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Characterization of Hydrophobic Residue Requirements for α-Synuclein Fibrillization[†]

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ABSTRACT: α-Synuclein is the major component of pathological inclusions characteristic of diseases like Parkinson's disease, dementia with Lewy bodies, and multiple-system atrophy. A role for α-synuclein in neurodegenerative diseases is further supported by point mutations and duplication and triplication of the α-synuclein gene (*SNCA*) that are causative of these disorders. The middle hydrophobic region of the α-synuclein protein, also termed the "non-Aβ component of Alzheimer's disease amyloid plaque (NAC)" domain, is required for α-synuclein to polymerize into amyloid filaments, which are the major components of α-synuclein pathological inclusions. In this study, we assessed the importance of specific stretches of hydrophobic residues in driving the intrinsic ability of α-synuclein to polymerize. Several small deletions, even one with as few as two amino acid residues (A76 and V77), dramatically impaired the ability of α-synuclein to polymerize into mature amyloidogenic fibrils, and instead, it preferentially formed oligomers. However, this inhibition of filament assembly was clearly dependent on the spatial context, since similar and larger hydrophobic deletions in other parts of the NAC domain reduced only the rate of fibril formation, without abrogating filament assembly. Further, mutation of residue E83 to an A rescued the ability of mutant Δ76−77 α-synuclein to polymerize. These findings support the notion that while both the location and hydrophobicity of protein segments are important elements that affect the propensity to form amyloid fibrils, the intrinsic ability of a polypeptide to fold structurally into amyloid is also critical.

Synucleopathies make up a group of neurodegenerative disorders sharing in common the presence of intracellular inclusions comprised predominantly of α-synuclein (α-syn)[1] amyloidogenic fibrils (*1*, *2*). These neuronal α-syn inclusions, termed Lewy bodies and Lewy neurites, comprise one of the defining characteristics of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) (*1*−*4*). Similarly, α-syn inclusions in oligodendrocytes are a hallmark of multiple-system atrophy (*5*, *6*). Furthermore, the presence of brain α-syn inclusions is associated with many other neurodegenerative diseases, including pure autonomic failure, neurodegeneration with brain iron accumulation type 1 (NBIA-1), Down's syndrome, and familial and sporadic Alzheimer's disease (*1*, *2*).

α-Syn is a highly soluble heat-stable and "natively unfolded" protein (*7*), predominantly expressed in central nervous system (CNS) neurons, where it is localized in the proximity of vesicles within presynaptic terminals (*1*, *8*, *9*). α-Syn is a small 140-amino acid protein characterized by an amino-terminal region containing several imperfect KTKEGV repeats, a hydrophobic center domain, and a highly negatively charged carboxy-terminal region (*1*) (see Figure 1A). Although the function of α-syn is still poorly understood, several studies suggest its involvement in modulating synaptic transmission, the density of synaptic vesicles, neuronal plasticity, vesicle recycling, and synaptic integrity (*1*, *9*−*12*).

Disease-causing alterations in the α-*syn* gene (*SNCA*) provide direct evidence of a fundamental role of α-syn in the pathogenesis of α-synucleopathies. Genetic studies have identified three autosomal dominant missense mutations (A30P, E46K, and A53T) in α-syn that are causative of PD and/or DLB (*13*−*15*). In addition, short chromosomal duplications or trisomies containing the *SNCA* gene, and relatively short flanking regions on chromosome 4, were discovered in patients with PD or DLB (*16*, *17*), indicating that a 50% increase in the level of expression of α-syn is sufficient to cause disease.

In vitro studies have shown that recombinant soluble α-syn can readily polymerize into amyloidogenic fibrils that are structurally similar to those observed in human brains (*18*−*21*). A53T and E46K α-syn exhibit an increased rate of self-assembly and fibril formation (*18*, *22*−*27*), suggesting that these mutants could be pathogenic because they promote inclusion formation (*28*). Several studies indicate that the polymerization of α-syn progresses from unordered monomers to partially folded intermediates that assemble into oligomers or protofibrils and finally elongate into "mature" amyloid filaments (*29*, *30*). This conversion of α-syn from monomer to amyloid fibrils is associated with a dramatic conformational change from random coil to predominantly β-pleated sheet (*21*, *30*, *31*). Although many studies support the notion that the formation of mature fibrillar α-syn inclusions is pathological (*28*, *32*, *33*), some evidence also indicates that certain types of intermediate species such as protofibrils may have toxic properties (*33*−*35*).

Limited proteolysis experiments (*21*, *36*), EPR spectroscopy measurements (*37*, *38*), and hydrogen−deuterium exchange

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[1]Abbreviations: α-syn, α-synuclein; NAC, non-Aβ component of Alzheimer's disease amyloid plaque; PD, Parkinson's disease; DLB, dementia with Lewy bodies; NBIA-1, neurodegeneration with brain iron accumulation type 1; CNS, central nervous system; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy; SD, standard deviation; SDS, sodium dodecyl sulfate; WT, wild-type.

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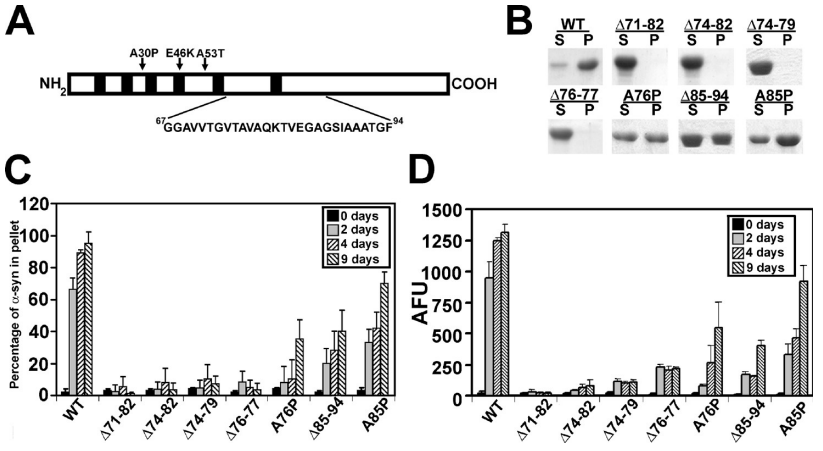

FIGURE 1: Studies of the effects of short deletions and P point mutations in the middle region of α-syn on polymerization. (A) Schematic of the structure of α-syn depicting the six imperfect "KTKEGV" repeats (black boxes), the three point mutations that cause disease (A30P, E46K, and A53T), and an expanded view of the amino acid sequence in the middle hydrophobic region. (B) Representative Coomassie Blue R-250-stained SDS−polyacrylamide gels showing the sedimentation state of WT α-syn or altered α-syn proteins (Δ71−82, Δ74−82, Δ74−79, Δ76−77, A76P, Δ85−94, and A85P) in supernatant (S) or pellet (P) after assembly incubation for nine days at 5 mg/mL. (C) Quantitative analysis of the polymerization of indicated α-syn proteins assayed by sedimentation after incubation for 0, 2, 4, and 9 days under assembly conditions, as described in Materials and Methods. (D) K114 fluorometry assessing the formation of amyloid from α-syn after incubation under assembly conditions as indicated. All proteins were incubated at 5 mg/mL. Data represent the average ± SD ($n = 6$) for all data sets.

experiments (*39*, *40*) show that residues ~30−110 are buried within the core of α-syn fibrils, suggesting that these residues are important for polymerization. Consistent with these findings, deletion of amino acid residues 71−82 within the hydrophobic region abrogated the ability of human α-syn to fibrillize (*21*). In this study, we sought to further assess the importance of specific stretches of residues in the middle hydrophobic region of α-syn in driving its polymerization into fibrils.

