

A Rationally Designed Six-Residue Swap Generates Comparability in the Aggregation Behavior of α -Synuclein and β -Synuclein

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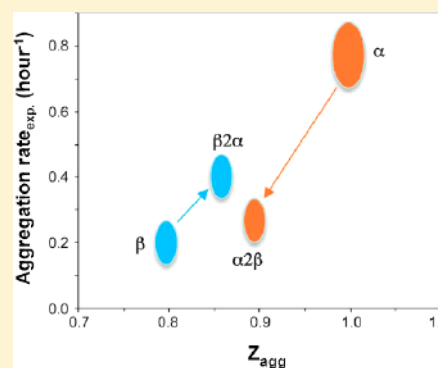
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S Supporting Information

ABSTRACT: The aggregation process of α -synuclein, a protein closely associated with Parkinson's disease, is highly sensitive to sequence variations. It is therefore of great importance to understand the factors that define the aggregation propensity of specific mutational variants as well as their toxic behavior in the cellular environment. In this context, we investigated the extent to which the aggregation behavior of α -synuclein can be altered to resemble that of β -synuclein, an aggregation-resistant homologue of α -synuclein not associated with disease, by swapping residues between the two proteins. Because of the vast number of possible swaps, we have applied a rational design procedure to single out a mutational variant, called $\alpha 2\beta$, in which two short stretches of the sequence in the NAC region have been replaced in α -synuclein from β -synuclein. We find not only that the aggregation rate of $\alpha 2\beta$ is close to that of β -synuclein, being much lower than that of α -synuclein, but also that $\alpha 2\beta$ effectively changes the cellular toxicity of α -synuclein to a value similar to that of β -synuclein upon exposure of SH-SY5Y cells to preformed oligomers. Remarkably, control experiments on the corresponding mutational variant of β -synuclein, called $\beta 2\alpha$, confirmed that the mutations that we have identified also shift the aggregation behavior of this protein toward that of α -synuclein. These results demonstrate that it is becoming possible to control in quantitative detail the sequence code that defines the aggregation behavior and toxicity of α -synuclein.



The analysis of the physicochemical properties of amino acid sequences has enabled very significant progress to be made toward understanding the principles of protein aggregation.¹ It has been demonstrated that the fundamental properties of the amino acids, such as their electrostatic charge and hydrophobicity, play a crucial role in determining the aggregation behavior of proteins *in vitro*,^{1–10} and increasing evidence suggests that this is also the case *in vivo*.^{11–13} It is particularly interesting to explore the use of this approach in the case of proteins involved in neurological disorders associated with protein deposition such as Alzheimer's and Parkinson's diseases.¹⁴ One such protein, α -synuclein, represents the primary component of Lewy bodies, the intracellular aggregates found in dopaminergic neurons of patients affected by Parkinson's disease.¹⁵ Considerable efforts have been devoted to the study of the behavior of this protein,^{16–20} in particular its aggregation process,^{21–26} which involves the formation of potentially neurotoxic oligomers.^{21,27–31}

A major aspect of this quest has been to understand why α -synuclein and β -synuclein, which are closely related natural homologues (Scheme 1) and both present in dopaminergic cells, exhibit dramatic differences in their aggregation

behavior,^{24,26,32–36} so that while α -synuclein readily aggregates both *in vitro* and *in vivo*, β -synuclein does so much more reluctantly. In order to understand the origin of this difference, systematic mutational studies have been carried out in which the effects on the aggregation rates of a range of amino acid substitutions have been monitored.^{5,26} The results of these studies revealed that the most prominent difference in the sequence of α -synuclein and β -synuclein—the absence in β -synuclein of an 11-residue aggregation-prone portion (residues 73–83) of the sequence of α -synuclein within the so-called NAC region (residues 61–95)—is not the primary cause of the different aggregation behavior.^{5,26} This result is particularly interesting as the NAC region is found within the cross- β structure in the fibrillar assemblies formed by α -synuclein.^{37–40}

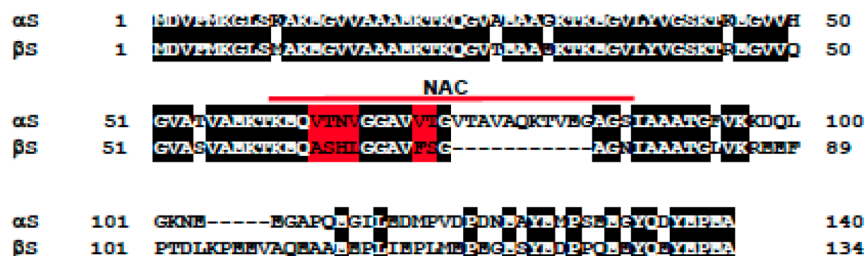
Progress toward resolving this problem has been made by showing that it is possible to alter very significantly the aggregation rates of α -synuclein or β -synuclein by specific amino acid substitutions that change profoundly the

