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The E46K Mutation in α-Synuclein Increases Amyloid Fibril Formation*

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The identification of a novel mutation (E46K) in one of the KTKEGV-type repeats in the amino-terminal region of α-synuclein suggests that this region and, more specifically, Glu residues in the repeats may be important in regulating the ability of α-synuclein to polymerize into amyloid fibrils. It was demonstrated that the E46K mutation increased the propensity of α-synuclein to fibrillize, but this effect was less than that of the A53T mutation. The substitution of Glu^{46} for an Ala also increased the assembly of α-synuclein, but the polymers formed can have different ultrastructures, further indicating that this amino acid position has a significant effect on the assembly process. The effect of residue Glu^{83} in the sixth repeat of α-synuclein, which lies closest to the amino acid stretch critical for filament assembly, was also studied. Mutation of Glu^{83} to a Lys or Ala increased polymerization but perturbed some of the properties of mature amyloid. These results demonstrated that some of the Glu residues within the repeats can have significant effects on modulating the assembly of α-synuclein to form amyloid fibrils. The greater effect of the A53T mutation, even when compared with what may be predicted to be a more dramatic mutation such as E46K, underscores the importance of protein microenvironment in affecting protein structure. Moreover, the relative effects of the A53T and E46K mutations are consistent with the age of onset of disease. These findings support the notion that aberrant α-synuclein polymerization resulting in the formation of pathological inclusions can lead to disease.

α-Syn[1] is a small (140-amino acid) protein characterized by an acidic carboxyl-terminal region and six repetitive imperfect repeats (KTKEGV-type) distributed throughout most of the amino-terminal half (1) (see Fig. 1). α-Syn is a highly soluble, heat-stable, natively unfolded protein (2, 3) predominantly expressed in the neurons of the central nervous system and localized at presynaptic terminals in close proximity to synaptic vesicles (1, 4–6). Whereas the function of α-syn has not been not fully characterized, several studies suggest that it is involved in modulating synaptic transmission, the density of synaptic vesicles, and neuronal plasticity (5–9).

α-Syn was directly implicated in neurodegenerative disease when the mutation A53T was identified in a large Italian family (Contursi) and three small Greek families with autosomal dominant Parkinson's disease (PD) (10). Thereafter, the A53T mutation was identified in an additional eight kindreds (11–14). Another autosomal dominant mutation (A30P) in α-syn has been identified in a German kindred (15). Recently, a short trisomy containing the *α-syn* gene plus flanking regions on chromosome 4 and a novel E46K mutation in α-syn have been identified in separate kindreds in which individuals manifest classical PD or the related disorder, dementia with Lewy bodies (DLB) (16–18).

It is now recognized that α-syn is the major building block of the filaments that form the proteinacious pathological inclusions characteristic of many disorders, including Lewy bodies and Lewy neurites in PD, DLB, and Lewy body variant of Alzheimer's disease (19–23). Approximately 10-nm-wide fibrils of α-syn also are the primary component of glial cytoplasmic inclusions in multiple system atrophy (24, 25). α-Syn pathological inclusions also are present in a subset of patients afflicted by several other neurodegenerative disorders, such as Down's syndrome, progressive autonomic failure, neurodegeneration with brain iron accumulation type-1, and both sporadic and familial Alzheimer's disease (25–33), leading to the term "synucleinopathies" (23, 34).

The finding that soluble recombinant α-syn readily polymerizes into ~10-nm filaments *in vitro* (35–38) has provided critical evidence supporting the notion that α-syn is the building block of Lewy bodies and related pathological inclusions. This finding also has provided important means to study this process *in vitro*. The polymerization of α-syn is nucleation-dependent (38, 39), and it is dependent on critical molecular interactions. The central hydrophobic region (known as the NAC region) in α-syn is necessary for fibrillization (40, 41). Amino acid deletions or differences in this region explain why other syn family members (*i.e.* β- and γ-syn) do not fibrillize under physiological conditions (40, 42). Other regions in α-syn also are important in modulating filament formation. The A53T mutation within the amino-terminal region has been consistently reported to increase the propensity of fibril formation (35, 36, 38). Further support for the important role of the amino-terminal region of α-syn comes from fibrillogenesis experiments in which the number of repeats was modified. Deletion of repeats 1 and 2 in the amino terminus of α-syn acceler-

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[1] The abbreviations used are: syn, synuclein; ATR-FTIR, attenuated total internal-reflection Fourier transform infrared; DLB, dementia with Lewy bodies; EM, electron microscopy; PD, Parkinson's disease; WT, wild-type.

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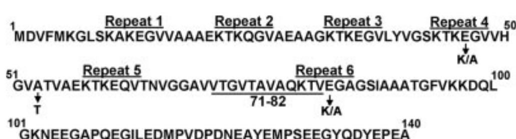

FIG. 1. **Sequence of human α-syn.** The amino acid sequence of human α-syn with the six KTKEGV-type repeats indicated *above* the sequences. The stretch of amino acid residues 71–82 required for filament assembly is identified. The positions of mutants of α-syn (E46K and A53T) causal of disease or the additional artificial mutants (E46A, E83A, and E83K) used in the studies are shown with *arrows*.

ates filament formation, whereas duplication of these repeats inhibits this process (43).

