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The Effect of Tyrosine Residues on α-synuclein Fibrillation

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Abstract

Aggregation of the intrinsically disordered protein α-synuclein into ordered amyloid fibrils is implicated in the pathogenesis of Parkinson's disease. To unravel the role of Tyr residues in α-synuclein fibrillation, we prepared recombinant N-terminal (Y39A) and C-terminal (Y(125,133,136)A) mutants of α-synuclein and examined their fibrillation propensities by thioflavin T and 1-anilinonaphthalene-8-sulfonate (ANS) fluorescent probes, SDS-PAGE and atomic force microscopy. We demonstrate that in contrast to wild-type α-synuclein, both mutants show large, but comparable delays in the fibrillation process and exhibit enhanced hydrophobicity during fibril-like assembly. Both Tyr mutants form fibril-like structures after prolonged incubation periods, which are morphologically distinct from those of the wild-type protein. Our results suggest that the N-terminal and C-terminal Tyr residues of α-synuclein are important primarily for the initiation of the fibrillation process.

Keywords: α-synuclein, fibrillation, Parkinson's disease, site-directed mutagenesis, ThT fluorescence, tyrosine

1. Introduction

α-Synuclein is an intrinsically disordered presynaptic protein.[1–3] Its amyloidogenic aggregation has been implicated in the onset of Parkinson's disease and several other neurodegenerative disorders.[4] The exact physiological and pathological roles of α-synuclein remain to be resolved, although they appear to involve protein-lipid interactions.[5,6] α-Synuclein fibrillation is a complex nucleation-dependent process, which is initiated with the structural conversion of the unfolded monomer into partially structured conformations.[7] Then, via the propagation of diverse oligomeric intermediates, cross-β-sheet fibrils are finally assembled.[8–10] The structural transformations of monomeric and oligomeric α-synuclein species are accompanied by increased hydrophobic exposure.[7,11,12] Despite the considerable medical interest, the molecular details of this process remain unknown.

The α-synuclein protein comprises 140 residues and three distinct regions (Figure 1): a positively charged N-terminus, a central hydrophobic non-Aβ component (NAC) region that has been proposed to be essential for aggregation,[13] and a highly negatively charged C-terminus. It has been shown that *in vitro*, and implied that probably also *in vivo*, α-synuclein is in dynamic equilibrium between its monomeric and oligomeric conformations.[14–16] Monomeric α-synuclein is part of an ensemble of rapidly inter-

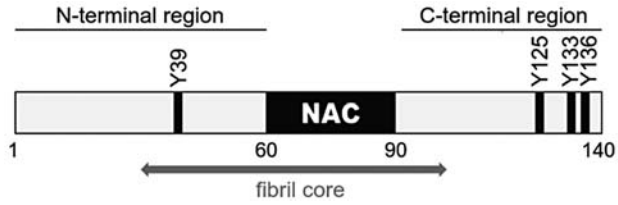

Figure 1. The α-synuclein sequence divided into the three structurally distinct parts. The N-terminal region, the middle NAC domain, and the C-terminal region are shown. The proposed fibril core-forming region (residues ~30 to ~100) and positions of the four Tyr residues are indicated.

converting conformations that are stabilized by transient long-range interactions between the α-synuclein C-terminus and its NAC and N-terminal regions.[17,18] Such interactions have been suggested to protect the highly amyloidogenic NAC region from self-association,[17,18] although this hypothesis has been questioned.[19–22]

Aromatic residues have been proposed to have important roles in molecular recognition and self-assembly in amyloid formation.[23] α-Synuclein has four Tyr residues, one in the N-terminal, and three in the C-terminal region (Figure 1). Several lines of evidence have highlighted their importance for α-synuclein fibrillation. First, both electrostatic and hydrophobic interactions are important for α-synuclein intramolecular contacts.[17] A recent computational study showed a high probability of long-range interactions between the region surrounding Y39 and the cluster of C-terminal Tyr residues of α-synuclein.[22] It has been previously suggested that disruption of these hydrophobic intramolecular interactions inhibits the fibrillation process.[20] Secondly, modifications of Tyr residues through nitration[24] or site-directed mutagenesis[20,25,26] have resulted in profound inhibitory effects on α-synuclein fibrillation capacity. Additionally, it has been recently suggested that the Tyr residues of α-synuclein are also involved in intermolecular interactions during fibrillation.[26]