MATERIALS AND METHODS

Expression and Purification of α*-Syn*. The human α-syn cDNA was cloned into the NdeI and HindIII restriction sites of bacterial expression vector pRK172. The cDNAs encoding the mutant A76P, A76G, A76V, and A85P α-syn proteins in the same vector were engineered by creating the corresponding nucleotide substitutions in the wild-type cDNA using complementary sets of synthetic single-stranded DNA containing the mutant sequence and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids expressing α-syn with A76, V77, or A78 deleted were generated by using the QuikChange site-directed mutagenesis kit and oligonucleotides lacking these specific codons. The 1−110 α-syn was created with the addition of a premature stop codon using QuickChange site-directed mutagenesis (*41*). The deletion of the nucleotide sequence encoding residues 71−82, 74−82, 74−79, 76 and 77, 67−71, 69 and 70, and 85−94 in α-syn cDNA was created using the Exsite PCR site-directed mutagenesis kit (Stratagene) and oligonucleotides that specifically bind to the DNA sequence adjacent to the targeted deleted sequence. Deletion of the nucleotide sequence encoding residues 73−83 was achieved by small modifications to the sequence with deletions in residues 71−82, using oligonucleotides which added two amino acids and removed one amino acid by QuickChange site-directed mutagenesis. The Δ76−77 α-syn construct with mutation Q79A or E83A was created with oligonucleotides corresponding to the amino acid substitutions by QuickChange site-directed mutagenesis. All mutations were confirmed by DNA sequencing.

α-Syn proteins were expressed in *Escherichia coli* BL21(DE3) and purified as previously described (*21*, *26*). Briefly, bacterial pellets harvested by centrifugation were resuspended in high-salt buffer [0.75 M NaCl, 50 mM Tris (pH 7.4), and 1 mM EDTA] containing a cocktail of protease inhibitors, heated to 100 °C for 10 min, and centrifuged at 70000*g* for 30 min. α-Syn proteins were purified by size exclusion chromatography followed by ion exchange chromatography. Supernatants were dialyzed into 100 mM NaCl and 20 mM Tris (pH 7.5) and applied to a Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ) and separated by size exclusion chromatography. The fractions were assayed for the presence of the α-syn proteins by SDS−polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue R-250 staining. All α-syn proteins [except for 1−110 α-syn (see below)] were concentrated using Centriprep-10 units (Millipore Corp., Bedford, MA), dialyzed against 10 mM Tris (pH 7.5), applied to a Mono Q column (GE Healthcare), and eluted with a 0 to 0.5 M NaCl gradient. For 1−110 α-syn, the fractions isolated by gel filtration were dialyzed against 25 mM 2-(morpholino)ethanesulfonic acid (pH 6.25), applied to a Mono S column (GE Healthcare), and eluted with a 0 to 0.5 M NaCl gradient. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Filament Assembly and Centrifugal Sedimentation. α-Syn proteins were assembled into filaments by incubation at 37 °C in 100 mM sodium acetate (pH 7.4) with continuous shaking. A fraction of each sample was set aside for K114 fluorometry and electron microscopic (EM) analysis. The remainder of each sample was centrifuged at 100000*g* for 20 min. SDS sample buffer [10 mM Tris (pH 6.8), 1 mM EDTA, 40 mM DTT, 1% SDS, and 10% sucrose] was added to pellets and supernatants, which were heated to 100 °C for 15 min. Equal volumes of α-syn proteins in the supernatants and pellets were separated by SDS−PAGE and were quantified by densitometry of Coomassie Blue R-250-stained gels.

K114 Fluorometry. α-Syn fibrils are amyloidogenic (*30*), and their formation can be quantified using the fluorescent amyloid binding dye K114 (*42*). This dye, derived from the structure of Congo Red, is soluble in aqueous buffers, and it demonstrates a tremendous increase in fluorescence upon binding to

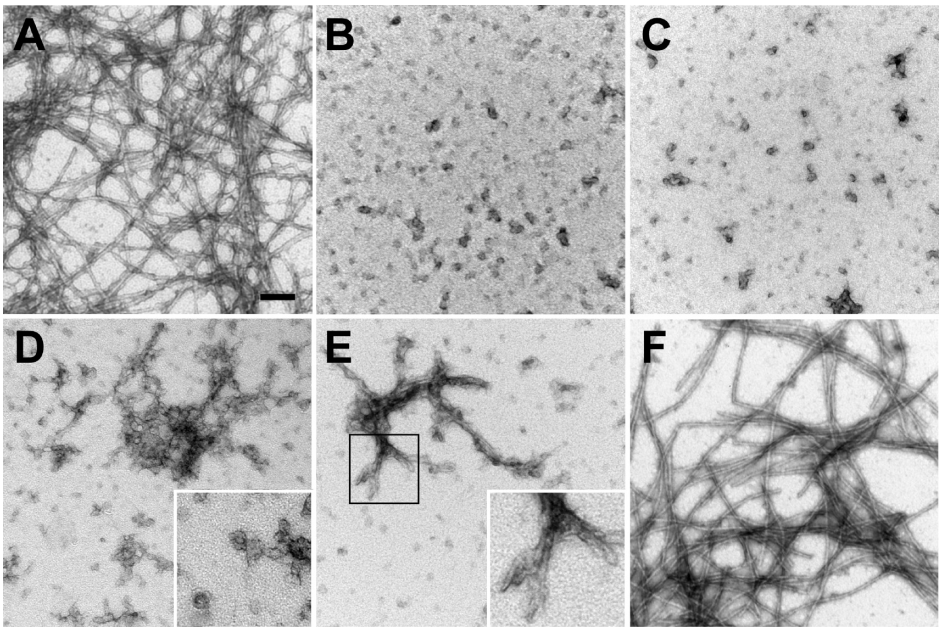

FIGURE 2: EM analysis of the assembly of α-syn proteins. Representative images of negative staining electron microscopy of α-syn proteins incubated at a concentration of 5 mg/mL under sedimentation conditions. The following proteins and time points were visualized: (A) WT α-syn for 2 days, (B) Δ71−82 for 9 days, (C) Δ74−79 for 9 days, (D) Δ76−77 for 4 days, (E) Δ76−77 for 9 days, and (F) A76P for 9 days. The scale bar is 100 nm. For the insets of panel D and E, scale bar is 50 nm.

amyloidogenic fibrils (*42*). We performed this assay, as previously described (*42*), by incubating a fraction of each sample with K114 (50 μM) in 100 mM glycine (pH 8.5) and measuring the fluorescence (λ_{ex} = 380 nm, λ_{em} = 550 nm, cutoff = 530 nm) with a SpectraMax Gemini fluorometer and SoftMax Pro version 4.0.

Negative Staining EM. Assembled α-syn filaments were absorbed onto 300 mesh carbon-coated copper grids, stained with 1% uranyl acetate, and visualized with a JEOL (Peabody, MA) 1010 transmission electron microscope. Images were captured with a Hamamatsu (Bridgewater, MA) digital camera using AMT (Danvers, MA) software. For the determination of diameters, the width of 100−120 filaments was measured using Image-Pro Plus (Media Cybernetics, Del Mar, CA).

Immuno-EM Analysis. Samples were applied to 300 mesh carbon-coated copper grids, and after being rinsed with PBS, the grids were blocked with 1% BSA for 30 min. Mouse monoclonal anti-α-syn Syn214, which reacts with C-terminal residues 130−140 in α-syn (*43*), was diluted in a PBS/1% BSA mixture and applied to the grids for 30 min. Following extensive washes, a goat anti-mouse antibody conjugated to 6 nm colloidal gold (Electron Microscopy Sciences, Hatfield, PA) diluted in a PBS/1% BSA mixture was applied to the grids for 30 min. Following washes with PBS, the samples were stained with 1% uranyl acetate.