The recent identification of the new (E46K) amino-terminal mutation in α-syn (18) in the fourth repeat of α-syn (Fig. 1) further suggests that the amino-terminal region may be important in modulating filament formation; however, the effect of this mutation had not been studied. Whereas our initial goal was to study the effect that this mutation had on the polymerization of α-syn, it was also noted that a Glu residue similar to Glu^{46} is present in five of the six degenerative repeats in α-syn. The only repeat that does not have such a residue (repeat 2) has Glu residues adjacent to each side of the repeat (see Fig. 1). The possibility that Glu residues in the repeats are important in modulating the ability of α-syn to polymerize was further investigated by generating the mutant E46A. The Ala mutation results in the loss of one negative charge, instead of a complete change in charge from negative to positive. The effects of the mutants E83A and E83K in the sixth repeat, closest to the amino acid stretch critical for polymerization, were also studied to assess the role of Glu residues.

EXPERIMENTAL PROCEDURES

Expression and Purification of α-Syn—Human α-syn cDNA was cloned into the NdeI and HindIII restriction sites of the bacterial expression vector pRK172. The cDNAs coding for the mutant α-syn protein E46A, E46K, E83A, and E83K 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). All mutations were confirmed by DNA sequencing. α-Syn proteins were expressed in *Escherichia coli* BL21(DE3). Bacterial pellets harvested by centrifugation were re-suspended in high-salt buffer (0.75 M NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA) containing a mixture of protease inhibitors, heated to 100 °C for 10 min, and centrifuged at 70,000 × *g* for 30 min. Supernatants were dialyzed into 100 mM NaCl, 20 mM Tris, pH 7.5 and applied onto a Superdex 200 gel filtration column (Amersham Biosciences) and separated by size exclusion. The fractions were assayed for the presence of the α-syn proteins by SDS-PAGE followed by Coomassie Blue R-250 staining. Proteins were concentrated using Centriprep-10 units (Millipore); dialyzed against 10 mM Tris, pH 7.5; applied to a Mono Q column (Amersham Biosciences); and eluted with a 0–0.5 M NaCl gradient. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce) and 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 7.0, with continuous shaking. A fraction of each sample was set aside for K114 fluorometry and electron microscopy (EM) analysis. The remainder of each sample was centrifuged at 100,000 × *g* for 20 min, and SDS sample buffer (10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM dithiothreitol, 1% SDS, and 10% sucrose) was added to pellets and supernatants, which were heated to 100 °C for 15 min. α-Syn proteins in the supernatants and pellets were quantified by densitometry of Coomassie Blue-stained SDS-polyacrylamide gels or ^{125}I quantitative Western blotting analysis (40, 44).

K114 Fluorometry—α-Syn can form amyloid fibrils (45), which can be quantified using the fluorescent amyloid binding dye K114 (46). 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 amyloid fibrils (46). This assay was conducted, as described previously (46), by incubating a fraction of each sample with K114 (50 μM) in 100 mM glycine, pH 8.5, and measuring fluorescence (λ_{ex} = 380 nm, λ_{em} = 550 nm, cutoff = 530 nm) with a SpectraMax Gemini fluorometer and SoftMax Pro 4.0 software.

CD Spectrometry—α-Syn proteins prepared at concentrations of 2.5 or 5 mg/ml in 100 mM phosphate buffer, pH 7.0, were analyzed by CD immediately upon or after incubation at 37 °C for 24, 48, or 96 h with constant shaking. Proteins were diluted to a final concentration of 5 μM into 5 mM phosphate buffer, pH 7.0. After dilution, the protein was transferred into a 1-mm quartz cuvette, and the CD spectra between 190 and 260 nm were recorded using an Aviv model 202 spectrophotometer (Lakewood, NJ.)

Attenuated Total Internal-Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy—Monomeric α-syn samples were dialyzed against 30 mM HEPES buffer in D_2O at pD = 7.4 (meter reading 7.0), adjusted to a protein concentration of 10 mg/ml, and drawn into a 15-μm flow cell with CaF_2 windows. This technique, employing ordinary transmission mode spectroscopy, was the best way to characterize the protein in solution and avoid consequences of any changes in physical condition. 512 co-added interferograms were collected on a Digilab FTS-60A equipped with a wide band MCT detector. All spectra were collected at room temperature at 20 kHz with a resolution of 2 cm^{-1}, undersampling ratio of 2, one level of zero-filling, and triangular apodization. The ratio of detector response to that obtained with protein-free buffer was calculated to yield the absorption spectrum. A flat baseline was subtracted from each spectrum, but no water vapor subtraction or smoothing operations were performed.

Assembled α-syn samples were dialyzed against 5 mM HEPES buffer in D_2O at pD = 7.4 and adjusted to a protein concentration of 5 mg/ml, and ~3 μl of this solution were placed on a 2 × 10 × 52-mm germanium internal reflection crystal with 45° facets and evaporated to dryness at room temperature. 1024 co-added interferograms were collected before and after application of each sample and processed as described above to yield the absorption spectrum. This technique, involving drying of the sample and probing with an evanescent field, was necessary because the assembled protein did not readily pass through the flow cell and would not otherwise remain in place throughout the 10-min period required to collect the spectrum. The six spectra of monomeric α-syn were analyzed simultaneously using Irfit (47), as were the six spectra of aggregated α-syn in a separate operation. Both sets of spectra are well fit by three components. Within each set, each component has the same frequency, half-width, and shape in each experimental spectrum, with only the amplitude of each component adjusted to fit the data.

