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Impact of Tyr to Ala mutations on α-synuclein fibrillation and structural properties

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ABSTRACT

Substantial evidence suggests that the fibrillation of α-synuclein is a critical step in the development of Parkinson's disease. *In vitro*, α-synuclein forms fibrils with morphologies and a staining characteristic similar to those extracted from disease-affected brain. Monomeric α-synuclein is an intrinsically disordered protein, with three Tyr residues in the C-terminal region, one in the N-terminus, and lacking Trp. It is thought that interactions between the C-terminus and the central portion of the molecule may prevent or minimize aggregation/fibrillation. To test this hypothesis we examined the importance of the Tyr residues on the propensity for α-synuclein to fibrillate *in vitro*. Fibril formation of α-synuclein was completely inhibited, in the timescale over which measurements were made, by replacing the three C-terminal Tyr residues with Ala. In addition, substitution of Tyr133 by Ala also resulted in the absence of fibrillation, whereas the individual Y125A and Y136A mutants showed limited inhibition. Replacement of Tyr39 by Ala also resulted in substantial inhibition of fibrillation. Structural analysis showed that the Y133A mutant had a substantially different conformation, rich in α-helical secondary structure, as compared with the wild-type and other mutants, although the formation of any tertiary structure has not been observed as can be judged from near-UV-CD spectra. These observations suggest that the long-range intramolecular interactions between the N- and C-termini of α-synuclein are likely to be crucial to the fibrillation process.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting 1–2% of the population over the age of 65. It involves the loss of dopaminergic neurons in the substantia nigra; a process that leads to decreased dopamine levels in the striatum causing symptoms such as tremors, rigidity of the muscles, and bradykinesia. While the exact mechanism of PD pathogenesis is not known, aggregation of the presynaptic protein α-synuclein is believed to play a critical role in the etiology of the disease [1–3]. The function of α-synuclein still remains unknown. However, a significant fraction of α-synuclein is localized within membrane fractions, especially synaptic vesicles associated with the vesicular transport processes. These observations suggest that α-synuclein plays a role in vesicular trafficking [4–8].

Wild-type α-synuclein is a 140 amino acid protein and is intrinsically disordered under physiological conditions *in vitro*. The overall structure of α-synuclein includes a highly conserved N-terminal domain (residues 1–95) containing seven 11-amino acid imperfect repeats with a consensus sequence of KTKEGV, a lipid binding motif. The highly acidic C-terminal domain contains three of the four tyrosine residues at positions 125, 133 and 136. The fourth Tyr residue is located at position 39. There are no Trp residues in α-synuclein. Recent studies have shown that monomeric α-synuclein has a more compact structure than expected for a completely unfolded polypeptide and this compactness has been linked to inhibition of fibrillation due to burial of the hydrophobic NAC domain [9–13]. Small angle X-ray scattering analysis showed that the radius of gyration, R_g, which is used to describe the dimensions of polypeptide chain, is ~40 Å of native α-synuclein, which is much larger than that predicted for a folded globular protein of 140 residues (15 Å), but significantly smaller than that for a fully unfolded random coil (52 Å) [14]. Recently, NMR studies have shown that α-synuclein adopts an ensemble of conformations that are stabilized by long-range interactions [9]. In particular, a long-range intramolecular interaction between the C-terminal region (residues 120–140) and the central part of α-synuclein (residues 30–100) was noted [9]. This interaction was proposed to inhibit fibrillation, and could arise from electrostatic or hydrophobic or both types of interactions. If hydrophobic interactions are important, then the cluster of three Tyr residues in the C-terminus is likely to play an important role in aggregation and fibrillation. To delve into the detailed mechanism the tyrosine residues play in the aggregation process, we created Tyr to Ala mutants of α-synuclein and assessed the impact of the mutations on the conformation and propensity to form fibrils.