RESULTS

It was previously demonstrated that the deletion of residues 71−82 in α-syn impairs the ability of this protein to polymerize into amyloid fibrils (*21*). To improve our understanding of the molecular requirements of hydrophobic residues in fibril formation, we generated and characterized α-syn with several smaller deletions between amino acids 71 and 82 or with deletions in adjacent hydrophobic stretches (^{67}GGAVV^{71} or ^{85}AGSIAAATGF^{94}). As previously reported, deletion of residues 71−82 impaired the polymerization of α-syn into amyloid fibrils as assayed by sedimentation analysis and K114 fluorometry (Figure 1B−D). Electron microscopy (EM) analysis demonstrated that unlike wild-type (WT) α-syn that can readily form smooth negatively stained fibrils (width of 10.7 ± 2.0 nm) within 2 days, Δ71−82 α-syn predominantly formed 19.4 ± 3.2 nm wide spherical oligomers that accumulate over 9 days (Figure 2A,B). Smaller deletions (Δ74−82 and Δ74−79) in this region also impaired the ability of α-syn to assemble into amyloidogenic polymers as assayed by sedimentation and K114 fluorometry (Figure 1B−D), while EM analysis demonstrated the formation of spherical oligomers 18 ± 3.2 and 15.6 ± 3.5 nm in width, respectively (data not shown and Figure 2C), and a paucity of fibrils. Even the deletion of only two amino acid residues, ^{76}AV^{77} (Δ76−77), dramatically impaired the polymerization of α-syn into amyloid fibrils (Figure 1C,D). Sedimentation analysis showed that this mutant was impaired in its ability to form large polymers, and K114 fluorometry demonstrated a small but consistent increase in signal after incubation for as few as 2 days. EM analysis revealed abundant 16.6 ± 3.4 nm spherical oligomers with Δ76−77 α-syn (Figure 2D,E). Nevertheless, these Δ76−77 α-syn oligomers appeared to frequently coalesce (Figure 2D), sometimes forming clusters with a fibrillar-like appearance after 9 days (Figure 2E). However, these clusters of Δ76−77 α-syn protein never form the smooth, elongated fibrils, which are observed with WT α-syn (Figure 2E, inset). The substitution of A76 for a P residue, an amino acid residue that strongly prevents formation of β-pleated sheet structure (*44*, *45*), reduced the propensity of α-syn to polymerize (Figure 1B−D). However, EM analysis showed that this mutant (A76P) could polymerize into 12.9 ± 2.4 nm wide amyloid fibrils (Figure 2F).

Previous studies showed that the substitution of residue A76 with a charged residue (R or E) delayed fibril formation (*21*). To further define the preferences of this residue, A76 was mutated to a less hydrophobic but nonpolar residue (G) or a more hydrophobic residue (V). Neither mutation significantly altered the rate of fibril formation (Figure 3A,B). By EM analysis, the A76G mutation created 12.0 ± 2.6 nm fibrils with a slightly altered morphology with fibrils that appear more directional than WT

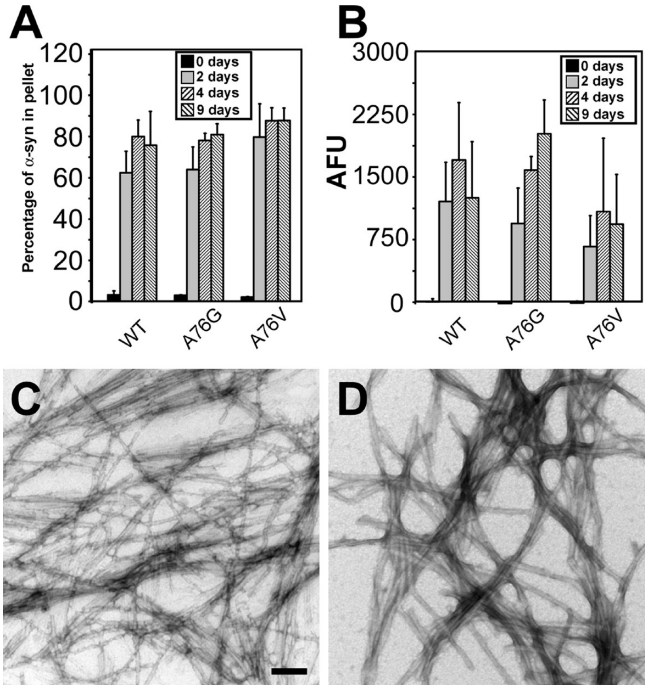

FIGURE 3: Assembly of α-syn protein with an A76G or A76V mutation. (A) Quantitative sedimentation analysis and (B) K114 fluorometry of WT α-syn versus that with the A76G or A76V mutation after 0, 2, 4, and 9 days at 5 mg/mL. Data represent averages ± SD for four independent experiments. (C and D) Representative images of negative staining electron microscopy of α-syn with A76G (C) and A76V (D) mutations. The scale bar is 100 nm.

α-syn (Figure 3C), and the A76V mutation created fibrils similar to WT α-syn [width of 10.8 ± 1.7 nm (Figure 3D)].

On the more C-terminal end of the hydrophobic domain, deletion of residues 85−94 (Δ85−94) or the substitution of P for A at position 85 (A85P) reduced the propensity of α-syn to form amyloidogenic fibrils as noted by sedimentation and K114 fluorometry (Figure 1B−D). Interestingly, the ultrastructure of the fibrils formed by Δ85−94 or A85P was different from that of typical WT α-syn fibrils (Figure 4A,B). Instead of a smooth profile, these fibrils appeared to have significant protrusions.

Deletion of hydrophobic residues ^{67}GGAVV^{71} (Δ67−71) also significantly reduced the ability of α-syn to form amyloidogenic fibrils (Figure 5A,B). Albeit not frequent, clusters of Δ67−71 α-syn fibrils (width of 14.5 ± 3.0 nm) with typical negative stained smooth profiles were observed by EM after incubation for 9 days (Figure 4C). Deletion of residues ^{69}AV^{70} (Δ69−70) also reduced the rate of amyloidogenic fibril formation, but to a much lesser degree than the Δ67−71 deletion, forming 13.2 ± 3.0 nm fibrils (Figures 4D and 5A,B). The deletion of these two hydrophobic residues, ^{69}AV^{70}, did not have the same drastic effect as deleting resides 76 and 77.

To further investigate the effect of the ^{76}AV^{77} deletion mutant, we generated single-amino acid deletion mutations A76 (Δ76), V77 (Δ77), and A78 (Δ78) to examine if a particular single residue was responsible for this dramatic inhibition of amyloidogenic fibrils. Δ78 α-syn was generated, since the ^{76}AV^{77} deletion mutant results in a single A residue between positions 75 and 79. Deletion of ^{76}AV^{77} is equivalent to ^{77}VA^{78}; therefore, the deletion of each of the three amino acids was important to investigate. However, none of these three single-deletion mutations significantly affected the ability of α-syn to polymerize into amyloid fibrils [Figures 4E,F and 5C,D; EM for Δ78 (data not shown)].

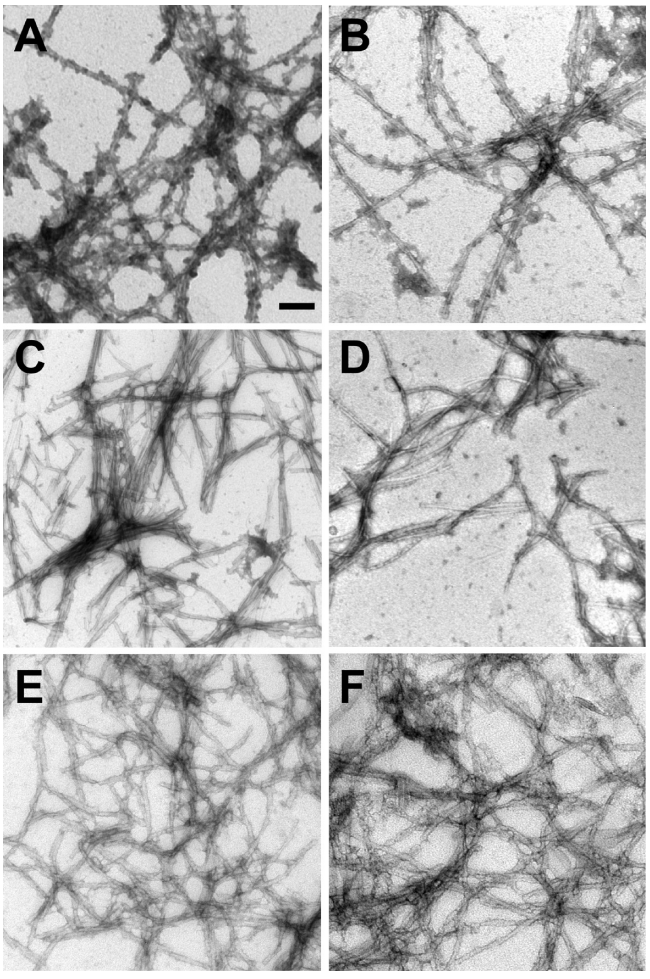

FIGURE 4: EM analysis of the assembly of additional α-syn mutants. Representative images of negative staining electron microscopy of α-syn proteins incubated at a concentration of 5 mg/mL under sedimentation conditions. The following proteins and time points were visualized: (A) Δ85−94 for 9 days, (B) A85P for 9 days, (C) Δ67−71 for 9 days, (D) Δ69−70 for 9 days, (E) Δ76 for 2 days, and (F) Δ77 for 2 days. The scale bar is 100 nm.