Despite the proposed involvement of Tyr residues in α-synuclein fibrillation, their exact contribution to this process remains unknown. The aim of the present study was to gain an insight into the role of the N-terminal and C-terminal Tyr residues in the mechanism of α-synuclein fibrillation. We prepared N-terminal single Y39A mutant and the C-terminal triple Y(125,133,136)A mutant of α-synuclein and examined their fibrillation propensities by a combination of extrinsic fluorescent probes (thioflavin T (ThT) and ANS), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and atomic force microscopy (AFM). Our results show that both mutant proteins display a significant delay in the fibrillation process *in vitro*. After a prolonged incubation period, both mutants form fibril-like structures, which appear to be morphologically distinct from the wild-type (WT) fibrils. The fibril growth of the Y39A and Y(125,133,136)A mutant proteins is accompanied by a higher hydrophobic exposure compared to that of the WT protein.

2. Materials and Methods

2. 1. Materials

Thioflavin T (ThT), ANS, glutaraldehyde and HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) were purchased from Sigma Aldrich (St.Louis, MO, USA). Solutions for protein preparation were prepared in deionized water (Braun, Melsungen, Germany). Protein loading buffer was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2. 2. Protein Expression and Purification

Expression and purification of the recombinant human WT α-synuclein and its Tyr to Ala mutants were performed as described previously,[20] with some modifications. The human WT α-synuclein cDNA was cloned into the pRK172 bacterial expression vector. The resultant construct was then used as the template for PCR-based mutagenesis (QuickChange mutagenesis kits, Stratagene, USA) to generate the α-synuclein constructs for the single mutant (Y39A) and the triple mutant Y(125,133,136)A. The Tyr to Ala mutations were accomplished by mutating the insert codons at selected locations (Y39A: TAT→GCT; Y125A: TAT→GCT; Y133A: TAT→GCT; Y136A: TAC→GCC). The insert sequences were confirmed with forward and reverse sequencing before protein expression. Transformations and selection were performed using *E. coli* BL-21(DE3) gold competent cells (Stratagene, USA) and carbenicillin (85 μg/mL) LB medium.

The cell culture at A_{500} ~1.4 was induced with isopropyl-β-D-thiogalactopyranoside and incubated for another 5 h. The cells were collected by centrifugation and the cell pellets were frozen and kept at –20 °C until used. The pellets were thawed at room temperature and redissolved in 40 mL (per 1 L of culture) of lysis buffer (50 mM NaCl, 20 mM Tris-HCl, 0.10 % Triton-X100, 200 μM phenylmethylsulfonyl fluoride, pH 7.4) and sonicated. The lysates were then clarified using a two-step ammonium sulfate precipitation of 30 % and 50 % saturation. Precipitate from the 50 % saturation of ammonium sulfate was collected by centrifugation at 4 °C and 22 000 × *g*, dissolved in 20 mM Tris, 50 mM NaCl, pH 7.4, and dialyzed overnight. The samples were purified using MonoQ anion exchange chromatography (GE Healthcare Biosciences) at 10 °C. The samples were eluted in 50 mM NaCl, 20 mM Tris, pH 7.4, using a linear gradient of NaCl from 50 mM to 500 mM. α-Synuclein typically eluated in a broad peak around 300 mM NaCl. The α-synuclein-containing fractions were pooled and dialyzed for a minimum of 36 h at 4 °C, against MilliQ ultrapure water (Millipore). After the dialysis, the precipitates were removed by centrifugation at 22 000 × *g*. The supernatants were lyophilized and stored at –20 °C.

Final confirmation of the molecular mass and homogeneity of the samples was achieved with matrix-assisted laser-desorption/ionization–mass spectrometry (MALDI-TOF MS) and SDS-PAGE.

2. 3. Protein Preparation

Protein stock solutions were prepared by dissolving the lyophilized proteins in 1 mM NaOH for 10 min. Samples were then adjusted to 150 mM NaCl, 20 mM HEPES pH 7.0, and filtered through 100-kDa molecular weight cut-off centrifugal filters (Amicon Ultra – 0.5 mL, Merck Millipore, Tullagreen, Ireland). The protein

concentrations were determined spectrophotometrically using a Cary Eclipse spectrophotometer (Varian, Mulgrave, Australia), as described previously.[20] The concentrations of the proteins were adjusted to 70 μM (~1 mg/mL).

2. 4. Prediction of Aggregation Propensities

Aggregation propensities of the proteins were predicted using the online program TANGO (http:/tango.crg.es/about.jsp).[27] Conditions: pH 7.0, 150 mM salt, 25 °C.