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Scheme 1. Sequence Alignment of α -Synuclein and β -Synuclein^a



^aThe sequence identity of these two proteins is 62%, with the large majority of the differences localized in the C-terminal region. The two stretches of residues in the NAC region (63–66 and 71–72) that were swapped between α -synuclein and β -synuclein to obtain the two mutational variants examined experimentally in this work, $\alpha 2\beta$ and $\beta 2\alpha$, are highlighted in red.

physicochemical properties of the sequences.^{5,26,41–43} For example, it has been demonstrated that the introduction of two contiguous electrostatic charges in α -synuclein inhibits dramatically the formation of fibrillar structures.⁵ A question that has remained unanswered, however, is exactly which of the amino acid substitutions that have occurred in nature and that have resulted in the divergence of the sequences of α -synuclein and β -synuclein are primarily responsible for the different behavior of these two proteins. In this work we make a significant step toward answering this question by systematically applying a rational design procedure to identify a single mutational variant, called $\alpha 2\beta$, and the complementary mutant, $\beta 2\alpha$, and then studying their aggregation and cytotoxicity properties.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutations at specific positions of the DNA sequences encoding human α -synuclein and β -synuclein were introduced by site-directed mutagenesis using the overlap-extension PCR strategy.⁴⁴ Restriction sites *Nde* I and *Bgl* II were used for subcloning into the pT7–7 plasmid for the α -synuclein and $\alpha 2\beta$ constructs or the pRK172 plasmid for the β -synuclein and $\beta 2\alpha$ constructs. Targeted mutations for the α -synuclein gene were V63A, T64S, N65H, V66L, V71F, and T72S, using the oligodeoxynucleotides pairs V63–66 for 5′gcaagcctcacatctgggaggagcagtgtg3′ and V63–66 Bac 5′cagatgtgaggtgtgtctttgtgtcttc3′, and V71–72 for 5′aggagcagtgttcggtgtgacagcag3′ and V71–72 Bac 5′acacccgagacagctcctccacatttg3′. Mutations for the β -synuclein gene were A63V, S64T, H65N, L66V, F71V, and S72T, using oligodeoxynucleotides A63–66 for 5′acaggtgacaaatgttgaggagcagtgtg3′ and A63–66 Bac 5′aacattgtcacctgttcctgtgttttcagc3′, and F71–72 for 5′agctgtgtgactggggcaggaacatcg3′ and F71–72 Bac 5′gccccagtcacacagctcctccagatg3′. The correct incorporation of the mutations was verified by DNA sequencing.

Protein Expression and Purification. Human recombinant α -synuclein and β -synuclein as well as the variants $\alpha 2\beta$ and $\beta 2\alpha$ were expressed in *E. coli* BL21(DE3) cells using the plasmid pT7-7 for α -synuclein and $\alpha 2\beta$ or the pRK172 plasmid for β -synuclein and $\beta 2\alpha$ and purified as described previously,⁴⁵ with minor modifications in the protocol, as follows. After transformation, BL21(DE3)-competent cells were grown in LB in the presence of ampicillin (100 μ g/mL). Protein expression was induced with 0.4 mM IPTG, and cells were harvested by centrifugation at 3500g after shaking at 30 °C overnight. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and EDTA-free protein inhibitor cocktail (Roche, West Sussex, UK) and lysed by multiple freeze–thaw cycles

and sonication. The cell suspension was boiled for 20 min and centrifuged at 20000g. Streptomycin sulfate was subsequently added to the supernatant to a final concentration of 10 mg/mL, and the mixture was stirred for 15 min at 4 °C. After centrifugation at 13 500 rpm, the supernatant was collected and ammonium sulfate was added (to 0.36 g/mL). The solution was stirred for 30 min at 4 °C and centrifuged again at 13 500 rpm. The pellet was resuspended in 25 mM Tris-HCl (pH 7.7) and loaded onto an HQ/M-column on an Applied Biosystems BIOCAD (Applied Biosystems, Framingham, MA) workstation. The proteins were eluted at ~300 mM NaCl with a salt gradient from 0 to 600 mM NaCl. The pure protein fractions were pooled together and dialyzed extensively at 4 °C against water. Protein purities were >95% as determined by SDS-PAGE, and molecular masses were confirmed by electrospray mass spectrometry. Protein concentrations were estimated from the absorbance measurements at 275 nm using an extinction coefficient of 5600 M^{−1} cm^{−1}. After purification, the protein samples were passed through a 0.22 μ m filter and then stored as a 400 μ M aqueous solution at −80 °C.