It was previously reported that WT α-syn assembly does not promote the formation of Δ71−82 α-syn fibrils when co-incubated (*21*). To determine if α-syn has the ability to stimulate assembly of the other fibrillization-incompetent α-syn mutants, we co-incubated the deletion mutants with an assembly-competent α-syn protein. To differentially resolve deletion mutants from fibril-competent α-syn by SDS−PAGE, we utilized C-terminally truncated α-syn 1−110, which is efficient in inducing WT α-syn amyloid formation (*41*). The 1−110 α-syn was co-incubated with Δ71−82, Δ74−82, Δ74−79, Δ76−77, or Δ67−71 α-syn. While Δ71−82 exhibited only a slight increase in the amount of sedimented protein (above that of incubation on its own), significant increases in the amount of sedimentation were observed with all of the other α-syn deletion mutants, such that between 35 and 60% of these proteins were sedimented (Figure 6A,B). Increases in K114 fluorometry above that of 1−110 α-syn alone supported an increase in amyloid formation of α-syn deletion mutants Δ74−82, Δ74−79, Δ76−77, and Δ67−71 when co-incubated with 1−110 α-syn (Figure 6C).

Immuno-EM analysis was performed to assess the presence and structure of the deletion mutants when incubated in the presence of 1−110 α-syn. Immunolabeling was performed with an antibody (Syn214) specific to the extreme C-terminus of

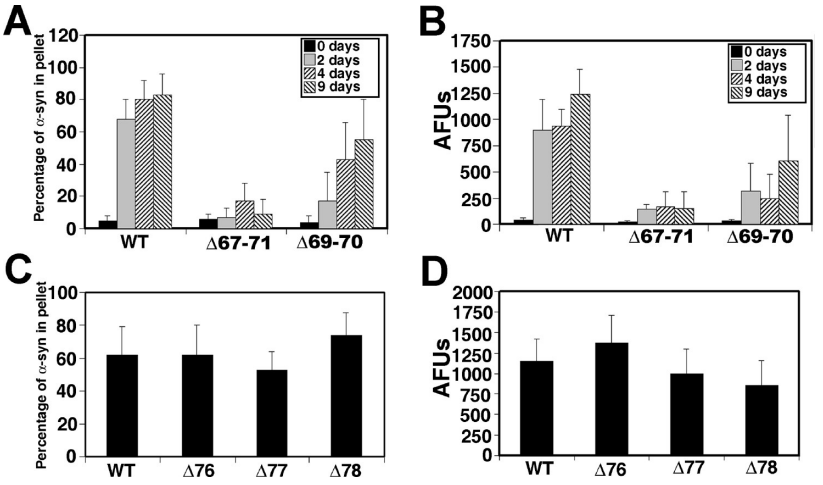

FIGURE 5: Analysis of the polymerization of α-syn deletion mutants. (A) Quantitative sedimentation analysis and (B) K114 fluorometry of α-syn deletion mutants Δ67−71 and Δ69−70 after 0, 2, 4, and 9 days under assembly conditions at 5 mg/mL. (C) Quantitative sedimentation analysis and (D) K114 fluorometry of α-syn with single-amino acid deletions Δ76, Δ77, and Δ78 after 2 days under assembly conditions at 5 mg/mL.

α-syn, so that the labeling would reveal only proteins with deletions in the hydrophobic region, rather than 1−110 α-syn. Fibrils labeled with antibodies were decorated with 6 nm gold particles, but their morphologies were also less distinct because of the binding of antibodies to the fibrils. Samples assembled from only 1−110 α-syn revealed abundant fibrils, but no labeling with gold particles (Figure 7A). In comparison, 1−110 α-syn samples assembled in the presence Δ71−82 α-syn demonstrated scant labeling of fibrils (Figure 7B), suggesting that possibly small levels of Δ71−82 protein were incorporated. Fibrils observed from samples with Δ74−82 α-syn, Δ74−79 α-syn, Δ76−77 α-syn, or Δ67−71 α-syn co-incubated with 1−110 α-syn demonstrated abundant immuno-gold labeling (Figure 7C−F), indicating that these proteins co-assembled with 1−110 α-syn.

Δ76−77 α-syn cannot form smooth elongated amyloidogenic fibrils on its own but can be partially incorporated into 1−110 α-syn fibrils. One possible explanation for these results is that Δ76−77 α-syn is deficient in nucleation. We therefore tested the ability of Δ76−77 α-syn to polymerize in the presence of WT α-syn seeds. WT α-syn protein was partially fibrillized at 5 mg/mL for 24 h, then removed, and incubated at a concentration of 0.5 mg/mL with Δ76−77 α-syn at 5 mg/mL. After 9 days, only 27 ± 5% (SD) (n = 6) of a combination of Δ76−77 α-syn and the WT seeds sedimented versus 63 ± 19% (SD) of WT α-syn alone (Figure 6D). The analysis of Δ76−77 with the WT seed was accompanied by an increase in K114 fluorometry by 484 ± 187 (SD) arbitrary fluorescence units (above that of Δ76−77 α-syn alone), versus 174 ± 49 (SD) arbitrary fluorescence units of the WT seed alone. The only modest sedimentation and incorporation of Δ76−77 α-syn, even in the presence of a seed, suggest that Δ76−77 α-syn is fundamentally impaired in its ability to polymerize into mature amyloidogenic fibrils, but as the data with 1−110 α-syn show, it can still be partially incorporated when incubated with an assembly-competent protein.

α-Syn with a deletion of amino acids 73−83 (Δ73−83) is capable of fibril formation (*46*), even though this alteration removes residues 76 and 77. We examined the ability of Δ73−83 α-syn to polymerize under our conditions and, as in previous reports (*46*), found this protein competent in fibril formation, creating fibrils of 7.7 ± 1.5 nm after incubation for 4 days [Figure 8A,B; EM (data not shown)]. We hypothesized that either residue Q79 (large, polar) or E83 (charged) may be preventing the polymerization of Δ76−77 α-syn, since both of these residues are absent in the Δ73−83 protein. α-Syn was created with amino acids 76 and 77 deleted and either Q79 or E83 mutated to an A (Δ76−77/Q79A or Δ76−77/E83A, respectively). Approximately 20% (n = 21) of Δ76−77/Q79A samples displayed increases in the level of sedimentation and K114 fluorometry, but only after incubation for 9 days (Figure 8A,B), and Δ76−77/E83A α-syn was capable of polymerization after incubation for 4 days (Figure 8A,B). EM studies showed that Δ76−77/Q79A α-syn formed oligomers, similar to that observed with Δ76−77 α-syn, and in some samples incubated for 9 days, occasional smooth fibrils (width of 12.2 ± 2.9 nm) were observed (Figure 8C). By EM, Δ76−77/E83A α-syn was observed to form abundant smooth fibrils (width of 9.3 ± 2.4 nm) after incubation for 4 days, but these fibrils had a tendency to "clump", creating fibrils shorter than those normally observed with WT α-syn (Figure 8D). These data demonstrate that the presence of the negatively charged E83 residue contributes to the inability of Δ76−77 α-syn to polymerize into mature amyloid fibrils.