2. 5. Fibrillation Studies

The fibrillation experiments were carried out in tightly sealed glass vials at 37 °C and with 700 rpm shaking using micro-stir bars. Each experiment was repeated at least three times.

2. 6. ThT and ANS Fluorescence Assays

The fluorescence assay measurements were performed in 10-mm-path-length cuvettes using a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Australia), with slits of 5 nm for both excitation and emission. For the ThT assays, aliquots of 10 μL were removed from the incubated protein solutions at several times and added to 1 mL of 10 μM ThT in 150 mM NaCl, 20 mM HEPES pH 7.0. After 5 min, the ThT fluorescence was recorded from 460 nm to 520 nm, with excitation at 444 nm. The signal was obtained as the ThT intensity at 480 nm. For ANS assays, aliquots of 50 μL were removed daily from the incubated protein solutions and added to 500 μL of ANS solution in 150 mM NaCl, 20 mM HEPES pH 7.0. The obtained ANS:protein molar ratio was 50. After 5 min incubations, the ANS fluorescence was recorded from 400 nm to 600 nm, with the excitation at 350 nm.

2. 7. Chemical Crosslinking and SDS-PAGE

At the end of each incubation period, the protein samples were centrifuged at 24 000 × *g* for 30 min, to separate the soluble fraction from the insoluble aggregated material. Centrifugation was omitted for the dissolved proteins before the incubations. The protein in the supernatant was: (i) electrophoresed; or (ii) chemically crosslinked with 2.5 % glutaraldehyde prior to electrophoresis. Briefly, 1.11 μL of the crosslinker was added to 10 μL of the protein solution and incubated at 37 °C for 30 min. The reaction was terminated with 1.11 μL of 1 M Tris-HCl (pH 8.0). Loading buffer with reducing agent (0.1 M dithiothreitol) was added to the crosslinked protein and the samples were boiled for 5 min. Non-crosslinked protein was mixed with loading buffer without reducing agent and with no heating prior to application. SDS-PAGE was performed on 15 % gels at 200 V, and after the electrophoresis, the gels were stained with Coomassie-blue. Protein bands were statistically analysed after densitometric quantification using image analyser system (ImageJ 1.29×, N.I.H., USA).

2. 8. AFM Imaging

Atomic force microscopy images were obtained with a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Tonawanda, NY, USA) operating in tapping mode. Here 20 μL to 30 μL of the protein solution (undiluted for mutant proteins, incubated for 3 days, and 10-fold diluted for all other samples) was spread across a freshly cleaved mica surface and incubated for 20 min. After the incubation, the samples were gently washed with deionized water and dried with a stream of nitrogen. Standard silicon cantilevers (AC160TS; Olympus, Tokyo, Japan) with a tip radius <10 nm and a resonance frequency around 300 kHz were used. The images were taken at a scan rate of 1 Hz, and the image resolution was 512 × 512 pixels.

3. Results and Discussion

3. 1. Tyr substitutions do Not Alter the Monomer/oligomer Equilibrium of α-synuclein

The substitution of Tyr residues with Ala could alter the structure and therefore the intrinsic aggregation propensity of α-synuclein in buffered solution (150 mM NaCl, 20 mM HEPES pH 7.0). Therefore, before subjecting the WT protein and the Y39A and Y(125,133,136)A mutants to prolonged incubation, we examined their initial aggregation tendencies. The fibrillation of α-synuclein is a process whereby a β-sheet structure is acquired.[28] To predict β-aggregation-prone regions in the mutants, we used the TANGO algorithm.[27] While the Y(125,133,136)A mutant had a similar aggregation tendency as the WT protein, a small decrease in aggregation propensity was observed for the Y39A mutant (Figure 2A).

On SDS-PAGE gels, the WT protein and the Tyr mutants migrated as monomers of apparent molecular weight of 15 kDa, with one very weak protein band corresponding to α-synuclein dimers (Figure 2B). Chemical crosslinking of the protein samples (2.5 % glutaraldehyde) revealed several dimeric and tetrameric species in addition to the monomeric forms, suggesting that the oligomers are sensitive to SDS (Figure 2B). Importantly, the pattern of electrophoretic mobility of the three proteins was very similar, with monomers accounting ~75 % of the total protein, dimers ~20 % and higher oligomers ~5 % (SD < 5 %). This suggests that the Tyr substitutions do not influence the initial aggregation propensity of the protein.