Abbreviations: PD, Parkinson's disease; ThT, Thioflavin T; CD, circular dichroism; ATR, attenuated total reflectance

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2. Materials and methods

2.1. Expression of wild-type and Tyr → Ala mutant recombinant human α-synuclein

Human wild-type α-synuclein cDNA was cloned into the bacterial expression vector pRK172. The resultant construct was then used as a template for PCR based mutagenesis. Tyr to Ala translations were accomplished by mutating insert codons, TAT to GCT, at selected locations. Mutagenesis and full vector PCR amplification were performed using the QuickChange mutagenesis kit (Stratagene, LaJolla, CA). Primer selection and thermocycling conditions were determined using the QuickChange primer design program. Primer synthesis was performed under standard conditions (Integrated DNA Technologies, Coralville, IA) and primers were used at a final concentration of 100 ng/μl. Transformations and selection were performed using *E. coli* BL-21(DE3) competent cells (Stratagene) and carbenicillin (75 μg/mL) LB media. Insert sequences were confirmed with forward and reverse sequencing using pRK172 flanking primers (UC Berkeley DNA sequencing facility) and pairwise aligned with the starting sequence. ESI-MS was performed on the protein products to confirm the mass changes with the associated mutation. Single mutant α-synuclein constructs (Y39A, Y125A, Y133A and Y136A) and triple mutant α-synuclein Y(125,133,136)A were generated.

2.2. Expression and purification of wild-type and Tyr → Ala mutant recombinant human α-synuclein

Induction of both wild-type and mutant synuclein was controlled under both the *lac* operon and T7 promoter in pRK172 transformed BL-21(DE3) cells. Induced cells were harvested by centrifugation and lysed using 0.1% Triton-X100 and sonication. Lysate was then clarified in a two-step ammonium sulfate precipitation of 30 and 50% saturation in the presence of 1 mM EDTA and 200 μM PMSF. Precipitate was removed by centrifugation at 4 °C and 13,000 rpm in a JA-20 rotor. Precipitate from the 50% saturation of ammonium sulfate was collected and dissolved in 20 mM Tris/50 mM NaCl pH 7.5 by overnight dialysis and this was α-synuclein-containing fraction used for the experiments. Samples were purified using HiPrep 16/10 DEAE FastFlow anion exchange chromatography (GE Healthcare Biosciences) at 4 °C. Samples were eluted in 0.02% sodium azide/20 mM Tris pH 7.5 using a linear gradient of NaCl from 50 mM to 250 mM in an elution volume of 200 mL at 2 mL/min. The eluate was collected in 4 mL fractions and the synuclein-containing fractions were located using the UV absorbance and SDS-PAGE. Final confirmation of the mutagenesis as well as homogeneity of the samples was confirmed with ESI-MS and SDS-PAGE. Synuclein-containing fractions were pooled, dialyzed overnight against MilliQ ultrapure water (Millipore), and lyophilized.

2.3. Protein preparation

Protein stock solutions were prepared by gravimetrically weighing an approximate weight of lyophilized protein and dissolving it in MilliQ water at basic pH. Once dissolved, samples were adjusted to 100 mM NaCl/100 mM $Na_XH_XPO_4$ pH 7.4, ultra-centrifuged to remove particulates, and the concentrations were quantitated at 25 °C using an extinction coefficient of $\varepsilon_{275\ nm}$=0.404 $cm^{-1}mg^{-1}mL$ for the wild-type; $\varepsilon_{275\ nm}$=0.303 $cm^{-1}mg^{-1}mL$ for single mutants and $\varepsilon_{275\ nm}$=0.101 $cm^{-1}mg^{-1}mL$ for triple mutant. A Bio-Rad Protein Assay (Bio-Rad, USA) was used to validate the values of extinction coefficients for the various mutants.

2.4. Materials

Thioflavin T (ThT) was obtained from Sigma (St. Louis, MO). All buffers and solutions were prepared with nanopure water. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was obtained from Sigma.

2.5. Mass spectrometry

The molecular weight of the α-synuclein mutants and their purity was confirmed by ESI-MS analysis. The proteins were run through a mini C18 column (Pharmacia) and eluted with 80% acetonitrile containing 1% formic acid before performing ESI-MS analysis (MicroMass ZMD).