SDS Aggregation Assays and Data Analysis. Before starting the aggregation experiments, NaOH was added to the protein solution to give a 10 mM final concentration, and the mixture was incubated on ice for at least 1 h; this treatment was carried out to dissolve possible pre-existing aggregates that could seed further aggregation. The pH of the protein solution was then neutralized using 10 mM HCl. Just prior to the start of the fluorescence experiment, a master mix was added to the different protein solutions, yielding a final concentration of 10 mM phosphate buffer (pH 7.4), 0.1 M NaCl, 0.02% NaN₃, 20 μ M ThT, 350 μ M SDS, and 70 μ M protein, as previously described.²⁶ The aggregation process was accelerated by continuous magnetic stirring at 37 °C with small magnetic stir bars, and ThT fluorescence was monitored using 1 mL quartz cuvettes (Hellma Analytics Ltd., UK) with sampling every 5 min, using a 10 s averaging time. Aggregation of the synuclein variants was monitored by Thioflavin T (ThT) fluorescence using a Cary Eclipse (Varian, Canada) fluorescence spectrophotometer with excitation and emission wavelengths of 450 and 480 nm, respectively. The aggregation reaction was followed in real time by measuring the ThT fluorescence, and the samples were taken out of the instrument when the ThT fluorescence had reached a steady state value.

Aggregation rates (*k*) were calculated from the maximum slopes of the normalized fluorescence intensities. The *t*_{1/2} values, defined as the times required to reach half of the final fluorescence intensity in the aggregation process, were also

calculated. N independent aggregation experiments were carried out, with the number depending on each variant: α -synuclein ($N = 6$), $\alpha 2\beta$ ($N = 5$), β -synuclein ($N = 4$), and $\beta 2\alpha$ ($N = 8$). The aggregation curves were first normalized, then the k and $t_{1/2}$ values were determined for each one, and finally all the values were averaged to define the aggregation rate for each sample.

Preparation of Synuclein Seeds. The four protein variants were processed as described above ("SDS aggregation assays" section), i.e., in the presence of 350 μ M SDS, in order to get mature fibrils formed. The samples were then centrifuged using an Optima Beckman-Coulter ultracentrifuge (Beckman-Coulter, UK) at 120 000 rpm using a TL-100 fixed angle rotor for 30 min. The supernatant was removed and used to quantify the remaining soluble protein by measuring the optical density at 280 nm in a spectrophotometer (Varian, Canada), with a molar extinction coefficient of 5960 $M^{-1} cm^{-1}$. The pellet was dissolved in 10 mM phosphate buffer pH 7.4, 100 mM NaCl, and centrifuged again using a TL-100 rotor and an Optima Beckman-Coulter centrifuge at 120 000 rpm. The supernatant was discarded, and the pellet was retained and dissolved in 10 mM phosphate buffer pH 7.4 and 100 mM NaCl. This fibril suspension was then sonicated using a Bandelin Sonoplus probe sonicator (Bandelin, Germany), which was set at the 10% power level, pulsing 30% of the time over a 5 min period. The resulting sonicated fibrils were used as seeds for further seeded aggregation reactions.

Seeded Aggregation Assays and Data Analysis. 500 μ L samples of each variant were prepared at a final concentration of 70 μ M in 10 mM phosphate buffer pH 7.4, 100 mM NaCl, supplemented with 20 μ M ThT and a total of 10% seeds (on a protein monomer basis) of the respective variant. Two replicas of each sample were applied on a Corning microtiter plate. The plate was subsequently placed in a FluoStar OPTIMA spectrophotometer (BMG LabTech, Ortenberg, Germany), which was previously equilibrated at 30 °C. ThT fluorescence with excitation and emission windows at 440 ± 10 and 480 ± 10 nm, respectively, was measured in real time over a 2 day period. The plate was subjected to continuous orbital shaking at 86 rpm, except during the time of the ThT fluorescence readings.

The seeded growth data were fitted using nonlinear least-squares algorithm implemented in the software Origin (MicroCal Inc., Northampton, MA), using the expression $F = F_0 + (F_\infty - F_0)(1 - e^{-kt})$, where F is the ThT fluorescence, F_0 is the initial ThT fluorescence, F_∞ is the ThT fluorescence after an infinite time, k is the elongation rate, and t is the time.