DISCUSSION

The primary amino acid sequences of polypeptides that are compatible with amyloid formation can demonstrate considerable variability and flexibility, yet several basic intrinsic properties such as conformational preferences, solubility, charge state, and the ability to pack into a fibrillar structure are important in influencing this process (*47*−*50*). This study aimed to characterize the amino acid requirements for α-syn fibril formation, focusing on deletions in key hydrophobic regions and providing a more exhaustive assessment of the role that these amino acid segments have in driving α-syn amyloid formation.

Our previous work demonstrated that deletion of residues 71−82 within the hydrophobic core of α-syn abrogated the ability of this protein to form amyloid fibrils (*21*). To further narrow the range of amino acids within this region that affect the ability of α-syn to form amyloid fibrils, shorter deletion mutations (Δ74−82, Δ74−79, and Δ76−77) were generated. All these deletion mutants impaired the ability of α-syn to form typical ~10 nm wide unbranched amyloid fibrils as observed with WT α-syn. Instead, these mutants demonstrated a propensity to form

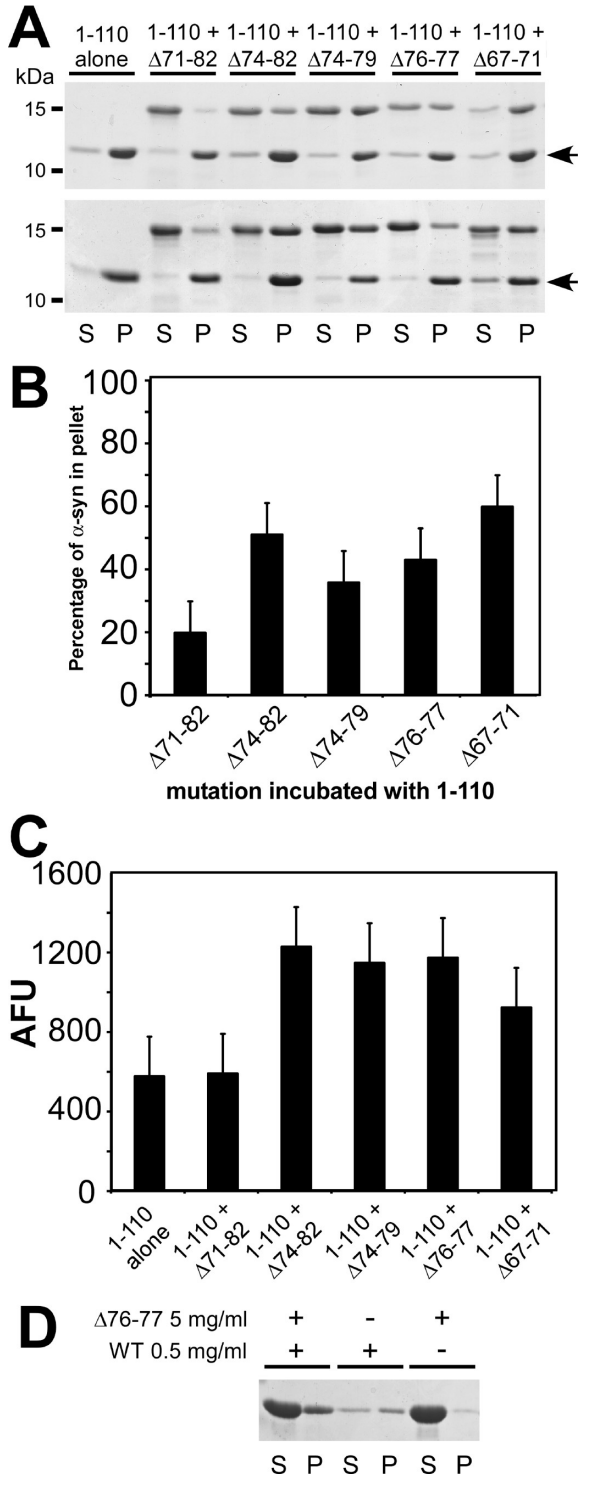

FIGURE 6: Co-assembly of 1−110 α-syn protein with deletion mutants. (A) Representative Coomassie Blue R-250-stained SDS−polyacrylamide gels showing the sedimentation state of 1−110 α-syn with Δ71−82, Δ74−82, Δ74−79, Δ76−77, or Δ67−71 in supernatant (S) or pellet (P) after incubation for 4 days with each protein at a concentration of 2.5 mg/mL. Arrows indicate the 1−110 α-syn protein. Two independent experiments are represented. (B) Quantitative sedimentation analysis of α-syn proteins with deletions in the hydrophobic region and (C) K114 fluorometry of co-assembly of deletion mutants with 1−110 α-syn at a concentration of 2.5 mg/mL after 4 days. Data represent averages ± SD for three independent experiments. (D) Representative Coomassie Blue R-250-stained SDS−polyacrylamide gel showing sedimentation of Δ76−77 α-syn at 5 mg/mL with or without a seed of partially fibrillized WT α-syn at 0.5 mg/mL after incubation for 9 days.

spherical oligomers that have diameters (16−20 nm) larger than those of typical mature fibrils (~10 nm). Typical "on filament pathway" oligomers/protofibrils are ~4−5 nm in size (*30*, *51*). Therefore, the oligomers generated by these deletion mutants are suggestive of "off pathway" oligomers.

Interestingly, the oligomers formed by deletions within the region of residues 71−82 are reminiscent in size of the spherical oligomers generated when α-syn fibril formation is inhibited by treatment with dopamine, baicalein, or EGCG (*52*−*54*) or to a small subpopulation of oligomers that form when α-syn is incubated under certain aqueous conditions (*55*, *56*). The inhibition of α-syn fibril formation by dopamine is due to the formation of dopamine-oxidized byproducts, which interact noncovalently to induce conformational changes in α-syn and form species that are unable to assemble into mature fibrils (*54*). It is possible that the deletion mutant proteins Δ71−82, Δ74−82, Δ74−79, and Δ76−77 α-syn form similar conformations or that they are fundamentally incompetent on their own to form the tertiary structures compatible with amyloid formation. Unlike the α-syn oligomers that are generated by dopamine treatment and that can become amyloid-compatible by denaturation and renaturation (*54*), mutants Δ71−82, Δ74−82, Δ74−79, and Δ76−77 α-syn intrinsically form these oligomers.

Nevertheless, co-assembly conditions with 1−110 α-syn promoted the partial copolymerization of Δ74−82, Δ74−79, and Δ76−77 α-syn. While small amounts of fibril immunolabeling with antibody Syn214 (specific for the C-terminus of α-syn) were observed following co-incubation of 1−110 α-syn with Δ71−82 α-syn, we cannot rule out the possibility that Δ71−82 was simply nonspecifically sticking to 1−110 α-syn. The much more robust Syn214 immunolabeling of fibrils obtained during the co-incubation of deletion mutants Δ74−82, Δ74−79, and Δ76−77 α-syn with 1−110 α-syn supports that at least some of these proteins were incorporated within fibrils. The ability of these proteins to be incorporated within 1−110 α-syn fibrils is further supported by sedimentation studies and by the increase in K114 fluorescence. These data suggest that these deletion proteins are not completely incapable of amyloid formation, but they require co-assembly with a more permissive amyloidogenic protein to drive their polymerization. Additional co-assembly experiments using seeds comprised of WT α-syn demonstrated that only a small portion of Δ76−77 α-syn could be induced to fibrillize. These data indicate that the lack of amyloid formation by Δ76−77 α-syn is not predominantly due to a deficiency in seeding, and the inefficiency of filament formation in the presence of WT α-syn seeds indicates a fundamental deficiency of Δ76−77 α-syn in elongation of amyloid fibrils. Nevertheless, the small increases in K114 fluorometry of Δ76−77 α-syn after incubation for 2 days, on its own, suggest that a small portion of protein is capable of forming β-sheets, even if the protein cannot extend to form smooth amyloid fibrils.