2.6. ThT fluorescence assay

The kinetics of fibril formation was monitored with ThT fluorescence in sample mixtures containing 72 μM α-synuclein, 100 mM NaCl/100 mM phosphate buffer, and 20 μM ThT at pH 7.4. Protein solutions were airfuged at 20,000 rpm for 40 min to remove oligomers and aggregates from solution. ThT was dissolved in water, filtered and the concentration determined at 25 °C using an extinction coefficient of $\varepsilon_{412\ nm}$=36,000 $M^{-1}cm^{-1}$. Plate reader measurements were carried out in 96-well plates at 37 °C, with 600 rpm shaking with 2 mm shaking diameter, and containing 1/8 in Teflon beads. The solution was excited at 444 nm, and emission was measured at 485 nm. The intensity at 485 nm was recorded in replicates of five to verify reproducibility, errors were <10%.

2.7. FTIR spectra

Attenuated total reflectance (ATR) Fourier Transform Infrared spectra were collected on a Nexus 670 FTIR instrument (Thermo-Nicolet, Madison, WT). Protein samples (0.5 mg/mL, 50 μL volume) were spread out and dried as a thin film on a germanium IRE, with a constant flow of nitrogen gas. 250 interferograms were collected for each sample. Data analysis was performed with the GRAMS32 software from Galactic Industries. Secondary structure components and percentages were estimated by curve fitting following Fourier self-deconvolution and second derivative [15].

2.8. Circular dichroism (CD) measurements

CD spectra were obtained with an AVIV 62DS spectrophotometer (Lakewood, NJ) using protein concentrations of 2.0 mg/mL at pH 7.4 (100 mM phosphate/100 mM NaCl buffer). Spectra were recorded in a 0.2 mm pathlength cylindrical for the far-UV (190–250 nm) and 10 mm quartz cell (Starna, Atascadero, CA) for the near-UV (250–310 nm) CD measurements with a step size of 0.5 nm, bandwidth of 1.5 nm, and an averaging time of 3 s. For all spectra, an average of 3 scans was obtained. CD spectra of appropriate buffers were recorded and subtracted from the protein spectra. The mean residue ellipticity, $[\Theta]_\lambda$, was calculated by using the relation

$$[\theta]_\lambda = \frac{M_o \theta_\lambda}{100 \cdot c \cdot l} \quad (1)$$

in which M_o is the mean residue molar mass (103.3 $g{\cdot}mol^{-1}$ for α-synuclein), Θ_λ is the measured ellipticity in degrees, c is the concentration in g/mL, and l is the path length in decimeters. $[\Theta]_\lambda$ was expressed in deg cm^2 $dmol^{-1}$. Secondary structure content was calculated from the far-UV-CD spectra using the CONTIN software package [16].

2.9. Native gel electrophoresis

Native PAGE was performed on a PHAST system (Amersham Pharmacia Biotech, Uppsala, Sweden) according to instructions provided by the manufacturer. Protein solutions were prepared as described above except the samples were not ultra-centrifuged. After 3 h, the protein samples with a concentration of 4.0 mg/mL were applied to the 20% gel. After electrophoresis, gels were Coomassie blue-stained and protein bands were quantified using

an UVItec documentation system (UVItec Limited, Cambridge, England).

3. Results and discussion

Protein conformation-dependent toxicity is an emerging theme in neurodegenerative disorders, including the synucleinopathies [17]. The native proteins are generally not thought to be pathogenic, however they are the reservoir providing the alternative conformations and quaternary structures that lead to toxic species, believed to be critical for neurodegeneration and neuroimpairment. The shift in equilibrium from benign to malignant conformations is currently thought to be the underlying cause or contributing factor to the pathogenesis. In this context, the more stable the native state, the lower the risk of the conformational change to the pathogenic conformer associated with the diseased state. The situation is somewhat more complex when dealing with an intrinsically disordered protein, such as α-synuclein. Several observations suggest that interactions between the C-terminal "tail" and the central region of α-synuclein may minimize aggregation/fibrillation, perhaps by preventing intermolecular interactions between the hydrophobic central NAC domain. The majority of hydrophobic interactions in the C-terminal 20 residues of α-synuclein emanate from the three Tyr residues. Thus, mutation of these residues to Ala provides insight into their contributions to the structure and aggregation of α-synuclein.