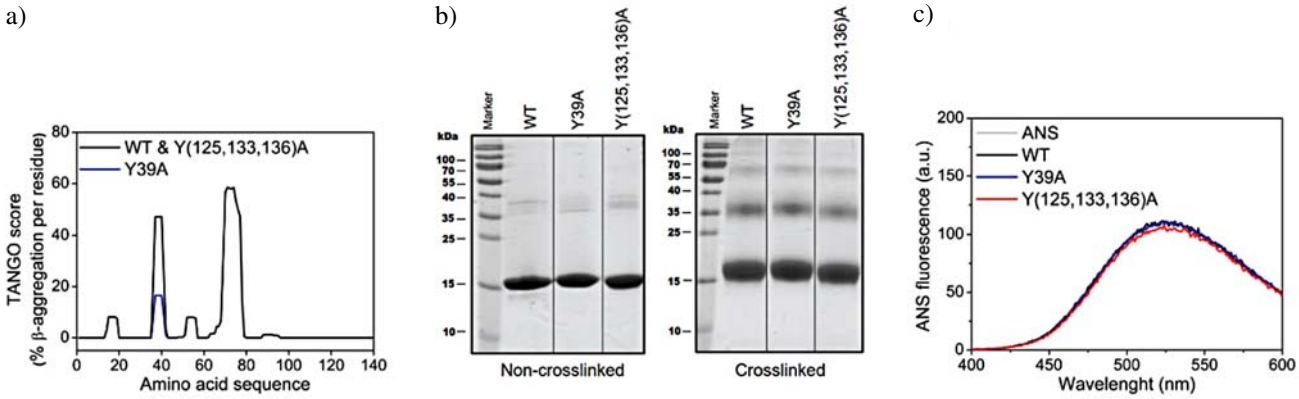

Figure 2. Characterization of the initial state of WT α-synuclein and its mutants. a) *In silico* prediction of β-aggregating regions using the TANGO algorithm. b) Oligomerization was analysed by SDS-PAGE of non-crosslinked and 2.5 % glutaraldehyde chemically crosslinked protein samples. c) Exposure of hydrophobic regions was analyzed by ANS fluorescence.

As the aggregation tendency of α-synuclein can be affected by its surface hydrophobicity, we next determined the degree of hydrophobic exposure. The fluorescent dye ANS exhibits an increase in fluorescence intensity and a blue shift in the fluorescence emission maximum upon interactions with exposed hydrophobic regions of proteins.[29] As shown in Figure 2C, neither the WT protein nor the two mutants evoked ANS fluorescence, which indicates the absence of any highly aggregation-prone species.

Therefore, as evident from our *in silico* analysis, SDS-PAGE and ANS fluorescence, it is likely that the Tyr to Ala mutations do not significantly alter the pre-fibrillation monomer/oligomer equilibrium of α-synuclein.

3. 2. Fibril Growth and Mature Fibril Formation

In order to determine the effect of Tyr residues on α-synuclein fibrillation, we characterized the fibrillation profiles of the WT protein and the Y39A and Y(125,133, 136)A mutants. The protein samples (70 μM) were incubated in 150 mM NaCl, 20 mM HEPES pH 7.0 at 37 °C with strong agitation for several days and analysed by ThT and ANS fluorescence, SDS-PAGE and AFM.

3. 2. 1. Tyr→Ala Mutations Inhibit α-synuclein Fibrillation

The fibrillation of WT protein and both mutant proteins was monitored as a function of time using ThT, a fluorescent dye that shows a large enhancement of its fluorescence intensity upon interactions with amyloid β-sheet-rich assemblies.[30] The α-synuclein fibrillation kinetics are typically sigmoidal, starting with a lag phase necessary for nuclei formation, followed by an exponential phase of fibril growth, and a final plateau state, established when the soluble monomers and intermediates are exhausted.[20] Figure 3A shows that substituting the sole N-terminal Tyr residue or all of the C-terminal Tyr residues with Ala significantly inhibited α-synuclein fibrillation. In the time period necessary for the WT protein to complete fibrillation (3 days), the ThT fluorescence of the mutant protein samples did not change significantly suggesting the absence of fibrillar aggregation. To determine whether the lack of Tyr residues in α-synuclein primarily affects the kinetics of nuclei formation, and whether these proteins might eventually produce fibrils, we prolonged the incubation period and followed the fibrillation of the proteins for up to 8 days. While WT α-synuclein displayed a sigmoidal fibrillation curve with a lag phase of less than 20 h, an increase in ThT fluorescence intensity for the Y39A and Y(125,133, 136)A mutants was observed only after ~100 h (day 4) of incubation (Figure 3A). The ThT fluorescence intensity of the Y39A and Y(125,133,136)A mutants was very low even at the end of this incubation period (day 8; Figure 3A), suggesting the absence of significant amounts of fibrillar structures.