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 Joel 1010 transmission electron microscope (Peabody, MA). Images were captured with a Hamamatsu digital camera (Bridgewater, MA) using AMT software (Danvers, MA). For diameter determination, the width of 100–120 filaments was measured using Image-Pro Plus software (Media Cybernetics, Del Mar, CA).

Calpain I Cleavage of α-Syn Proteins—Calpain I (Calbiochem) cleavage of purified recombinant human α-syn proteins was carried out using methods similar to those described previously (48). Briefly, α-syn protein was incubated with calpain I in buffer containing 40 mM HEPES, pH 7.5, and 5 mM dithiothreitol at 37 °C. Calpain cleavage was initiated by the addition of calcium (1 mM final concentration). Aliquots were removed from the reaction mixture and added to an equal volume of 2× Invitrogen SDS-stop buffer at various time points, heated in a boiling water bath, and stored at −20 °C.

Western Blotting Analysis—Proteins were resolved on slab gels by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in buffer containing 48 mM Tris, 39 mM glycine, and 10% methanol. Membranes were blocked with a 1% solution of powdered skimmed milk dissolved in Tris buffered saline-Tween 20 (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), incubated with anti-α-syn antibodies Syn 303, SNL-1, or Syn 102 (49, 50). After incubation with either goat anti-mouse or a goat anti-rabbit antibodies conjugated to horseradish peroxidase, the blots were developed with Renaissance Enhanced Luminol Reagents (PerkinElmer Life Sciences) and exposed onto X-Omat Blue XB-1 films (Eastman Kodak Co.).

RESULTS

The effects of two α-syn mutations causal of disease (A53T and E46K) and three synthetic mutations (E46A, E83A, and A83K) were studied. The ability of these proteins to polymerize was compared with that of wild-type (WT) α-syn as assayed by sedimentation analysis and K114 fluorometry (Fig. 2, *A* and *B*).

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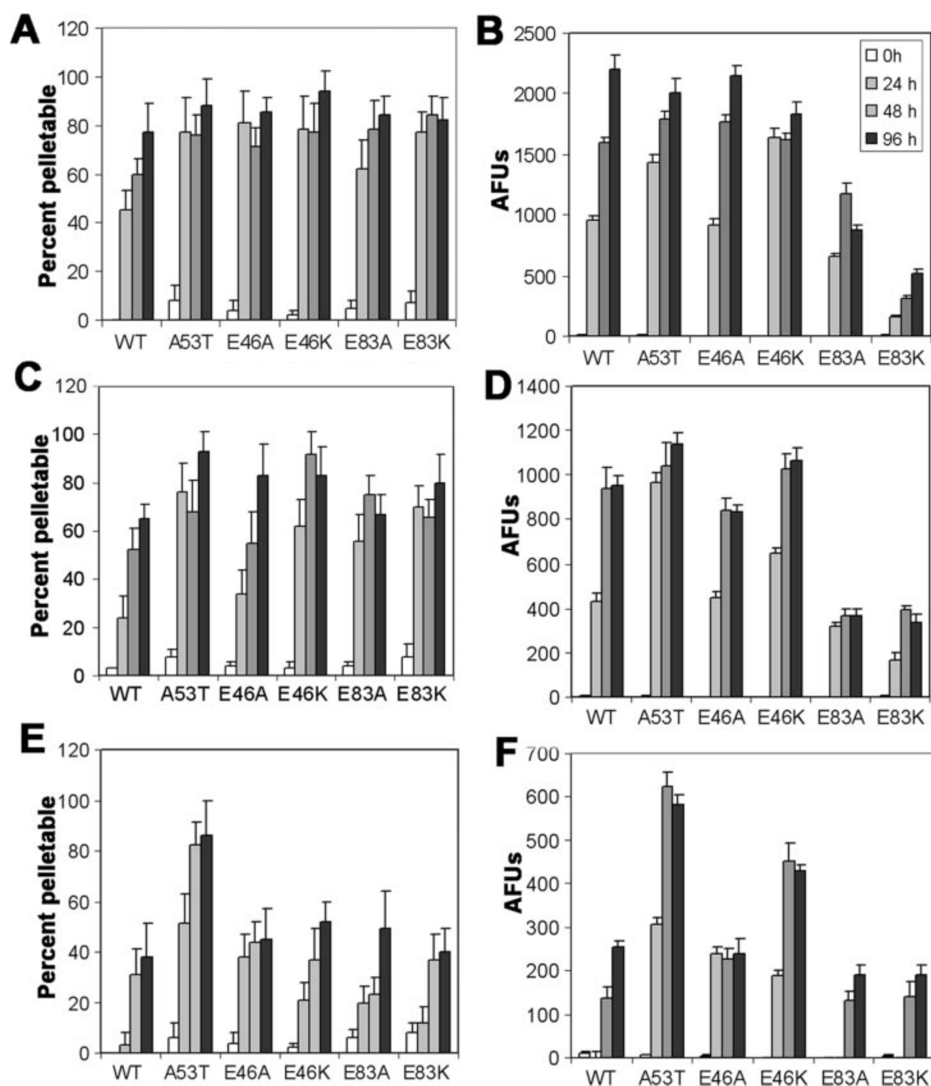