3.1. Impact of Tyr → Ala mutations on the fibrillation of human α-synuclein

Protein aggregation and fibrillation kinetics typically appear sigmoidal, usually attributed to a nucleated polymerization process in which the initial lag phase corresponds to the requirement for formation of a critical nuclei, the subsequent exponential growth phase corresponds to fibril elongation, and the final plateau is ascribed to exhaustion of soluble monomers and intermediates. The kinetics of fibrillation for wild-type and Tyr → Ala mutant recombinant human α-synucleins were monitored using Thioflavin T (ThT) fluorescence. The histological dye, ThT, is widely used for the detection of amyloid fibrils which give rise to a new excitation maximum at 444 nm and enhanced emission at 485 nm. Unbound ThT in solution is essentially non-fluorescent at these wavelengths. ThT is believed to bind specifically to the crossed β-sheet structure associated with amyloid-type fibrils and the binding is independent of the primary

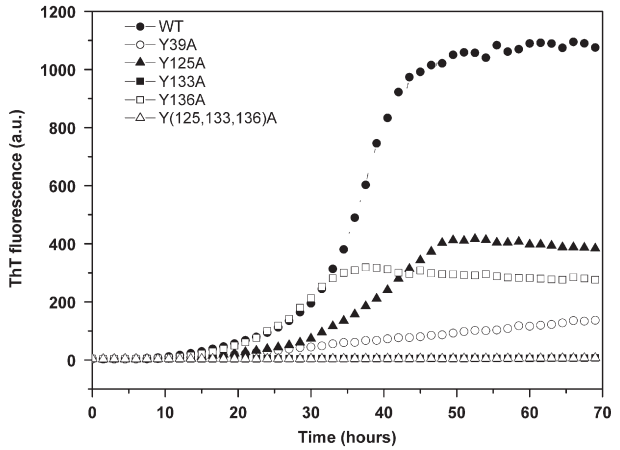

Fig. 1. Kinetics of fibril formation by wild-type α-synuclein and its mutants: Y39A, Y125A, Y133A, Y136A and Y(125,133,136)A monitored by ThT fluorescence at 37 °C. Key: WT (●), Y39A (○), Y125A (▲), Y133A (■), Y136A (□) and Y(125,133,136)A (△). Conditions: 100 mM NaCl, 100 mM phosphate buffer pH 7.4, 20 μM ThT, protein concentration 1.0 mg/mL.

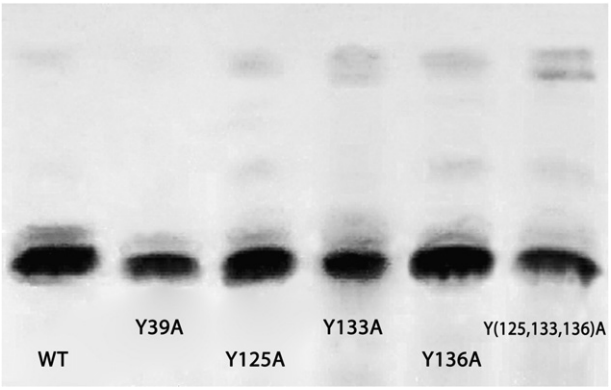

Fig. 2. Native PAGE electrophoretogram of wild-type α-synuclein and its mutants: Y39A, Y125A, Y133A, Y136A and Y(125, 133, 136)A. Protein concentrations were 4 mg/mL. Protein solutions were prepared 3 hours before the samples were applied to the 20% gel.