Since the fibrillation process takes place through incorporation of soluble protein species into the growing fiber, we used SDS-PAGE to monitor the relative amounts of soluble α-synuclein. After 3 days of incubation, SDS-PAGE of the supernatant fractions showed depletion of the soluble WT protein (Figure 3B; compare with day 0, Figure 2B), thus confirming the incorporation of the WT α-synuclein into insoluble aggregates. On the other hand, there were no significant reductions in the amount of soluble protein remaining in the supernatants of the Tyr mutant samples after 3 days of incubation (Figure 3B). These results confirm the absence of higher molecular weight insoluble aggregates of the Y39A and Y(125,133,136)A mutants during the 3-day incubation and are in accordance with the results of the ThT fluorescence. After 8 days of incubation, depletion of soluble protein was observed in the supernatant fractions of the Y39A and Y(125,133, 136)A mutants (Figure 3C), suggesting the formation of

insoluble aggregates. The observed minor degradation of the α-synuclein mutants (Figure 3C) can be attributed to prolonged incubation conditions.

3. 2. 2. Tyr→Ala Mutants Show Enhanced Hydrophobic Exposure During Aggregation

Partially folded intermediates and transient on-pathway oligomers of α-synuclein have been shown to expose hydrophobic patches on their surface,[7,11] which can be detected by ANS fluorescence. In agreement with previous reports for amyloidogenic proteins,[7,31] the ANS fluorescence intensity of WT α-synuclein (Figure 3D) increased concomitantly with the increasing ThT fluorescence and reached its maximum when the fibrillation process entered the stationary phase (day 2). On the contrary, the Y39A and Y(125,133,136)A mutants did not react with ANS until day 4 (Figure 3D and Table 1), which is in agreement with the absence of ThT fluorescence intensity increase (Figure 3A).

After 4 days of incubation (~100 h), the ANS fluorescence intensity of the Y39A mutant increased (Figure 3D), which parallels with a simultaneous small change in ThT fluorescence (Figure 3A). The Y(125,133,136)A mutant also showed changes in ANS fluorescence after 100 h of incubation (Figure 3D). During the next two days of the incubation (days 4–6), the ANS fluorescence intensity of both mutants progressively increased and surpassed the maximal intensity of the WT α-synuclein (Figure 3D and Table 1). These results suggest the exposure of a higher fraction of ANS-binding surfaces in the Y39A and Y(125,133,136)A mutants compared with that of the WT protein. Additionally, the ANS fluorescence intensity maximum of the two mutants exhibited a greater shift to lower wavelengths (from ~525 nm on days 0–4 to 488 nm on day 6) in comparison with that of the WT protein (from 525 nm on day 0 to ~500 nm at day 2 and 3) (Table 1), which suggests that the mutant proteins provide a more hydrophobic environment for the binding of the ANS dye.[32]

3. 2. 3. Tyr→Ala Mutants Form Fibril-like Structures After Prolonged Incubation Period

To get a better insight into the morphology of the Y39A and Y(125,133,136)A mutant aggregates compared with WT fibrillar structures, AFM imaging was performed. WT α-synuclein, but not its Tyr mutants, forms characteristic fibrillar structures after 3 days of incubation (Figure 3E), which is in accordance with the ThT fluorescence results at this time point (Figure 3A). However, AFM analysis revealed the presence of small rod-like structures in the case of the Y39A mutant, and of larger, spherical to oval-shaped oligomers in the case of the Y(125,133,136)A mutant (Figure 3E). As no significant depletion of soluble proteins was observed (Figure 3B; compare with day 0, Figure 2B) and no changes in ANS fluorescence were detected during the first 3 days of incubation of the mutants (Table 1), we conclude that these oligomeric species represent a minor fraction of the total protein (note that undiluted samples were imaged here). However, AFM images of the mutant proteins incubated for 8 days showed considerable amounts of fibril-like structures (Figure 3F). The mutant fibrils appeared morphologically similar to those of the WT α-synuclein, with heights in the range of 6–9 nm, which is in agreement with previous reports.[33]