Preparation of Synuclein Oligomers. The preparation and isolation of synuclein oligomers was based on previously published protocols,^{46,47} with some modifications. Lyophilized purified preparations from a solution in water of the four synuclein variants, namely α -synuclein, β -synuclein, $\alpha 2\beta$, and $\beta 2\alpha$, were dissolved in phosphate-buffered saline (PBS) (0.01 M sodium phosphate buffer [pH 7.4], 150 mM NaCl) to obtain concentrations of 700–900 μ M. Each stock solution was centrifuged at 16 000g for 5 min, and the supernatant was incubated overnight (16–18 h) at 37 °C without shaking. In order to remove any large aggregates, the solution was again centrifuged at 16 000g for 5 min. To isolate the oligomeric species specifically, the supernatant was filtered by centrifugation at 14 000g for 2 min using a 100 kDa cutoff Amicon Ultra membrane, 0.5 mL (Millipore, Billerica, MA) at room temperature. In order to eliminate the remaining monomeric

species, 150 μ L aliquots of PBS were gently added to about 40 μ L of retentate, and centrifugation was performed as before. This rinsing process was repeated three more times. The presence of a high molecular weight oligomeric fraction and the absence of any significant monomeric fraction were verified by electrophoresis with 4%–12% Bis-Tris NuPAGE gels (Invitrogen, Paisley, UK) followed by Western blotting using N19 anti- α/β synuclein polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) according to the manufacturer's specifications. In addition, the oligomer preparations were tested by dot blot against the specific antioligomeric polyclonal antibody A11 (Invitrogen, Paisley, UK) following the manufacturer's protocol and with 2.5 μ g of protein spotted onto a nitrocellulose membrane. Purified oligomeric fractions were stored at 4 °C for up to 24 h. The protein concentration of the fractions was determined by Micro BCA Reagent Kit (Pierce, Rockford, IL).

Transmission Electron Microscopy (TEM). Samples were prepared from 10 μ L aliquots using negative staining by 2% (w/v) uranyl acetate on Formvar/carbon-coated copper grids (Agar Scientific, Essex, UK). Images of fibrils and oligomers were obtained at magnifications of 25 000 \times and 180 000 \times , respectively, using a Phillips CM-10 transmission electron microscope (CITIUS, University of Seville, Spain).

Cytotoxicity Assays. The cytotoxic effects of the four synuclein variants considered in this work were assayed with the MTT reduction technique⁴⁸ on SH-SY5Y neuroblastoma cells in culture as follows. SH-SY5Y cells were cultured in 96-well plates in 200 μ L of complete DMEM-F12 medium and allowed to undergo a maximum of 20 passages for all cytotoxicity assays. Upon reaching a 70% confluent state, the medium was replaced by 100 μ L of complete DMEM-F12 medium containing either oligomers or monomers of the protein variants at a final concentration of 15 μ M. Cells treated with medium alone, and with medium containing 2% Triton X-100 served as maximum (100%) and negative (0%) viability controls, respectively. All wells (including controls) contained the same amount of added PBS to the medium (<12% v/v in all assays). After a 24 h incubation of the cells, their viability was assayed with the MTT Cell Proliferation Kit (Roche, West Sussex, UK) according to the manufacturer's instructions. Cell viability (%) was calculated from four independent experiments ($N = 4$) performed with different oligomer preparations in parallel, each containing sample duplicates.

Rational Design of the $\alpha 2\beta$ and $\beta 2\alpha$ Mutants. In order to design the $\alpha 2\beta$ mutant, we identified a small set of amino acid substitutions capable of decreasing the aggregation rate of α -synuclein to a value comparable to that of β -synuclein. For this purpose we used the Zyggregator method⁴ to predict the aggregation propensity of a large number of mutants obtained by swapping residues between the α -synuclein and β -synuclein sequences, by taking advantage of previous observations that indicated that this method is capable of making accurate predictions of the aggregation propensity of various synuclein forms.²⁶ Neglecting the gaps in the alignment of the α -synuclein and β -synuclein sequences (Scheme 1), we considered 43 residues that can be swapped between the two sequences. We systematically considered swaps of an increasing number of residues, ranging from 1 to 10 (Supporting information Table 1). Depending on the number of residues involved in the swaps, either all the possible swaps or a random subset of 10 000 ones were considered. Among all the mutational variants that we considered, the $\alpha 2\beta$ mutant

emerged as a clear candidate since the difference between its Z_{agg} score and that of β -synuclein (0.10, Table 1) was