structure of the protein. Only the fibrillar forms give rise to significant fluorescence upon ThT binding. The binding of ThT to α-synuclein fibrils is therefore an effective and specific method for monitoring the fibrillation process. The data in Fig. 1 indicate that changing the tyrosine residues in α-synuclein to alanine significantly impedes fibril formation. For example, complete inhibition of fibril formation was observed after replacing Tyr with Ala at all three positions 125, 133 and 136 simultaneously, or at position 39 alone. These observations are consistent with a critical interaction between the cluster of three Tyr residues at the C-terminus and Tyr39. We propose that Tyr39 and the C-terminal Tyr residues form an aromatic cluster, which could also involve additional hydrophobic residues, such as the two C-terminal Met residues. Interestingly, comparison of the effects of the individual Tyr to Ala substitutions shows that Y133 has a much more significant contribution than either Y125 or Y136. This could mean a more specific hydrophobic interaction between Y133 and Y39. These data clearly indicate the importance of Tyr's located at these positions for intra- or intermolecular interactions between the N- and C-termini of α-synuclein. Although the interactions between the N- and C-termini are expected to be mostly electrostatic, the C-terminus may form hydrophobic interactions with the central NAC domain of α-synuclein [9,13], as well as with Y39. Several observations suggest that the critical fibrillogenic region of α-synuclein involves the relatively hydrophobic NAC region, residues 61–92. Although it is reasonable to propose that the intramolecular interactions between the C-terminus and Y39 and other parts of the central or N-terminal region, will lead to potential steric occlusion of parts of the NAC region from intermolecular interactions, thus preventing aggregation/fibrillation, our data clearly argue against this. In fact, decreasing these interactions by replacing the Tyr's with Ala leads to decreased fibrillation; this could result from (i) stabilization of the monomeric state, (ii) stabilization of the oligomeric states or (iii) from decreased intermolecular interactions leading to fibrillation. Since the Tyr to Ala mutations lead to increased exposure of the hydrophobic central domain of the molecule, we favor the latter explanation. However, the observation of some oligomeric states for Tyr → Ala mutants on native-PAGE electrophoretogram (Fig. 2) suggest the presence of some intermolecular interactions which could lead to the formation of soluble nonfibrillating oligomers.

3.2. Impact of Tyr → Ala mutations on the secondary structure of α-synuclein

The secondary structure of α-synuclein and its Tyr to Ala mutants was investigated with FTIR, which is more sensitive to β-structure than CD. FTIR spectra for wild-type and the mutant α-synucleins are shown in Fig. 3A. The spectra of all the Tyr → Ala mutants had less

pronounced shoulders in the vicinity of 1630 cm^{-1}, corresponding to less β-structure as compared to the wild-type protein.

Deconvoluted amide I spectra for each protein were generated by second-derivative analysis (Fig. 3B). The peaks in the vicinity of 1638 and 1625 cm^{-1} can be assigned to β-sheet components, and the major band at 1657 cm^{-1} is assigned to a disordered structure [18–20]. In the spectrum of the wild-type α-synuclein, the main band for β-structure is at 1625 cm^{-1}, whereas for all the mutants the major β-structure band is in the vicinity of 1638 cm^{-1} (Fig. 3B). In the absence of local environmental effects this suggests that there is a major change from antiparallel β-sheet in the wild-type to parallel β-sheet in the mutants. Insight into the Fig. 3B reveals that the most intense band at position 1638 cm^{-1} is for Y133A, while the least intense peak is for wild-type α-synuclein.

The most intense band in all the second-derivative spectra is at ~1656 cm^{-1} and is assigned to a disordered (and possibly helical) structure and is strongest for the Y39A mutant. The bands located at 1688, 1680, 1672, and 1666 cm^{-1} are assigned to various types of loop and turn structures [18]. These peaks are much more pronounced in the mutated forms of α-synuclein, suggesting a more complex structure than for the wild-type. The deconvoluted FTIR spectra show that the secondary structure for the mutant α-synucleins is significantly different from that of wild-type. It is presumably this change in conformation that is responsible for the different propensity of the mutants compared to wild-type for fibrillation.