FIG. 2. **Analyses of the polymerization of α-syn and mutants thereof.** WT, A53T, E46A, E46K, E83A, and E83K α-syn were incubated at 5 (*A* and *B*), 2.5 (*C* and *D*), or 1.0 mg/ml (*E* and *F*) under filament assembly condition as described under "Experimental Procedures." Samples at 0, 24, 48, and 96 h were assayed by sedimentation analysis (*A*, *C*, and *E*) and K114 fluorometry (*B*, *D*, and *F*). $n = 6$ for all data sets.

At the highest concentration (5 mg/ml) of α-syn proteins used for these analyses, all α-syn proteins readily formed polymers that could be sedimented at $100,000 \times g$. Whereas all mutant α-syn proteins tested formed amyloid structures as revealed by K114 fluorometry, the E83A and E83K mutants generated much less K114 fluorescence. Experiments using a low concentration of proteins (2.5 or 1 mg/ml) showed that the E46K mutant polymerized more readily than the WT protein but that the effect was not as dramatic as the change seen in the A53T mutant. As quantified by sedimentation assay, the synthetic mutants E46A, E83A, and E83K also showed an increased propensity to polymerize.

To determine the conformational changes associated with the polymerization of α-syn proteins, CD and ATR-FTIR spectrometry were performed. CD spectrometry on monomeric α-syn proteins showed a minimum between 195 and 200 nm, indicating predominantly random coil or unstructured conformation (Fig. 3*A*). After incubation under assembly conditions, WT, A53T, E46K, and E83K α-syn proteins demonstrated CD spectra characterized by negative deflection at 215–225 nm and positive signal at 195–205, indicating that they had acquired predominantly β-pleated sheet conformation (Fig. 3*B*). Despite positive results in sedimentation assays (Fig. 2) and EM (see Fig. 5), the assembled proteins E46A and E83A yielded relatively low CD signal compared with the other mutants tested.

The infrared spectra of monomeric α-syn proteins exhibit a maximum at 1646.2 cm^{-1}, with a predominant component comprising 60–70% of the signal that is centered at 1643.9 cm^{-1}. This suggests that the proteins are unstructured under these conditions (Fig. 3*C*). Most of the remaining signal, ~30%, is accounted for by a broad component centered at 1668.1 cm^{-1}; however, conformational assignment of this component is not reliable. The five mutant proteins were indistinguishable from WT protein. The spectra for the aggregated α-syn proteins were quite distinct from those of monomeric proteins (Fig. 3*D*). The maximum was at 1627.9 cm^{-1}, with the dominant component centered at 1625.0 cm^{-1} comprising 44–55% of the signal. The position of this component suggests that substantial amounts of β-sheet structure have formed in the course of aggregation. The component at 1643.9 cm^{-1} in monomeric proteins appears to have shifted slightly to 1641.4 cm^{-1} and decreased to between 7% and 24% of the signal. As with the monomeric proteins, ~30% of the signal is accounted for by a high frequency component centered, in this case, at 1661.4 cm^{-1}. Again, spectra from the five mutated proteins were similar to that of the WT protein. The difference between A53T and E46K α-syn and