Table 1. Comparison between the Predicted Aggregation Propensities (Z_{agg}) and the Experimentally Observed and Aggregation Rates (k) and Aggregation Half-Times ($t_{1/2}$) for α -Synuclein, β -Synuclein, and the Two Mutational Variants ($\alpha 2\beta$ and $\beta 2\alpha$) Analyzed in This Study, in the Presence of 350 μM SDS^a

| variant | Z_{agg} | k (h^{-1}) | $t_{1/2}$ (h) |
|---------------------|------------------|-------------------------|-----------------|
| α -synuclein | 0.98 | 0.78 ± 0.07^b | 1.27 ± 0.12 |
| $\alpha 2\beta$ | 0.90 | 0.26 ± 0.01^c | 5.9 ± 0.8 |
| β -synuclein | 0.80 | 0.20 ± 0.03^d | 18.1 ± 8.1 |
| $\beta 2\alpha$ | 0.86 | 0.41 ± 0.02^e | 6.73 ± 1.1 |

^aThe values of k and $t_{1/2}$ represent the average \pm standard deviation:

^b $N = 6$. ^c $N = 5$. ^d $N = 4$. ^e $N = 8$.

significantly smaller than that of all the other mutants obtained with a 6-residue swap (0.161 ± 0.006 , Supporting Information Table 1). Although by swapping more residues even more similar values in the Z_{agg} scores of the mutants and β -synuclein could be obtained (e.g., on average 0.139 ± 0.005 with 10-residue swaps, Supporting Information Table 1), we limited our swaps to 6 residues for simplicity. Similarly, the difference between the Z_{agg} scores of the $\beta 2\alpha$ mutant and α -synuclein (-0.12) was significantly smaller in absolute value than that of all the other mutants obtained with a 6-residue swap (-0.165 ± 0.005 , Supporting Information Table 2). We note that the values reported here for the Z_{agg} scores of α -synuclein and β -synuclein differ from those in previous publications because we used an upgraded version of the Zyggregator algorithm.

RESULTS AND DISCUSSION

The goal of this study was to identify a small set of amino acid substitutions required to make the aggregation propensity of α -synuclein comparable to that of β -synuclein. To achieve this goal, we used a rational approach in which we generated *in silico* all the possible mutational variants of α -synuclein obtained by swapping between α -synuclein and β -synuclein a set of N residues located at positions in which the two proteins differ in sequence. Starting from $N = 1$, the aggregation propensities of all possible mutational variants were calculated using the Zyggregator method.⁴ By considering increasing values of N , we eventually found that for $N = 6$ it was possible to single out a particular mutant, called $\alpha 2\beta$, with a predicted aggregation propensity, as measured by the Z_{agg} score,⁴ close to that calculated for β -synuclein (Table 1). Although by considering more than six residues the aggregation propensities of the designed mutants can be made even closer to that of β -synuclein, we have limited our substitutions to six residues because our aim has been to identify a very small set of sequence substitutions required to lower the aggregation rate of α -synuclein to that of β -synuclein. Our results indicate that, at least from the point of view of aggregation propensity, the two stretches in the amino acid sequence that we identified (residues 63–66 and 71–72, Scheme 1, red segments), which are both in the NAC region, represent the greatest differences between α -synuclein and β -synuclein. We also considered the complementary mutant, which we called $\beta 2\alpha$, in order to test the effect of the corresponding mutations in β -synuclein; this mutant was not, therefore, specifically designed to have an aggregation propensity similar to that of α -synuclein (Table 1).

The predictions for the aggregation behavior of $\alpha 2\beta$ were then tested by *in vitro* aggregation studies. Given that β -synuclein does not readily aggregate *in vitro*^{5,32,34,42,49,50} except under certain conditions,^{26,50} we first performed our aggregation assays in the presence of a low concentration (350 μM) of sodium dodecyl sulfate (SDS), a well-characterized lipid-mimetic widely applied in α -synuclein biophysical investigations and known to accelerate the protein aggregation process.^{26,51–56} By measuring ThT fluorescence intensities (Figure 1A), we found, as predicted by the Z_{agg}