The secondary structure of wild-type and Tyr → Ala mutant α-synucleins was also examined by CD (100 mM sodium phosphate pH 7.4/100 mM NaCl buffer) (Fig. 4A). Circular dichroism is much more sensitive to a helical structure than FTIR, in the latter bands for disordered and helical structures are very close and frequently difficult to separate. The spectrum of α-synuclein at pH 7.4 is characterized by a negative CD band near 200 nm (Fig. 4A). Whereas the spectra of Y39A and Y125A had a minimum at 200–202 nm, two other mutants showed an increased minima: Y136A at 204 nm and Y(125,133,136)A at 205 nm, indicative of some increased structure/compactness. In marked contrast, the Y133A mutant has a far-UV-CD spectrum, which is characteristic of α-helix. Based on CONTIN analysis, α-synuclein in aqueous solution at pH 7.4 contains 19±1% α-helix, Y39A 12±2% α-helix, Y125A 25±2 α-helix, Y133A 88±6% α-helix, Y136A 20±2% α-helix and Y(125,133,136)A 26±2% α-helix. Replacement of Tyr with Ala at position 39 leads to a decrease in the quantity of α-helical structures but an increase in the amount of turns, while the replacement of Tyr with Ala at position 133 drastically increases the amount of helical structures."; This major conformational change also correlates with the largest effect on fibrillation. It is interesting that the triple Tyr → Ala mutant did not have significantly more helix than the wild-type α-synuclein.

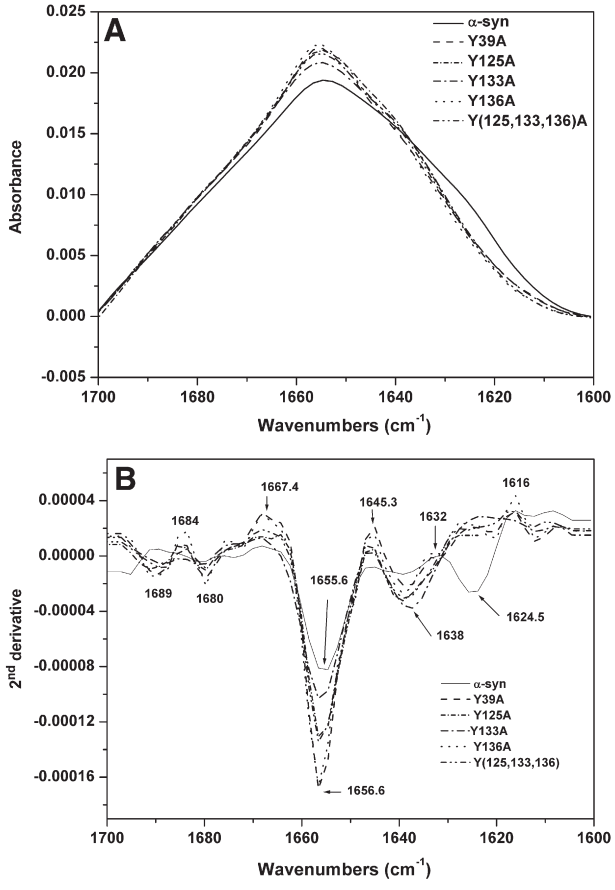

Fig. 3. FTIR spectra (A) and their 2nd derivative (B) of wild-type α-synuclein and its mutants: Y39A, Y125A, Y133A, Y136A and Y(125, 133, 136)A. Conditions: 100 mM NaCl, 100 mM phosphate buffer pH 7.4, protein concentration 0.5 mg/mL.

Analysis of the CD spectra shows that the other mutants have similar amounts of β-sheet structure as wild-type α-synuclein. Application of different secondary structure prediction algorithms on the primary structure of α-synuclein did not predict much increase in the amount of α-helical structures for the mutants. However, the program AGADIR, designed for the prediction of a secondary structure for peptides [21], predicted an increase in the amount of α-helix from 46% to approx. 75%, regardless of the position of the Tyr to Ala mutation. Based on the FTIR and CD data we conclude that replacement of the Tyr to Ala decreases the amount of β-structure and induces the formation of more α-helical structure. The near-UV-CD spectra did not show any induced tertiary interactions (Fig. 4B), although some higher oligomeric states of Tyr → Ala mutants were detected on native-PAGE electrophoretogram (Fig. 2).

