrillogenic protein \( \beta \)-syn fibrillogenic by using designed mutations; (ii) we have demonstrated that \( \beta \)-syn can be rendered fibrillogenic by the use of two simple but distinct algorithms that calculate physicochemical properties; and (iii) we have derived a molecular model that reflects the 2-fold nature of the algorithms and their relative mathematical simplicity.

We hypothesize that filament formation of synucleins is primarily a thermodynamic process of protein condensation that incorporates an association stage, followed by the evolution of...
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