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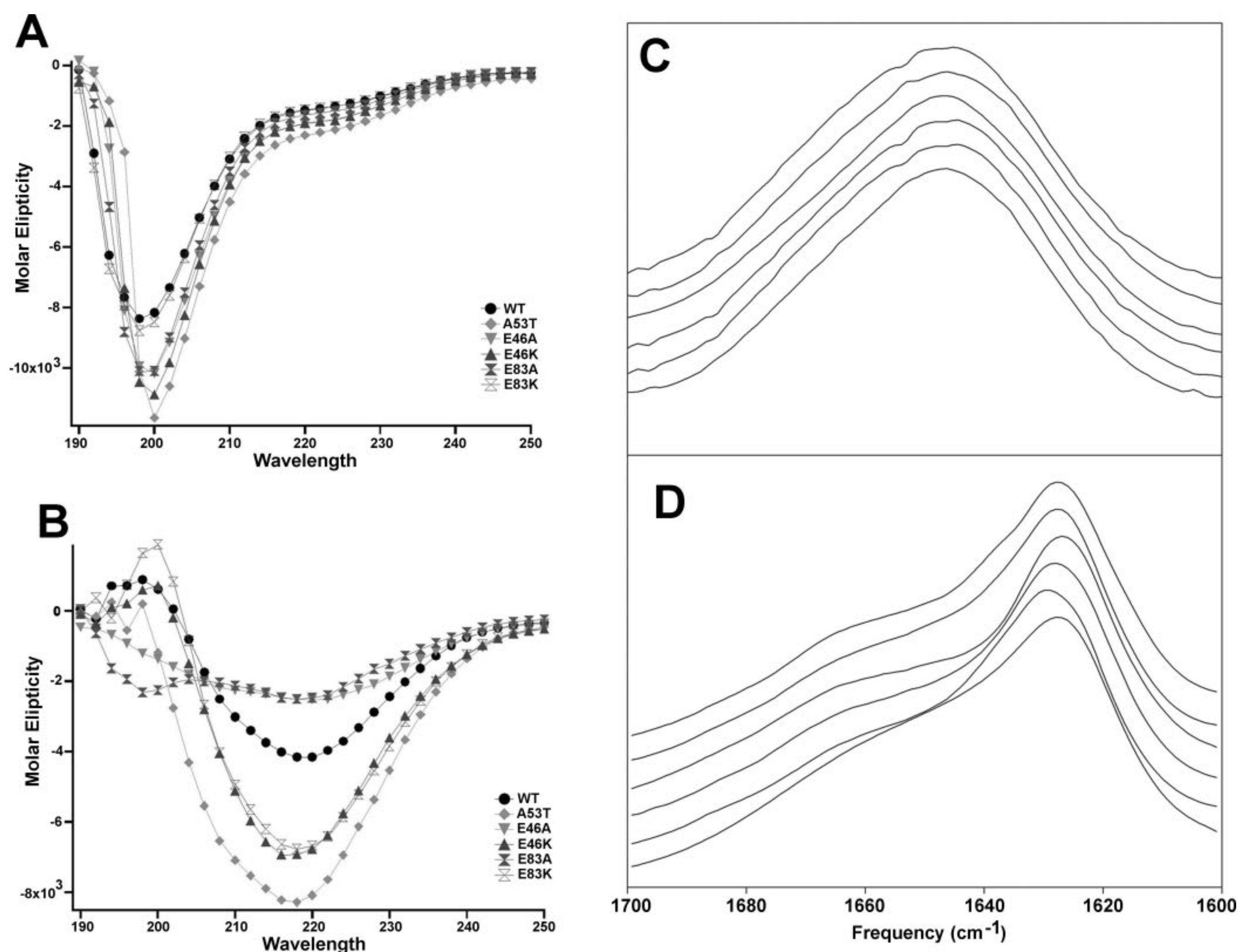

FIG. 3. **Biophysical analysis of conformation changes associated with polymerization of α-syn proteins.** CD analysis of unassembled (*A*) and assembled α-syn proteins (*B*) measured at 5 μM in 5 mM phosphate buffer, pH 7.0, and expressed in molar ellipticity. In *A* and *B*, the spectrum corresponding to each protein is indicated in each graph. ATR-FTIR spectra in the amide I region of unassembled (*C*) and assembled (*D*) α-syn proteins as described under "Experimental Procedures." In *C* and *D*, the spectra (from *top* to *bottom*) were obtained with E83K, E83A, E46K, E46A, A53T, and WT α-syn.

the other proteins is minimal and therefore difficult to interpret as a significant conformational change.

In order to obtain more detailed information regarding the conformational changes accompanying the fibrillization of WT and E46K α-syn, samples prepared at 2.5 mg/ml were allowed to fibrillize for 0, 24, 48, or 96 h. At each time point, CD spectra were collected for WT and E46K α-syn. The changes in CD spectra over time indicated that E46K α-syn converts from random coil conformation to β-sheet more readily than the WT protein (Fig. 4, *A* and *B*). These changes can be more easily compared by plotting assembly time *versus* molar ellipticity at 200 nm. The signal at this wavelength is important for differentiating between random coil and β-sheet because they generate negative and possible values, respectively. This analysis demonstrated that the E46K mutant undergoes earlier acquisition of β-sheet structure than the WT protein (Fig. 4*C*).

To directly assess the ultrastructure of assembled α-syn proteins and to try to explain the difference in CD characteristics of the mutant proteins E46A and E83A, EM studies were performed (Fig. 5). These studies revealed that all proteins have the ability to form negatively stained fibrils that resemble those formed by the WT protein. The E46K mutant protein had a tendency to form compact bundles of fibrils (Fig. 5*D*). The Ala mutant proteins, notably E46A, often formed amorphous, elongated polymers that had a very distinct morphology compared with typical α-syn filaments (Fig. 5*C*). These polymers did not have the normal dark-edged contrast; instead, they were evenly electron dense, reminiscent of nonspecific protein aggregation. Mutants at residue Glu^{83} also yielded fibrils that were narrower than the WT α-syn protein: WT (9.2 ± 2.0 nm), E46A (11.5 ± 2.4 nm), E46K (11.3 ± 2.1 nm), E83A (6.2 ± 1.6 nm), and E83K (6.9 ± 1.9 nm).