Consistent with previous studies (*21*, *46*), we showed that while Δ71−82 α-syn was deficient in fibril formation, Δ73−83 α-syn was able to polymerize into mature amyloid, albeit at a rate slower than that of WT α-syn. These findings are apparently counterintuitive since both of these deletions remove a similar region and they both include residues 76 and 77, as well as other deleted segments (Δ74−79 and Δ74−82) that seem to be necessary for amyloid formation of α-syn. To try to explain these findings, the bulky, noncharged polar residue (Q79) and negatively charged residue (E83) in this region were mutated to A residues within Δ76−77 α-syn. The Q79A amino acid

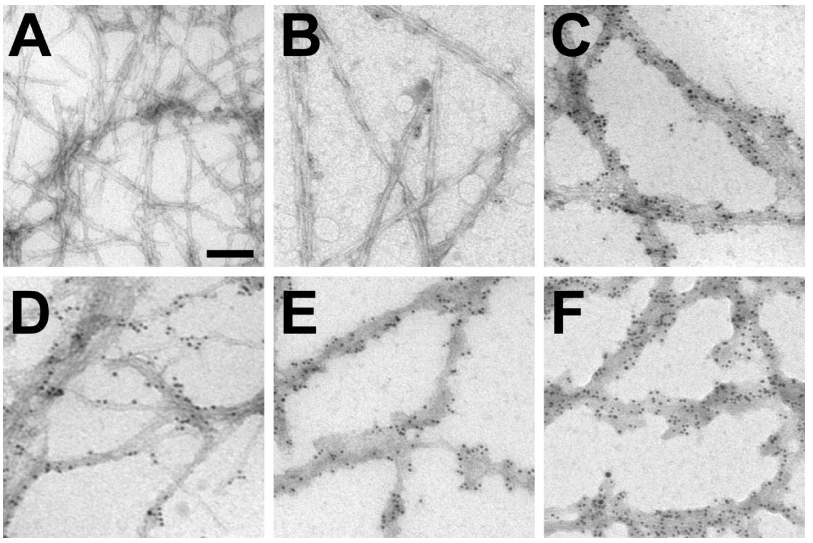

FIGURE 7: Immuno-EM analysis of coassembly of α-syn with deletions in the hydrophobic region with 1−110 α-syn. Immunolabeling was performed with antibody Syn214, which reacts with the extreme C-terminus of α-syn, followed by immuno-gold labeling. (A) The 1−110 α-syn protein assembled on its own did not exhibit immuno-gold labeling, while (B) Δ71−82, (C) Δ74−81, (D) Δ74−79, (E) Δ76−77, and (F) Δ67−71 presented levels of immuno-gold labeling consistent with the levels of sedimentation and K114 fluorometry indicated in Figure 6. The scale bar is 100 nm.

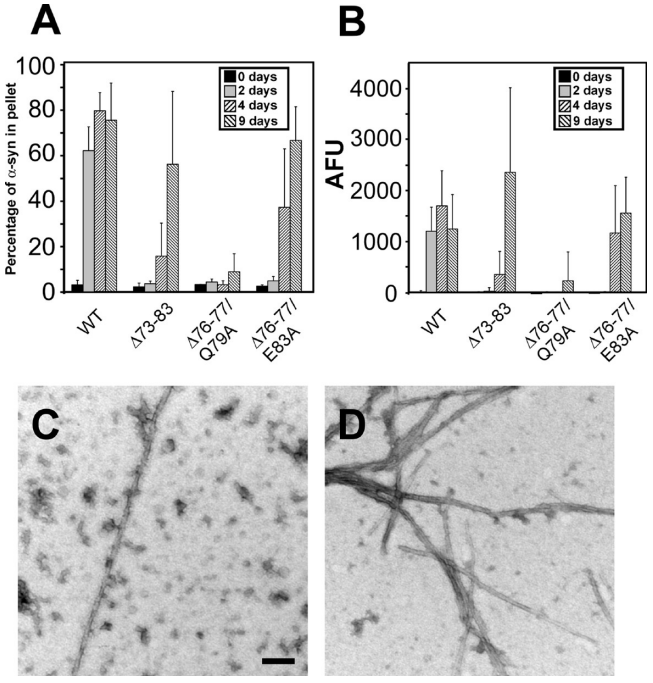

FIGURE 8: Study of the assembly of Δ76−77 α-syn with a Q79A or E83A mutation. (A) Quantitative sedimentation analysis and (B) K114 fluorometry of WT α-syn vs α-syn with deletion of residues 73−83 (Δ73−83) or deletion of residues 76−77 with a Q79A (Δ76−77/Q79A) or E83A (Δ76−77/E83A) mutation after incubation for 0, 2, 4, and 9 days at 5 mg/mL. Data represent averages ± SD for four independent experiments. (C and D) Representative images of negative staining electron microscopy of Δ76−77/Q79A α-syn (C) and Δ76−77/E83A α-syn (D). The scale bar is 100 nm.

substitution was able to promote the polymerization of Δ76−77 α-syn into amyloid, although inefficiently and only in a subset of samples. Conversely, the presence of the E83A mutation with Δ76−77 α-syn was able to sufficiently drive significant amyloid formation after 4 days of incubation. It was previously shown that mutating E83 to an A in WT α-syn increases the propensity of α-syn to form amyloid (*26*). This E residue may reduce the extent of amyloid formation due to the size of the residue and its negative charge as well as its focal inhibition of *β*-pleated sheet formation (*26*, *44*, *45*). Indeed, modeling studies have suggested "a possible role of E83 as a gatekeeper residue to reduce the aggregation propensity" (*57*). The presence or lack of E83 likely explains some of the differences between some of the deletion proteins. Δ73−83 α-syn has a significant ablation of hydrophobic residues, but this is partially compensated by the removal of E83. Furthermore, the deletion of residues 73−83 results in a new hydrophobic stretch from the adjacent upstream and downstream sequences (^{67}GGAVVT^{72}-^{84}GAGSIAAATGF^{94}). Other deletion proteins such as Δ74−82, Δ74−79, and Δ76−77 have smaller deletions of hydrophic residues but still include the E83 residue. Our data, therefore, suggest that the presence and placement of the highly charged E83 residue play a large role in the competency of hydrophobic deletion mutants to polymerize and can explain the differences in polymerization between the Δ71−82 and Δ73−83 deletion mutants. However, the propensity of Δ73−83 α-syn and Δ76−77/E83A α-syn to polymerize is significantly reduced when compared to that of WT α-syn, and Δ76−77/E83A α-syn exhibited altered morphology, as observed by EM. Therefore, the presence of the E83 residue is not the only determining factor, and conformational constraints resulting from deletion of particular hydrophobic residues also contribute to the propensity and ability of these proteins to polymerize into amyloid.

The substitution of A76 with a P residue, which is a potent *β*-pleated sheet inhibitor, did not result in as dramatic of an effect as that of the deletion of residues 76 and 77. The A76P mutation reduced the propensity for amyloid formation, similar to the A76E mutation (*21*), but it maintained the ability to form mature fibrils, demonstrating a remarkable flexibility in the structure of amyloid formation at this position. These findings further suggest that deletion of residues 76 and 77 likely causes significant structural constraints that differ from the missense mutations of the A76 residue, preventing the protein from naturally folding into amyloid. Further, mutation of A76 to either G or V did not significantly alter polymerization, suggesting an overall propensity of α-syn to fibrillize despite modifications to this residue. Nevertheless, the A76G mutation exhibited slight changes to fibril morphology, appearing more directional than WT α-syn

protein. While the alterations to the structure resulting from this mutation are currently unknown, these data suggest that a small, less hydrophobic residue may modify the packing of α-syn fibrils during amyloid formation.

Deletions of hydrophobic amino acid stretches flanking the segment of amino acids 71−82 also reduce the propensity to form amyloid. Interestingly, deletion of residues 85−94 did not prevent fibril formation; Δ85−94 α-syn and the A85P mutation resulted in fibrils with abundant short protrusions rather than the typical smooth profile. This alteration in ultrastructure may be due to "stuttering" during polymerization, changes in the packing of the protein, or a compensatory structure resulting from conformational constraints.

