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The effect of amino acid substitution in the imperfect repeat sequences of α-synuclein on fibrillation

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Keywords:

Parkinson’s disease, α-synuclein, fibrillation, E46K, imperfect repeat sequences
Abstract

Human α-synuclein is the causative protein of several neurodegenerative diseases, such as Parkinson’s disease (PD) and dementia with Lewy Bodies (DLB). The N-terminal half of α-synuclein contains seven imperfect repeat sequences. One of the PD/DLB-causing point mutations, E46K, has been reported in the imperfect repeat sequences of α-synuclein, and is prone to form amyloid fibrils. The presence of seven imperfect repeats in α-synuclein raises the question of whether or not mutations corresponding to E46K in the other imperfect KTKE(Q)GV repeats have similar effects on aggregation and fibrillation, as well as their propensities to form α-helices.

To investigate the effect of E(Q)/K mutations in each imperfect repeat sequence, we substituted the amino acid corresponding to E46K in each of the seven repeated sequences with a Lys residue. The mutations in the imperfect KTK(E/Q)GV repeat
sequences of the N-terminal region were prone to decrease the lag time of fibril formation. In addition, AFM imaging suggested that the Q24K mutant formed twisted fibrils, while the other mutants formed spherical aggregates and short fibrils. These observations indicate that the effect of the mutations on the kinetics of fibril formation and morphology of fibrils varies according to their location.
1. Introduction

α-synuclein is a natively unfolded protein of unknown function that is highly expressed in the neurons of the central nervous system. Since the major fibrillar protein component of Lewy Bodies (LBs) in both sporadic and familiar PD is α-synuclein, a causative factor for α-synuclein in PD pathogenesis is strongly supported. This 140-amino-acid protein consists of three regions: an N-terminal region (1-60), the non-amyloid component of Alzheimer’s disease (NAC) region (61-95), and a C-terminal region (96-140). The NAC region is recognized as the core region responsible for the formation of fibrils [1-4], having cross β-structures in which individual β-strands run perpendicular to the fiber axis [5]. The N-terminal and NAC regions contain seven imperfect repeats (XKTKEGVXXXX) that include a highly conserved KTKEGV hexameric motif. Similarities in these imperfect repeats
with those found in apolipoproteins have suggested a possible role in the association

of α-synuclein with lipids through the formation of an α-helical secondary structure

[6, 7]. Furthermore, when mixed with lipid vesicles, including negatively charged

phospholipids, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) or sodium dodecyl sulfate

(SDS), the N-terminal region of α-synuclein adopts an α-helical conformation [8, 9].

The α-helical form of α-synuclein has been reported not to fibrillate in vitro [10, 11].

These repeat sequences also have the effect of decreasing the tendency of α-synuclein
to form β-sheet structures, such as fibrils [12]. Previously, we have suggested that

KTKEGV motifs play a significant role in maintaining the natively unfolded status of

α-synuclein [13].

Rare familial forms of early-onset PD have been linked to the three independent

missense mutations A53T [14], A30P [15] and E46K [16] in the N-terminal region of
\(\alpha\)-synuclein. The E46K mutation in the fourth imperfect repeat sequence increases the ability of fibril formation and aggregation \textit{in vitro} [17, 18] and in cultured cells [19].

Examinations of PD-linked and synthetic mutants [20] suggest that the N-terminal region may be important in modulating aggregation and fibrillation. The presence of seven imperfect repeats in \(\alpha\)-synuclein raises the question of whether or not the mutations in the corresponding to E46K in the other imperfect KTKE(Q)GV repeats cause similar effects on aggregation and fibrillation, as well as their propensities to form \(\alpha\)-helices. Therefore, we attempted to introduce amino acid substitutions at the position corresponding to E46 in each of the seven imperfect repeats (Fig. 1). We evaluated their propensity to form fibrils, aggregates and \(\alpha\)-helices, as well as their morphology, using thioflavin-T (TfT) fluorescence, light scattering, circular dichroism (CD) and atomic force microscopy (AFM). Our findings suggest that the N-terminal
KTK(E/Q)GV hexameric repeats are important for modulating the kinetics of fibrillation and the morphology of fibrils. We show that the impact of the mutations in the imperfect repeats on the kinetics of fibril formation and the morphology of fibrils differs according to their location.
2. Materials and Methods

2.1. Chemicals

Thioflavin-T (TfT) was purchased from Sigma. 1,1,1,3,3,3-Hexafluoro-2-propanol
(HFIP) was purchased from Wako.