The A53T α-syn mutation was previously shown to affect one of the major calpain I cleavage sites in α-syn (48). There also are major differences in the cleavage patterns of assembled *versus* monomeric α-syn cleaved by calpain I (48). With these findings in mind, the effects of E/A and E/K mutations on the processing of monomeric and polymerized α-syn by calpain 1 were investigated. No significant differences in the overall rate of cleavage of soluble or fibrillized WT, E/A, or E/K mutant α-syn proteins by calpain were observed (Fig. 6, *A* and *D*; data not shown). However, cleavage of soluble E46A and E46K α-syn appeared to result in a slight increase in the mobility of product #4 (corresponding to residues 1–57; Ref. 48). A more thorough analysis was conducted by resolving the cleavage products on the same SDS-polyacrylamide gel (Fig. 6*B*) and by immunoblotting analysis with antibody Syn 303 that specifically reacts with the amino terminus of α-syn (*i.e.* residues 2–4). This

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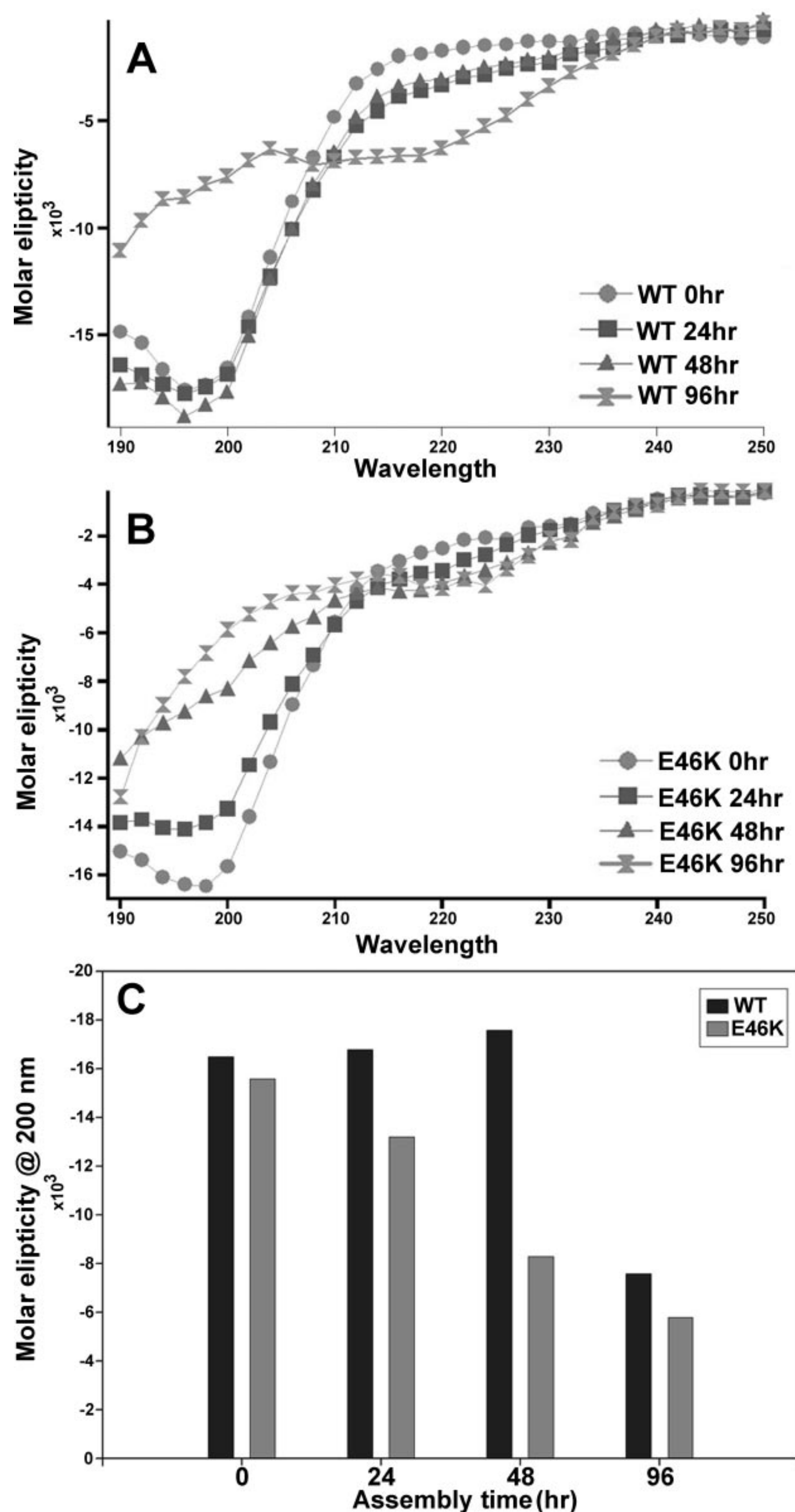

FIG. 4. **Kinetic analysis of β-sheet formation monitored by CD spectroscopy**. In order to observe the transition to β-sheet over time, CD spectra of WT (*A*) and E46K (*B*) at 2.5 mg/ml were taken after 0, 24, 48, and 96 h of incubation as described under "Experimental Procedures." In *A* and *B*, the spectrum corresponding to each time point is indicated in the graphs. In *C*, the absolute value of the molar ellipticity at 200 nm for each protein is compared at the various time points collected.

analysis demonstrated that calpain cleavage of E46A and E46K α-syn does not affect fragments 1, 2, or 3 but slightly increases the mobility of fragment 4. Fragment 1 (residue 1–57) and fragment 4 (residues 58–140) of WT α-syn are likely generated from a single cleavage by calpain (48). As previously shown, the A53T mutation significantly reduces cleavage at this site (Fig. 6, *A*–*C*) (48). The Glu^{46} mutations do not affect the generation of fragment 1. Therefore the more likely effect of the Glu^{46} mutants is to induce additional cleavage by calpain around amino acid residue 46, thereby resulting in a new fragment (indicated by *asterisks* in Fig. 6) with slightly greater mobility than product 4. The cleavage of fibrillized α-syn by calpain is different from that of soluble α-syn. Fibrillized α-syn is cleaved at two major sites in the carboxyl-terminal region: fragment 5 (residues 1–122) and fragment 6 (residues 1–114) (48). Similar to fibrillized WT α-syn, fibrillized mutants of α-syn demonstrated a similar cleavage pattern in the carboxyl terminus (Fig. 6*D*; data not shown).