The deletion of residues 67−71 resulted in a significant reduction in the extent of amyloid formation, although filament assembly was not completely abolished. Other studies demonstrate that the deletion of residues 66−74 completely impairs amyloid formation, while deletion of residues 71−74 also significantly reduces the extent of polymerization, resulting in the formation of only short fibrils (*58*). The inability of Δ66−74 α-syn versus the ability of Δ67−71 α-syn (albeit significantly reduced) to polymerize may result from the conformational constraints imposed by the deletion of these particular residues. Alternatively, the deletion of the four extra hydrophobic residues in Δ66−74 α-syn may pass the threshold required to prevent fibril formation. However, the variable propensity of α-syn to form amyloid, depending on residue context and steric conditions, is further underscored by the ability of α-syn 1−74, where most of the hydrophobic middle region is removed, to still form abundant amyloid fibrils (*58*). In our studies, deletion of residues 67−71 or residues 69 and 70 did not alter fibril morphology or structure as determined by EM analysis (Figure 4C,D). Quantitative assays revealed that deletion of residues A69 and V70 resulted in abundant amyloid fibrils after incubation for 9 days (Figure 5A,B), while deletion of the same residues in an analogous hydrophobic stretch (A76 and V77) abrogated the formation of mature amyloid. The dramatic differences in the effects of deleting these short residues highlight the fact that it is both the nature of the residues and the spatial context that influence the process of amyloid formation. Single-residue deletions (Δ76, Δ77, and Δ78) as well as the A76P mutation did not completely prevent amyloid formation. This suggests that even within a spatial context, inhibition of amyloid formation requires alterations to particular or a minimal number of amino acids.

Our data are consistent with solid-state NMR studies and algorithms predicting β-sheet formation (*59*−*61*), which identify the major extended β-sheet region around residues 69−82. These predictions are based on an analysis of hydrophobic and charged residues in the core of the α-syn protein. While these models are informative, our data suggested considerable flexibility in the ability of α-syn to form amyloid.

Our studies indicate that not all hydrophobic regions of a polypeptide are equally important in determining its amyloidogenic aggregation tendency, despite the ability of very short specific amino acid stretches to facilitate amyloid fibril formation (*62*). Amyloid formation appears to have several influential factors, including the composition of its residues and the conformational tendency to pack into amyloid tertiary and quaternary structures. However, there is also considerable flexibility in this process. While the hydrophobic core of α-syn holds the key for the ability of this protein to form amyloid, this study supports the complexity of this statement as demonstrated by the variability in amyloid formation between particular amino acid deletions. A better understanding of the basic molecular requirement and constrains associated with amyloid formation will provide important insights that may enable the generation of therapeutic agents capable of preventing the assembly of amyloid.

REFERENCES

1. Norris, E. H., Giasson, B. I., and Lee, V. M. (2004) α-Synuclein: Normal function and role in neurodegenerative diseases. *Curr. Top. Dev. Biol. 60*, 17–54.
2. Goedert, M. (2001) α-Synuclein and neurodegenerative diseases. *Nat. Rev. Neurosci. 2*, 492–501.
3. Spillantini, M. G., Schmidt, M. L., Lee, V. M. Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) α-Synuclein in Lewy bodies. *Nature 388*, 839–840.
4. Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M. Y., Trojanowski, J. Q., and Iwatsubo, T. (1998) Aggregation of α-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol. 152*, 879–884.
5. Spillantini, M. G., Crowther, R. A., Jakes, R., Cairns, N. J., Lantos, P. L., and Goedert, M. (1998) Filamentous α-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neurosci. Lett. 251*, 205–208.
6. Tu, P. H., Galvin, J. E., Baba, M., Giasson, B., Tomita, T., Leight, S., Nakajo, S., Iwatsubo, T., Trojanowski, J. Q., and Lee, V. M. Y. (1998) Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble α-synuclein. *Ann. Neurol. 44*, 415–422.
7. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T. (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry 35*, 13709–13715.
8. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H. A., Kittel, A., and Saitoh, T. (1995) The precursor protein of non-Aβ component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron 14*, 467–475.
9. Clayton, D. F., and George, J. M. (1999) Synucleins in synaptic plasticity and neurodegenerative disorders. *J. Neurosci. Res. 58*, 120–129.
10. Cabin, D. E., Shimazu, K., Murphy, D., Cole, N. B., Gottschalk, W., Mcllwain, K. L., Orrison, B., Chen, A., Ellis, C. E., Paylor, R., Lu, B., and Nussbaum, R. L. (2002) Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking α-synuclein. *J. Neurosci. 22*, 8797–8807.
11. Murphy, D. D., Rueter, S. M., Trojanowski, J. Q., and Lee, V. M. Y. (2000) Synucleins are developmentally expressed, and α-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. *J. Neurosci. 20*, 3214–3220.
12. Chandra, S., Gallardo, G., Fernández-Chacón, R., Schlüter, O. M., and Südhof, T. C. (2005) α-Synuclein cooperates with CSPα in preventing neurodegeneration. *Cell 123*, 359–361.
13. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science 276*, 2045–2047.
14. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L., and Riess, O. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet. 18*, 106–108.
15. Zarranz, J. J., Alegre, J., Gomez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez, T. E., del Ser, T., Munoz, D. G., and de Yebenes, J. G. (2004) The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol. 55*, 164–173.
16. Chartier-Harlin, M. C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., Waucquier, N., Defebvre, L., Amouyel, P., Farrer, M., and Destee, A. (2004) α-Synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet 364*, 1167–1169.
17. Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M. R., Muenter, M., Baptista, M., Miller, D., Blancato, J.,