Amino acid bulkiness, the ratio of side chain volume to its length, predicts clearly observable features reporting on the local conformational behavior of intrinsically disordered proteins. Alanine has a higher α-helical preference than the bulkier side chain of tyrosine, which prefers to form a beta sheet structure. Favorable long-range intramolecular interactions of α-synuclein have been observed between a hydrophobic cluster that comprises the C-terminal part of the highly hydrophobic NAC region (residues 85–95) and the C-terminus (residues 110–130) probably mediated by Met^{116}, Val^{118}, Tyr^{125} and Met^{127} [9]. Within the C-terminal domain, residues 120–130 contact residues 105–115, and the region above residue 120 also interacts with the N-terminus above residue 20. These interactions presumably inhibit spontaneous α-synuclein oligomerization and are compatible with the influence of fibrillation exerted by methionine oxidation, tyrosine nitration and phosphorylation of Ser 129 [22–27]. The significant stabilization of the natively unfolded conformation has been shown to represent a contributing factor to the inhibition of methionine-oxidized α-synuclein fibrillation [23]. Nitration of tyrosines leads to formation of partially folded conformations, which are stabilized by formation of specific oligomers, most likely octamers. These octamers are stable and possess decreased conformational flexibility, as seen by the increase in resistance towards limited proteolysis [24,25].

The findings that Tyr to Ala mutants do not form any partially folded conformations but they exhibit differing folding flexibility, a dissimilar secondary structure and differing aggregation propensity are in agreement with the our current understanding that α-synuclein can adopt diverse conformations. From comparison of the effects of specific Tyr → Ala mutations in terms of the conformational effects versus the kinetics of fibrillation it is clear that there are several different factors involved. The individual mutation having the largest effect on the structure, Y133A, also has the largest effect in inhibiting fibrillation. This observation suggests that inducing the helical conformation observed in this mutation prevents fibrillation, thus, low

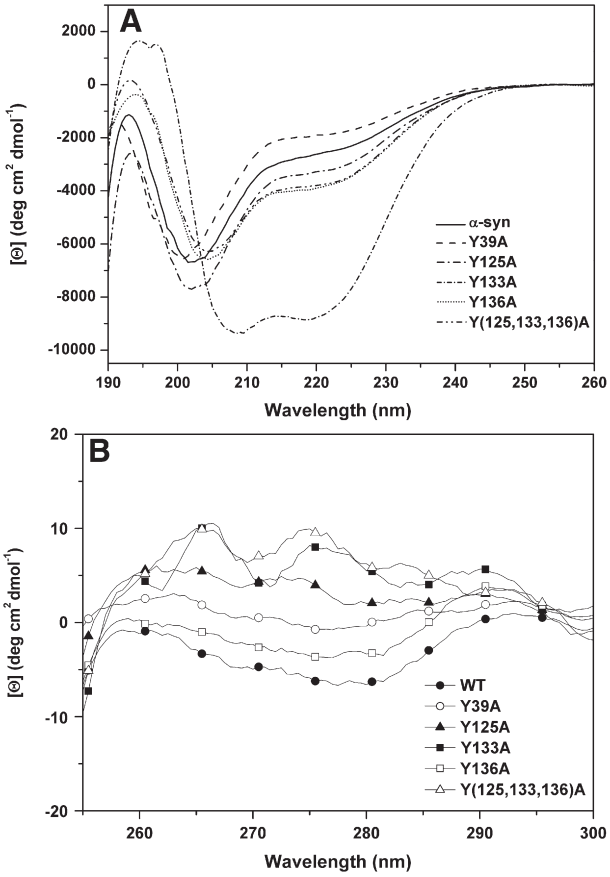

Fig. 4. Far-UV (A) and near-UV (B) CD spectra of wild-type α-synuclein and its mutants: Y39A, Y125A, Y133A, Y136A and Y(125, 133, 136)A. Conditions: 100 mM NaCl, 100 mM phosphate buffer pH 7.4, protein concentration 2.0 mg/mL.

molecular weight compounds that would induce a similar conformation might be effective at preventing α-synuclein fibrillation in vivo.

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