DISCUSSION

The identification of a novel mutation (E46K) in a kindred with PD/DLB in one of the repeats of α-syn suggested that Glu

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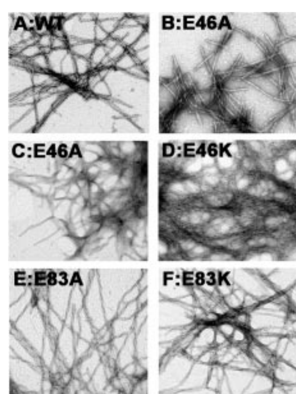

FIG. 5. **EM analysis of α-syn filaments assembled *in vitro*.** WT, E46A, E46K, E83A, and E83K α-syn proteins at 5 mg/ml were incubated under assembly conditions and analyzed by negative staining EM as described under "Experimental Procedures." *Scale bar*, 200 nm.

residues present in or close to all six repeat regions may be important factors modulating the ability of α-syn to polymerize into fibrils. Theoretically, because Glu residues show very little β-sheet propensity, it could be predicted that Glu residues may act to reduce the propensity of α-syn to fibrillize by preventing the local formation of β-pleated sheets (51, 52). This follows logically if one considers that conversion from random coil to β-sheet accompanies α-syn amyloid formation (36, 40, 53). It also would be expected that the E46K mutation would have a greater effect than the A53T mutation, considering the chemical properties of the substituted amino acids.

To study these possibilities, in addition to the E46K mutation, the effects of the synthetic mutation E46A were also analyzed. Similar to E46K, this mutation is expected to locally increase β-sheet propensity. It also results only in the ablation of a negative charge as opposed to a complete change in charge. In parallel to the mutations at amino acid residue 46, similar mutations at amino acid residue 83 located in the sixth repeat and closest to the region critical for filament assembly, were studied.

All of the mutant α-syn proteins studied demonstrated random coil conformation as monomers, and they could all assemble into fibrils reminiscent of WT α-syn. The E46K mutation increased the propensity to fibrillize compared with the WT protein. The E46A mutation also increased the propensity to polymerize, but this substitution resulted in two types of mature fibrils. Some fibrils had the typical morphology of WT α-syn filaments, but other fibrils appeared to share properties common to nonspecific protein aggregation. It has been proposed that α-syn polymerization progresses from unordered monomers to partially folded intermediates and, finally, into "mature" elongated filaments (54). It is possible that the E46A mutation hinders the ability of α-syn to proceed from partially folded intermediates to fully folded subunits, resulting in "hybrid" fibrils that consist of both types of building blocks. The mixture of nonspecific aggregates with fibrils may account for the lack of interpretable CD signal due to a shadowing effect. Despite this finding with CD, it was still possible to determine that these aggregates contained predominantly β-sheet structure by utilizing ATR-FTIR. Measurements for ATR-FTIR are performed by applying the samples to a germanium internal reflection crystal, and therefore this method is less affected by the presence of protein aggregates than the liquid phase measurement used for CD. Taken together, the data obtained by these two methods show that residue Glu^{46} has an important effect on the fibrillogenesis of α-syn.

The mutations of residue Glu^{83} had unexpected results. These substitutions increased the ability of α-syn to polymer-