Hardy, J., and Gwinn-Hardy, K. (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science 302*, 841.
18. Conway, K. A., Harper, J. D., and Lansbury, P. T. (1998) Accelerated in vitro fibril formation by a mutant α-synuclein linked to early-onset Parkinson disease. *Nat. Med. 4*, 1318–1320.
19. Giasson, B. I., Uryu, K., Trojanowski, J. Q., and Lee, V. M. Y. (1999) Mutant and wild type human α-synucleins assemble into elongated filaments with distinct morphologies in vitro. *J. Biol. Chem. 274*, 7619–7622.
20. Hashimoto, M., Hsu, L. J., Sisk, A., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1998) Human recombinant NACP/α-synuclein is aggregated and fibrillated in vitro: Relevance for Lewy body disease. *Brain Res. 799*, 301–306.
21. Giasson, B. I., Murray, I. V., Trojanowski, J. Q., and Lee, V. M. Y. (2001) A hydrophobic stretch of 12 amino acid residues in the middle of α-synuclein is essential for filament assembly. *J. Biol. Chem. 276*, 2380–2386.
22. Li, J., Uversky, V. N., and Fink, A. L. (2001) Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human α-synuclein. *Biochemistry 40*, 11604–11613.
23. Narhi, L., Wood, S. J., Steavenson, S., Jiang, Y., Wu, G. M., Anafi, D., Kaufman, S. A., Martin, F., Sitney, K., Denis, P., Louis, J. C., Wypych, J., Biere, A. L., and Citron, M. (1999) Both familial Parkinson's disease mutations accelerate α-synuclein aggregation. *J. Biol. Chem. 274*, 9843–9846.
24. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both α-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. U.S.A. 97*, 571–576.
25. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Harper, J. D., Williamson, R. E., and Lansbury, P. T. (2000) Accelerated oligomerization by Parkinson's disease linked α-synuclein mutants. *Ann. N.Y. Acad. Sci. 920*, 42–45.
26. Greenbaum, E. A., Graves, C. L., Mishizen-Eberz, A. J., Lupoli, M. A., Lynch, D. R., Englander, S. W., Axelsen, P. H., and Giasson, B. I. (2005) The E46K mutation in α-synuclein increases amyloid fibril formation. *J. Biol. Chem. 280*, 7800–7807.
27. Choi, W., Zibaee, S., Jakes, R., Serpell, L. C., Davletov, B., Crowther, R. A., and Goedert, M. (2004) Mutation E46K increases phospholipid binding and assembly into filaments of human α-synuclein. *FEBS Lett. 576*, 363–368.
28. Giasson, B. I., and Lee, V. M. Y. (2003) Are ubiquitination pathways central to Parkinson's disease? *Cell 114*, 1–8.
29. Uversky, V. N., Li, J., and Fink, A. L. (2001) Evidence for a partially-folded intermediate in α-synuclein fibril formation. *J. Biol. Chem. 276*, 10737–10744.
30. Conway, K. A., Harper, J. D., and Lansbury, P. T. (2000) Fibrils formed in vitro from α-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry 39*, 2552–2563.
31. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-β conformation. *Proc. Natl. Acad. Sci. U.S.A. 97*, 4897–4902.
32. Mazzulli, J. R., Mishizen, A. J., Giasson, B. I., Lynch, D. R., Thomas, S. A., Nakashima, A., Nagatsu, T., Ota, A., and Ischiropoulos, H. (2006) Cytosolic catechols inhibit α-synuclein aggregation and facilitate the formation of intracellular soluble oligomeric intermediates. *J. Neurosci. 26*, 10068–10078.
33. Waxman, E. A., and Giasson, B. I. (2008) Molecular mechanisms of α-synuclein neurodegeneration. *Biochim. Biophys. Acta 1792*, 616–624.
34. Volles, M. J., Lee, S.-J., Rochet, J.-C., Shtilerman, M. D., Ding, T. T., Kessler, J. C., and Lansbury, P. T. (2001) Vesicle permeabilization by protofibrillar α-synuclein: Implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry 40*, 7812–7819.
35. Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science 300*, 486–489.
36. Miake, H., Mizusawa, H., Iwatsubo, T., and Hasegawa, M. (2002) Biochemical characterization of the core structure of α-synuclein filaments. *J. Biol. Chem. 277*, 19213–19219.
37. Der-Sarkissian, A., Jai, C. C., Chen, J., and Langen, R. (2003) Structral organization of α-synuclein fibrils studied by site-directed spin labeling. *J. Biol. Chem. 278*, 37530–37535.
38. Chen, M., Margittai, M., Chen, J., and Langen, R. (2007) Investigation of α-synuclein fibril structure by site-directed spin labeling. *J. Biol. Chem. 282*, 24970–24979.
39. Del, M. C., Greenbaum, E. A., Mayne, L., Englander, S. W., and Woods, V. L., Jr. (2005) Structure and properties of α-synuclein and other amyloids determined at the amino acid level. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15477–15482.
40. Vilar, M., Chou, H. T., Luhrs, T., Maji, S. K., Riek-Loher, D., Verel, R., Manning, G., Stahlberg, H., and Riek, R. (2008) The fold of α-synuclein fibrils. *Proc. Natl. Acad. Sci. U.S.A. 105*, 8637–8642.
41. Murray, I. V., Giasson, B. I., Quinn, S. M., Koppaka, V., Axelsen, P. H., Ischiropoulos, H., Trojanowski, J. Q., and Lee, V. M. (2003) Role of α-synuclein carboxy-terminus on fibril formation in vitro. *Biochemistry 42*, 8530–8540.
42. Crystal, A. S., Giasson, B. I., Crowe, A., Kung, M. P., Zhuang, Z. P., Trojanowski, J. Q., and Lee, V. M. (2003) A comparison of amyloid fibrillogenesis using the novel fluorescent compound K114. *J. Neurochem. 86*, 1359–1368.
43. Giasson, B. I., Jakes, R., Goedert, M., Duda, J. E., Leight, S., Trojanowski, J. Q., and Lee, V. M. Y. (2000) A panel of epitope-specific antibodies detects protein domains distributed throughout human α-synuclein in Lewy bodies of Parkinson's disease. *J. Neurosci. Res. 59*, 528–533.
44. Chou, P. Y., and Fasman, G. D. (1974) Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochemistry 13*, 211–222.
45. Chou, P. Y., and Fasman, G. D. (1974) Prediction of protein conformation. *Biochemistry 13*, 222–245.
46. Zibaee, S., Jakes, R., Fraser, G., Serpell, L. C., Crowther, R. A., and Goedert, M. (2007) Sequence determinants for amyloid fibrillogenesis of human α-synuclein. *J. Mol. Biol. 374*, 454–464.
47. Pawar, A. P., Dubay, K. F., Zurdo, J., Chiti, F., Vendruscolo, M., and Dobson, C. M. (2005) Prediction of "aggregation-prone" and "aggregation-susceptible" regions in proteins associated with neurodegenerative diseases. *J. Mol. Biol. 350*, 379–392.
48. Chiti, F., Stefani, M., Taddei, N., Ramponi, G., and Dobson, C. M. (2003) Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature 424*, 805–808.
49. Uversky, V. N., and Fink, A. L. (2004) Conformational constraints for amyloid fibrillation: The importance of being unfolded. *Biochim. Biophys. Acta 1698*, 131–153.
50. Bemporad, F., Calloni, G., Campioni, S., Plakoutsi, G., Taddei, N., and Chiti, F. (2006) Sequence and structural determinants of amyloid fibril formation. *Acc. Chem. Res. 39*, 620–627.
51. Apetri, M. M., Maiti, N. C., Zagorski, M. G., Carey, P. R., and Anderson, V. E. (2006) Secondary structure of α-synuclein oligomers: Characterization by Raman and atomic force microscopy. *J. Mol. Biol. 355*, 63–71.
52. Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat. Struct. Mol. Biol. 15*, 558–566.
53. Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A. L. (2004) The flavonoid baicalein inhibits fibrillation of α-synuclein and disaggregates existing fibrils. *J. Biol. Chem. 279*, 26846–26857.
54. Norris, E. H., Giasson, B. I., Hodara, R., Xu, S., Trojanowski, J. Q., Ischiropoulos, H., and Lee, V. M. (2005) Reversible inhibition of α-synuclein fibrillization by dopaminochrome-mediated conformational alterations. *J. Biol. Chem. 280*, 21212–21219.
55. Lashuel, H. A., Petre, B. M., Wall, J., Simon, M., Nowak, R. J., Walz, T., and Lansbury, P. T. Jr. (2002) α-Synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol. 322*, 1089–1102.
56. Fredenburg, R. A., Rospigliosi, C., Meray, R. K., Kessler, J. C., Lashuel, H. A., Eliezer, D., and Lansbury, P. T. Jr. (2007) The impact of the E46K mutation on the properties of α-synuclein in its monomeric and oligomeric states. *Biochemistry 46*, 7107–7118.
57. Rivers, R. C., Kumita, J. R., Tartaglia, G. G., Dedmon, M. M., Pawar, A., Vendruscolo, M., Dobson, C. M., and Christodoulou, J. (2008) Molecular determinants of the aggregation behavior of α- and β-synuclein. *Protein Sci. 17*, 887–898.
58. Du, H. N., Tang, L., Luo, X. Y., Li, H. T., Hu, J., Zhou, J. W., and Hu, H. Y. (2003) A peptide motif consisting of glycine, alanine, and valine is required for the fibrillization and cytotoxicity of human α-synuclein. *Biochemistry 42*, 8870–8878.
59. Heise, H., Hoyer, W., Becker, S., Andronesi, O. C., Riedel, D., and Baldus, M. (2005) Molecular-level secondary structure, polymorphism, and dynamics of full-length α-synuclein fibrils studied by solid-state NMR. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15871–15876.
60. Heise, H., Celej, M. S., Becker, S., Riedel, D., Pelah, A., Kumar, A., Jovin, T. M., and Baldus, M. (2008) Solid-state NMR reveals

structural differences between fibrils of wild-type and disease-related A53T mutant α-synuclein. *J. Mol. Biol. 380*, 444–450.
61. Zibaee, S., Makin, O. S., Goedert, M., and Serpell, L. C. (2007) A simple algorithm locates *β*-strands in the amyloid fibril core of α-synuclein, A*β*, and tau using the amino acid sequence alone. *Protein Sci. 16*, 906–918.
62. Ventura, S., Zurdo, J., Narayanan, S., Parreno, M., Mangues, R., Reif, B., Chiti, F., Giannoni, E., Dobson, C. M., Aviles, F. X., and Serrano, L. (2004) Short amino acid stretches can mediate amyloid formation in globular proteins: The Src homology 3 (SH3) case. *Proc. Natl. Acad. Sci. U.S.A. 101*, 7258–7263.