The formation of fibril-like structures in the mutant proteins (Figure 3F) is accompanied by a lower increase in ThT fluorescence intensity in comparison with that of the WT protein (Figure 3A). The discrepancy between ThT staining and fibril occurrence has also been reported by others.[34–36] Given that ThT might preferentially bind to aromatic side-chain-rich channels on the fibril surface,[30,37] it is possible that the absence of Tyr residues is directly responsible for the low ThT signal. However, according to the proposed α-synuclein fibril structure,[38–40] only Y39 participates in the β-structured fibril core and can potentially contribute to ThT binding (Figure 1). As both mutant proteins showed a similarly low affinity for ThT (Figure 3A), we can ascribe the ThT fluorescence discrepancy to structural differences in the fibril assemblies of

Table 1. The ANS binding profile of WT α-synuclein and its Y39A and Y(125,133,136)A mutants incubated at 37 °C and pH 7.0 for the period of 8 days. The results are an average of three measurements (SD was below 5 %). The fluorescence intensity maximum, λ_{max}, and maximal intensity of ANS fluorescence in the absence of proteins were 525 nm and 110 a.u., respectively.

	WT		Y39A		Y(125,133,136)A	
Days	**λ_{max}(nm)**	**Intensity (a.u.)**	**λ_{max}(nm)**	**Intensity (a.u.)**	**λ_{max}(nm)**	**Intensity (a.u.)**
0	525	111	525	111	525	108
1	515	133	526	109	525	109
2	501	176	525	111	525	108
3	500	193	525	110	525	110
4	/	/	500	166	508	116
5	/	/	491	206	498	170
6	/	/	488	291	488	282
7	/	/	490	288	487	280
8	/	/	492	279	493	272