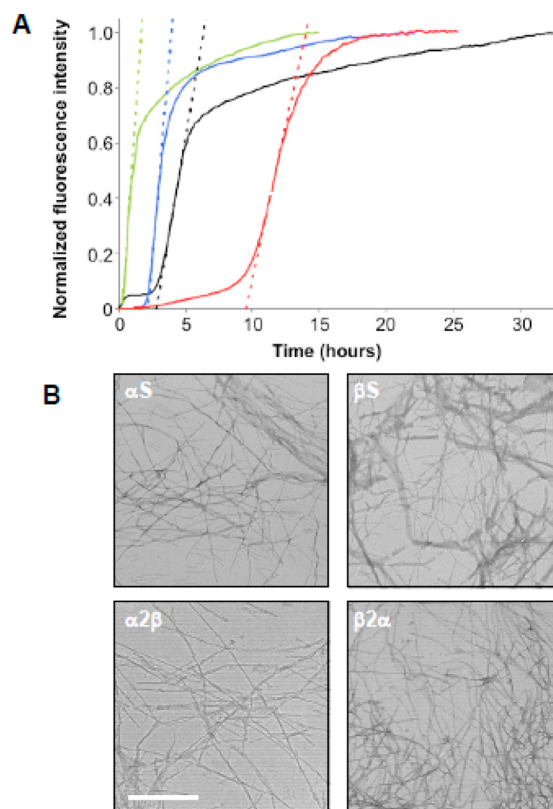


Figure 1. (A) Representative kinetic traces of the aggregation of α -synuclein (green), β -synuclein (red), $\alpha 2\beta$ (black), and $\beta 2\alpha$ (blue). Aggregation was obtained in the presence of 350 μM SDS and monitored by ThT fluorescence. Aggregation rates are indicated by dashed lines, which represent the maximum slope in the aggregation curves. (B) TEM images from negatively stained samples corresponding to the fibrillar assemblies formed by α -synuclein (αS), β -synuclein (βS), $\alpha 2\beta$, and $\beta 2\alpha$ at the end of the aggregation process. Scale bar: 500 nm.

scores, that the aggregation rate of $\alpha 2\beta$ was shifted from that of α -synuclein to be very close to that of β -synuclein (Table 1 and Figure 1A). Moreover, we also found that the aggregation rate of $\beta 2\alpha$ was shifted in the opposite direction to be intermediate between those of α -synuclein and β -synuclein, as expected from the predictions (Table 1 and Figure 1A). In addition, the same aggregation data were analyzed by calculating the $t_{1/2}$ values (Table 1), with similar results. In order to verify that the newly described mutants—similarly to α -synuclein and β -synuclein—are able to form fibrillar structures after appropriate incubation periods, we used transmission electron microscopy (TEM) from samples corresponding to the end of the aggregation reactions and confirmed that both $\alpha 2\beta$ and $\beta 2\alpha$ variants are able to form amyloid-like fibrils (Figure 1B).

In addition, we measured the fibrillation rates for the synuclein variants from seeded aggregation reactions,^{45,57,58} in the absence of SDS. We found that under the conditions that we used β -synuclein did not form fibrils (Table 2), while for the

Table 2. Comparison between the Predicted Aggregation Propensities (Z_{agg}) and the Experimentally Observed Elongation Rates (k_e) for α -Synuclein, β -Synuclein, and the Two Mutational Variants ($\alpha 2\beta$ and $\beta 2\alpha$) Analyzed in This Study, from Seeded Aggregation Assays^a

| variant | Z_{agg} | k_e (h^{-1}) |
|---------------------|------------------|---------------------------|
| α -synuclein | 0.98 | 0.135 ± 0.001 |
| $\alpha 2\beta$ | 0.90 | 0.025 ± 0.002 |
| β -synuclein | 0.80 | |
| $\beta 2\alpha$ | 0.86 | 0.017 ± 0.002 |

^aThe values of k and $t_{1/2}$ represent the average \pm standard deviation ($N = 2$).

other protein variants, we observed an absence of the lag phase and a good exponential fit (Supporting Information Figure 1). From these experiments we derived elongation rates (k_e) for α -synuclein and the $\alpha 2\beta$ and $\beta 2\alpha$ variants, to be compared with the predicted Z_{agg} scores (Table 2). These rates for the two mutants in relation to α -synuclein again showed a good correlation with the Z_{agg} scores (Table 2). These results, therefore, further support the conclusion that when the six amino acid residues that we identified in β -synuclein (Scheme 1, red regions) replace the equivalent residues in the sequence of α -synuclein, the aggregation rate of the latter decreases significantly. The complementary changes, which were used to obtain the $\beta 2\alpha$ variant, increases the aggregation rate of β -synuclein to an extent similar to that expected from the aggregation propensity predictions (Tables 1 and 2).

We next wondered whether, in addition to the aggregation properties of α -synuclein and β -synuclein having changed dramatically by mutation according to the Z_{agg} scores, the general properties of the early oligomers would have changed as well. Recently, exogenous α -synuclein oligomers have received increasing attention as extracellular α -synuclein oligomers, which are thought to be relevant for the pathology of Parkinson's disease because of their potential neurotoxicity,^{28,59} have been detected in biological fluids.⁶⁰ We therefore decided to compare the morphology and toxicity of the synuclein oligomers accumulated at the initial stages of the aggregation process of the four forms of synuclein considered in this study. In order to obtain an enriched preparation of early oligomeric species for their study, we processed the four protein variants similarly as follows. We used high protein concentrations to induce aggregation and isolated the oligomers by size exclusion, an approach that has been shown to favor the accumulation of soluble α -synuclein oligomers^{22,47,61–63} with the ability to bind to and permeabilize membranes.^{46,64} After incubation of the four protein forms at a high concentration, we separated the soluble oligomers from the monomers with a 100 kDa cutoff filter. The isolation of a homogeneous oligomeric fraction was confirmed by native gel electrophoresis followed by Western blot (Supporting Information Figure 2) as well as by sandwich ELISA (Supporting Information Figure 3). The yield of oligomers for the four synuclein variants was calculated and found to be relatively low (Supporting Information Table 3). By analyzing numerous preparations, however, α -synuclein and $\beta 2\alpha$ were found to generate a higher yield of oligomers than β -