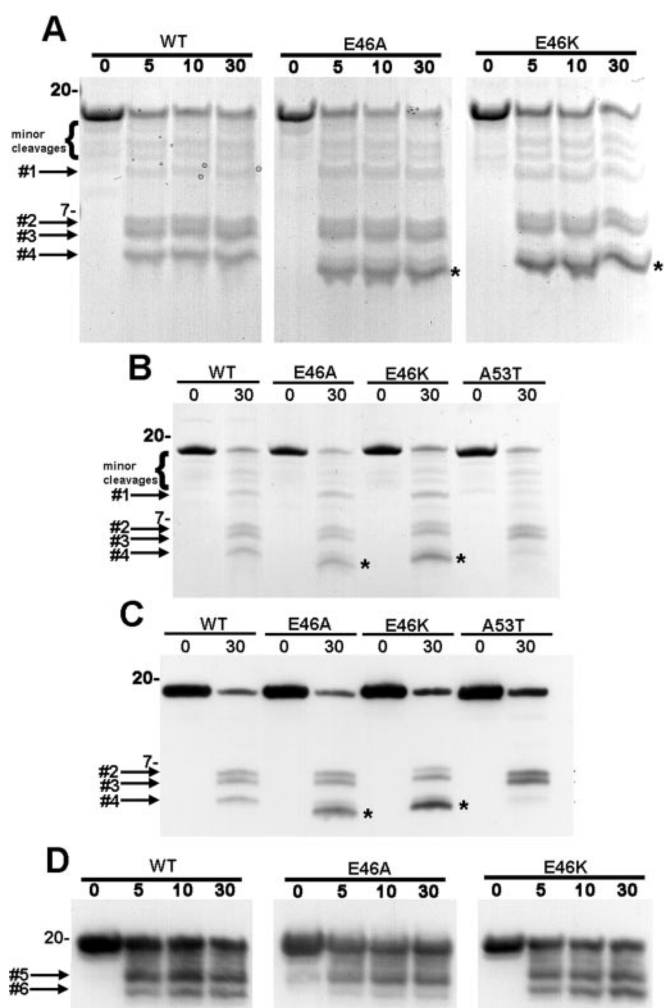

FIG. 6. **Analysis of calpain I cleavage of α-syn proteins.** *A*, Coomassie Blue stain of SDS/Tris-Tricine gel of soluble WT, E46A, and E46K α-syn incubated with calpain for 0–30 min as indicated above each lane. (*B*) Coomassie Blue stain of SDS/Tris-Tricine gel and (*C*) immunoblot analysis with antibody Syn 303 of soluble WT, E46A, E46K, and A53T α-syn untreated (0 min) or treated with calpain for 30 min. Samples of each reaction were electrophoretically resolved on the same gels. *D*, immunoblot analysis with antibody Syn 303 of fibrillized WT, E46A, and E46K α-syn incubated with calpain for 0–30 min as indicated above each lane. Major cleavage products previously identified (48) are labeled as follows: *#1*, residues 58–140; *#2*, residues 1–83; *#3*, residues 1–75; *#4*, residues 1–57; *#5*, residues 1–122; and *#6*, residues 1–114. Proteolytic products resulting from the cleavage of E46A and E46K α-syn and with a slightly greater mobility than product 4 are identified by *asterisks*.

ize, but the resulting filaments showed reduced characteristics of true amyloid, as shown by lower K114 fluorescence. The observed reduction in K114 fluorescence could be due to direct effects of the mutations on K114 binding or alterations in the packing of fibrils resulting in reduced K114 fluorescence upon interaction between the dye and the filaments. Ultrastructural analysis revealed that filaments comprised of E83A and E83K α-syn were significantly different as reflected by their much narrower width. It is likely that these mutations, so close in proximity to the amino acid stretch (residues 71–82) needed for filament assembly, affect the organization of subunits in the fibrils, thereby diminishing their amyloidogenic properties. It is interesting to note that other synthetic mutations close to this region such as A76E and A76R reduce the fibrillogenicity of α-syn but do not affect the ultrastructure of the fibrils (40).

Given the nature of the A53T and E46K mutations, it was expected that the E46K mutation would have enhanced the

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polymerization of α-syn more than the A53T mutation, based on the predicted effects of this mutation on the ability to form β-pleated structure. Nevertheless, under the conditions used here, the A53T mutant increased the propensity of α-syn to fibrillize to a greater extent than the E46K mutation. A recent study by Choi *et al.* (68) also showed that the E46K mutant demonstrates increased amyloid formation compared with WT α-syn, but the rate of amyloid formation for the E46K and A53T mutants was similar. The reason for these observed differences is not clear, but they may depend on the assembly conditions used or the assay utilized (*i.e.* thioflavin T fluorescence). However, our results indicate that although both mutations are in close proximity, the specific micro-environment of an amino acid can be more important than the type of mutation. It also demonstrates that the context of the amino acid change may be more important for protein structure than the theoretical secondary structural propensity of a specific amino acid. Interestingly, the *in vitro* findings of the effects of the A53T and E46K mutations on α-syn polymerization are consistent with pathogenesis in human subjects. Patients with the A53T mutation have an earlier age of disease onset (average age of onset, 45 years) (10, 13, 14, 55, 56) than those carrying the E46K mutation (average age of onset, 60 years) (18).

The findings presented here further underscore the importance of the amino-terminal region of α-syn in modulating filament formation. Despite the robustness of these results, one must not overlook the effect of the middle region on this process (40) or the ability of the carboxyl-terminal region to influence α-syn fibril formation (53, 57, 58). For example, incremental carboxyl-terminal deletions of the negatively charged carboxyl-terminal domain of α-syn increase its propensity to fibrillize (53, 57, 58), indicating an inhibitory role of this region on polymerization. Although no mutations have been identified in the carboxyl-terminal region, phosphorylation of α-syn at Ser^{129} by casein kinase I or II (59) can also increase the propensity of α-syn to fibrillize (60, 61).

Our finding that the *in vitro* fibrillogenesis rates of A53T and E46K mutant α-syn parallel the age of onset of disease in humans is consistent with other studies that indicate that aberrant polymerization of α-syn results in toxicity. This notion is supported by studies showing that the density of α-syn pathological inclusions correlates with disease severity in demented PD and DLB patients (62–66). Moreover, transgenic mouse models suggest that α-syn inclusions impair cellular function by obstructing normal cellular trafficking and disrupting cell morphology (44, 67). The studies of Glu^{46} and Glu^{83} mutant α-syn also provide new insights into the complexity and intricacies of amyloid fibril formation. These findings underscore the fact that a better understanding of this process will be needed to develop effective mechanism-based therapeutics.

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