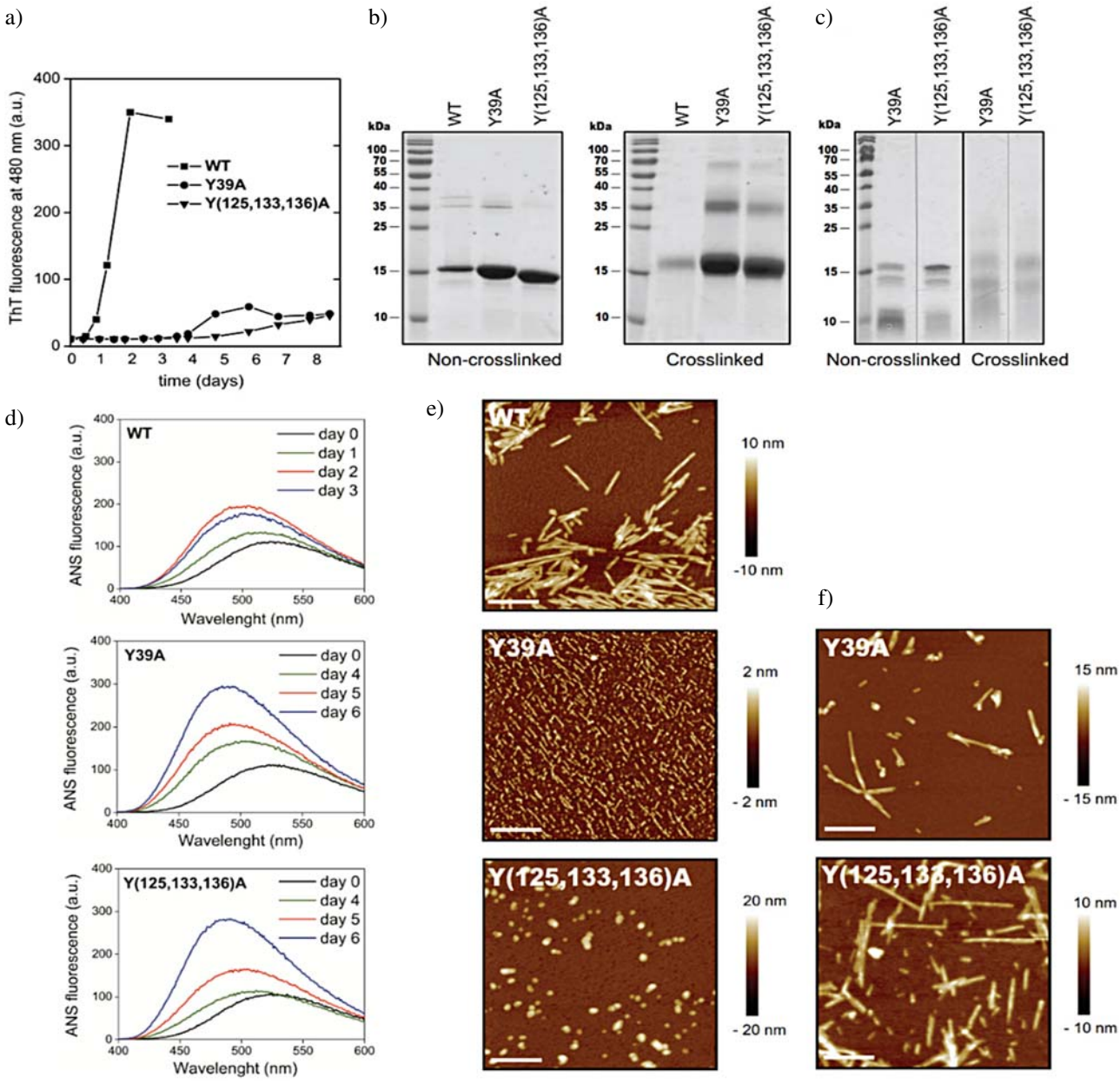

Figure 3. Characterization of the fibrillation of WT α-synuclein and its Tyr mutants. Aliquotes were taken from the incubated protein solutions at distinct times, and subjected to ThT and ANS fluorescence analysis, SDS-PAGE, and AFM imaging. a) Fibrillation kinetics of the proteins, as monitored by ThT fluorescence. (b) SDS-PAGE of non-crosslinked and crosslinked protein supernatant samples after 3 days of incubation. c) SDS-PAGE of non-crosslinked and crosslinked protein supernatant samples after 8 days of incubation. d) ANS emission spectra for the incubated protein mixtures monitored over time. The ANS emission spectra of the mutant proteins over the first 3 days and the last 2 days of incubation showed almost superimposable profiles and were therefore omitted for clarity. e) AFM images of the aggregated proteins after 3 days of incubation. Scale bar, 300 nm. f) AFM images of the aggregated proteins after 8 days of incubation. Scale bar, 300 nm.

the mutant proteins in comparison with those of WT α-synuclein, which are not detectable by AFM.

3. 3. Tyr Residues Participate in Early Events of α-synuclein Fibrillation

The molecular mechanisms underlying α-synuclein fibrillation are poorly understood, representing a major obstacle in the development of novel therapeutic agents for Parkinson's disease and related neurodegenerative disorders. Most α-synuclein fibrillation research in the last 15 years has focused on the role of the most amyloidogenic NAC region of the protein. Tyr residues have also been addressed by several studies to have an important role in α-synuclein fibril formation. Namely, nitration of Tyr residues has been found to redirect the aggregation process

towards oligomeric non-fibrillar structures.[24] However, the specific role of unmodified Tyr residues in native protein fibrillation has been investigated by only a few studies,[20,25,26] which independently showed that substituting the sole N-terminal Tyr residue (Y39) or C-terminal Tyr residue(s) (Y125,Y133,Y136), with Ala, leads to complete/substantial inhibition of α-synuclein fibril formation. However, none of these studies addressed specifically which step in the fibrillation process is affected and/or which molecular mechanism is responsible for the inhibitory effect. Here, we confirm previous observations that Y39A and Y(125,133,136)A mutants do not fibrillate in the time scale sufficient for WT α-synuclein to form mature fibrils (3 days) (Figure 3E). However, both two mutants form fibril-like structures after a prolonged incubation period (8 days) (Figure 3F), displaying an approximately 5-fold increase in the fibrillation lag phase (Figure 2A). This is the first time fibril-like structures of these two mutant proteins have been observed, suggesting that Tyr→Ala substitutions in α-synuclein (Y39A and Y(125,133,136)A) do not prevent fibril formation, as previously proposed by us and others,[20,25,26] but only delay the onset of fibrillation. Therefore, our results suggest that Tyr39 and the C-terminal Tyr residues contribute to the initial steps of α-synuclein fibrillation. Also intriguing, the process of fibril formation by mutant proteins was accompanied by exposure of more hydrophobic regions than in the case of WT protein (Figure 3D, Table 1).

The WT α-synuclein and both mutants show no significant differences in their initial aggregation tendencies (Figure 2). In addition, far-UV circular dichroism and Fourier transform infrared spectroscopy measurements have shown that the structures of Y39A and Y(125,133, 136)A mutants remain predominantly disordered,[20] and a NMR study of the Y39A mutant has revealed only small local structural changes.[25] Therefore, the observed delay in the fibrillation of α-synuclein mutants is most probably due to a lack of the usual function of the aromatic residues in this process.