synuclein and $\alpha 2\beta$ (Supporting Information Table 3). We then sought to compare the ability of the four oligomer preparations to be recognized by the anti- β -amyloid A11 antibody⁶⁵ by Dot blot (Supporting Information Figure 4). This conformation-specific antibody has been reported to recognize an as yet unknown generic polypeptide backbone epitope⁶⁶ that is independent of the amino acid sequence and is present in different prefibrillar protein oligomers.^{22,30,67,68} While α -synuclein and $\beta 2\alpha$ oligomers were virtually not recognized by this antibody under the experimental conditions used, the β -synuclein and $\alpha 2\beta$ oligomers were strongly and specifically recognized by the A11 antibody (Supporting Information Figure 4), suggesting a degree of conformational similarity between the β -synuclein and $\alpha 2\beta$ oligomers.

In order to compare the morphology of the oligomers generated from the four protein variants, we used transmission electron microscopy (TEM) together with negative staining at high magnification (180 000 \times) (Figure 2A). In agreement with previous reports, α -synuclein oligomers appeared to form 10–15 nm rounded-like aggregates assembling into higher order annular species,²² while the accumulated β -synuclein oligomers, which looked similar but somewhat smaller (5–10 nm), were seen to have a tendency to assemble into larger clusters (Figure 2A). A comparison between the overall images as well as the detailed sections (insets) suggests that the general morphology of the $\alpha 2\beta$ oligomers is closer to that of the β -synuclein species, namely, the somewhat lower size and the observed tendency to form large clusters. Moreover, the inverse change was also observed, as the general morphology of the $\beta 2\alpha$ oligomers appears to be closer to that of the α -synuclein oligomers (Figure 2A).

Finally, in order to obtain a comparative assessment of the cytotoxic effect of the different oligomers, we added freshly purified aggregates at a final concentration of 15 μM to SH-SY5Y human neuroblastoma cells in culture (Figure 2B). After 24 h, the state of the cells was monitored by the MTT reduction inhibition assay, a generic biochemical test used to monitor cell viability.⁴⁸ As shown previously for the case of α -synuclein,^{69–71} the monomeric fractions of all four protein variants did not significantly alter the cell viability, as compared to untreated controls (α -synuclein, $P = 0.078$; $\alpha 2\beta$, $P = 0.118$; β -synuclein, $P = 0.754$; $\beta 2\alpha$, $P = 0.835$) and between themselves (P values provided in the legend of Figure 2B). Also, in accordance with previous reports from similar experiments,^{63,72} α -synuclein oligomers showed moderate but significant cytotoxic effects, as shown by a decrease in MTT reduction to $78 \pm 5\%$ ($P = 0.026$) of that of untreated cells, while exposure of cells to β -synuclein oligomers produced no reduction in cell viability relative to controls ($108 \pm 5\%$; $P = 0.122$), indicating that the β -synuclein oligomers are not toxic to cells under these conditions (Figure 2B). Remarkably, the $\alpha 2\beta$ oligomers were found to be nontoxic ($110 \pm 5\%$; $P = 0.191$) as were the β -synuclein oligomers, while the $\beta 2\alpha$ oligomers displayed significant toxic effects ($76 \pm 2\%$; $P = 0.002$), comparable to the toxicity of oligomeric α -synuclein (Figure 2B). These changes in cytotoxicity might be caused by differential conformational rearrangements in the different protein oligomers, as recently shown for HypF-N and α -synuclein.^{73,74}

CONCLUSIONS

We have shown that it is possible to alter the aggregation rate of α -synuclein and to bring it to a value close to that of β -

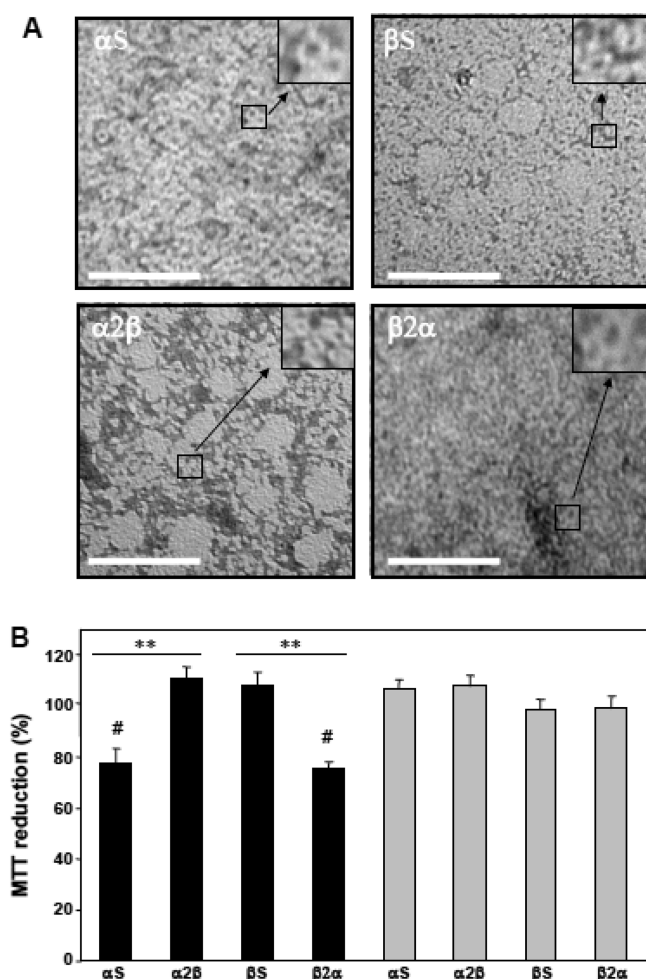


Figure 2. Morphology and toxicity of α -synuclein (α S), β -synuclein (β S), $\alpha 2\beta$, and $\beta 2\alpha$ soluble oligomers accumulated after overnight incubation at 37 °C of a 700–900 μ M solution in PBS. (A) TEM images from negatively stained samples obtained after coating EM grids with the corresponding oligomers. Scale bars: 100 nm. Inset: 2.5 \times zoomed image. (B) MTT reduction on SH-SY5Y cells treated with 15 μ M oligomers (black), or monomers (gray), for 24 h, compared to untreated (control) cells. α S: α -synuclein; β S: β -synuclein. Error bars correspond to the SEM from four independent experiments ($N = 4$). Statistically significant differences within the values of the oligomers group (** $P < 0.01$) were observed for α S vs $\alpha 2\beta$ ($P = 0.004$) and β S vs $\beta 2\alpha$ ($P = 0.003$), as opposed to α S vs $\beta 2\alpha$ ($P = 0.734$) and β S vs $\alpha 2\beta$ ($P = 0.750$). Statistically significant differences between the values of the oligomers and the corresponding monomers (# $P < 0.01$) were observed for α S ($P = 0.001$) and $\beta 2\alpha$ species ($P = 0.003$), as opposed to $\alpha 2\beta$ ($P = 0.667$) and β S species ($P = 0.153$). Differences between the monomers values were found to be statistically not significant in all cases (α -synuclein vs β -synuclein: $P = 0.120$; α S vs $\beta 2\alpha$: $P = 0.163$; $\alpha 2\beta$ vs β S: $P = 0.128$; $\alpha 2\beta$ vs $\beta 2\alpha$: $P = 0.168$). Statistical differences were calculated by applying the Student t test.

synuclein by swapping between the two proteins a set of just six residues chosen in a rational manner. We achieved this result not by performing extensive *in vitro* studies of the aggregation rates of a large number of mutational variants of α -synuclein but by the rational design of just one mutational variant, called $\alpha 2\beta$. This variant was identified by a computational screen that indicated a very small set of substitutions reflecting directly the evolutionary divergence of these two proteins. As predicted, our experiments confirmed that the $\alpha 2\beta$ variant closely resembles

β -synuclein in terms of aggregation kinetics. Remarkably, the corresponding $\beta 2\alpha$ variant also shifted its aggregation kinetics toward those of α -synuclein. Furthermore, possibly caused by a switch in the conformational properties or nature of the early prefibrillar oligomers, the six selected mutations in the $\alpha 2\beta$ -based oligomers were sufficient to abolish the cytotoxic effect observed for α -synuclein oligomers in a cell-based model, while the mutations present in the $\beta 2\alpha$ -based oligomers showed to produce an increase in the cytotoxicity as compared to β -synuclein. These results demonstrate that the overall strategy based on Zyggregator predictions that we have presented here can be used to modify the aggregation properties of proteins in a highly quantitative manner.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Figures 1–4 and Supporting Tables 1–3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy.

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