The reported NMR derived structures of the α-synuclein ensemble have shown that the N-terminal and C-terminal parts of the protein participate in transient intramolecular[17,18] and also intermolecular[15] interactions. In particular, long-range intramolecular interactions between the C-terminal residues at positions 120–140 and the central region comprising residues 30–100,[17] as well as antiparallel interchain N/C to C/N contacts between residues 35–50 and 110–140,[15] have been noted. Although electrostatic interactions have been proposed to govern these contacts,[15,17] the interacting regions also contain Tyr residues that can form a hydrophobic aromatic cluster and stabilize the interactions. Although C/N intramolecular contacts were suggested to shield the amyloidogenic NAC region from self-association,[17] several studies contradict this hypothesis.[19–22] A computational study of experimentally obtained structural data has recently suggested that the NAC region is positioned in a solvent-exposed conformation in a substantial fraction of the α-synuclein conformational ensemble.[22] Also, electrostatic repulsion between the highly acidic C-terminal[41] and the fast internal dynamics of the peptide backbone[42] hinder rapid α-synuclein aggregation. However, under specific conditions, the α-synuclein equilibrium can be shifted to more aggregation-prone conformations. Therefore, it is likely that the replacement of Tyr(s) with Ala(s) interrupts specific aromatic interactions between the N-terminal and C-terminal regions of α-synuclein, resulting in a delayed onset of fibrillation. These N/C aromatic-aromatic interactions may confer energetically favourable on-pathway transitions from the intrinsically disordered monomeric α-synuclein to the aggregation-prone oligomeric intermediate. If these stabilizing interactions are missing, then the time for spontaneous conformational conversion and for interactions between the NAC regions is prolonged and the on-pathway oligomeric species are substantially less packed (Figure 3D), which leads to a delayed onset of fibrillation and an altered fibril packing.

4. Conclusions

It has been previously suggested that Tyr residues contribute to α-synuclein fibril formation; however, their role in this process has not been characterized. Our study demonstrates that both the N-terminal and C-terminal Tyr residue(s) of α-synuclein participate in the early events of its fibrillation and the lack thereof leads to a significant delay, but does not abolish completely, the fibrillation potential of α-synuclein. The characterization of α-synuclein fibrillation at the residue level is important in terms of the design of novel therapeutic agents. Anti-amyloidogenic drugs that would specifically target α-synuclein Tyr residues, as already proposed for certain compounds,[25,34] may therefore prevent the undesired fibrillation-promoting N/C aromatic-aromatic interactions. However, this anti-fibrillation therapeutic strategy must be considered with caution, since it may only halt, but not prevent, the onset of fibril formation. Additionally, the fibrillation process could than include formation of more hydrophobic and therefore potentially cytotoxic species,[31] and therefore this therapeutic approach could eventually do more harm than good. However, cell studies are necessary to address this issues, since delaying the aggregation onset could prove to be efficient in the cellular context, where cell repairing mechanism would be more able to efficiently intervene.[12]

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Povzetek

Agregacija nativno nezvitega presinaptičnega proteina α-sinukleina v urejene amiloidne fibrile naj bi imela ključno vlogo v patogenezi Parkinsonove bolezni. Da bi ugotovili vpliv tirozinskih ostankov (Tyr) na proces fibrilacije α-sinukleina, smo pripravili rekombinantno N-terminalno (Y39A) in C-terminalno mutanto (Y(125,133,136)A) proteina. Proces fibrilacije proteinov smo preučili z uporabo dveh fluorescentnih prob (tioflavin T in 1-anilinonaftalen-8-sulfonat (ANS)), poliakrilamidne gelske elektroforeze v prisotnosti natrijevega dodecilsulfata (NaDS-PAGE) in mikroskopije na atomsko silo. V primerjavi z divjim tipom α-sinukleina odsotnost N-terminalnega Tyr ali vseh treh C-terminalnih Tyr močno inhibira fibrilacijo proteina. Po podaljšanem času inkubacije obe mutanti tvorita fibrilam-podobne strukture, ki pa se morfološko razlikujejo od fibril divjega tipa proteina, proces fibrilacije pa spremlja tudi zvišana hidrofobnost. Glede na dobljene rezultate sklepamo, da N-terminalni in C-terminalni Tyr ostanki α-sinukleina prispevajo predvsem k začetnim stopnjam fibrilacije.