2.2. Recombinant technology

The human α-synuclein structural gene was subcloned into pET-28a(+) expression
and E83K were used to carry out site-directed mutagenesis using the QuikChange®
Site-Directed Mutagenesis Kit (Stratagene) with the following oligonucleotides:

5’-GGACTTTCAAAGGCCAAGAAAAGGAGTTGTGGCTGC-3’ (E13K),

5’-GCTGCTGAGAAAACCAAAAAAGGTGTGGCAGAAGC-3’ (Q24K),

5’-GAAGCAGCAGGAAAGACAAAAAAAGGTGTGCTCTACATGTAGGC-3’

(E35K),
5'-AGGCTCCAAAACCAAGAAGGGAGTGGTGCATGG-3' (E46K),
5'-GTGGCTGAGAAGACCAAAAAACAAGTGACAAATGTTGG-3 (E61K) and
5'- GTAGCCCAGAAGACAGTGAAAGGAGCAGGGAGCATTGC -3' (E83K).

All mutations were confirmed by DNA sequencing (PerkinElmer ABI Model 310).

□ □ -Synuclein proteins were purified and prepared for circular dichroism (CD) measurements, Tff fluorescence assay, light scattering assay, and atomic force microscopy (AFM) imaging, as reported previously [21].

2.3. Circular dichroism measurements

□ The CD spectra of 0.2 mg/ml α-synuclein at 25°C were recorded from 190 to 250 nm in a 0.1-cm cell, at a 1.0-nm bandwidth, a 4-s response time and a scan speed of 20 nm/min using a J-720 spectropolarimeter (JASCO). Each spectrum was generated from the average of five scans. The CD spectrum of 10 mM potassium phosphate buffer, pH 7.0, was used as the background and subtracted from the protein spectra.
The data are presented as the mean residue molar ellipticity (deg cm$^2$ dmol$^{-1}$).

2.4. Fibril formation analysis

All protein samples were concentrated to ca. 3.0 mg/ml by using Amicon Ultra-15 filters (Millipore) in phosphate buffer saline, pH 7.3, centrifuged at 150,000 g for 1 h to remove any aggregates, and adjusted to 2.0 mg/ml. For the thioflavin T (TfT) fluorescence assay, the protein concentration was 1 mg/ml (70 µM). The α-synuclein samples were incubated with 25 µM TfT in 96-microwell plates with flat bottom (Nunc) in a sample volume of 200 µl for each well. Teflon spheres (As-One) were placed into each well of the 96-microwell plates, which were sealed by tape (Nunc) and incubated at 37°C with shaking at 500 rpm. Our preliminary studies in which we removed aliquots over time for analysis demonstrated that the presence of 25 µM TfT in the reaction mixture does not interference with the kinetics of amyloid formation.
(data not shown). Fibril formation was monitored by TfT fluorescence. The TfT fluorescence was recorded at 486 nm with excitation at 450 nm using an ARVO MX 1420 multilabel counter (PerkinElmer). The TfT fluorescence measurements were plotted as a function of time and fitted to the sigmoidal curve described using Graphpad Prism 4. A light scattering assay at 330 nm was carried out to monitor the total aggregation.

2.5 1,1,1,3,3,3-Hexafluoro-2-propanol titration

HFIP was added to α-synuclein solution in 10 mM phosphate buffer, pH 7.0, and diluted with the same buffer to obtain final concentrations of 0 to 10% (v/v) HFIP and 0.2 mg/ml α-synuclein. Samples were analyzed by CD spectroscopy after incubation at room temperature for 24 h.
2.6. AFM imaging of WT and mutant α-synuclein

For the AFM analysis, 2-μl samples of α-synuclein were incubated and placed on freshly cleaved mica. After air drying, the mica surfaces were rinsed three times with Milli-Q water. The samples were then allowed to air-dry. Tapping mode imaging was performed on a Nanowizard II™ BioAFM (JPK Instruments, Berlin, Germany) using a silicon probe (OMCL-AC160TS-C2, Olympus, Tokyo, Japan). The scanning parameters varied with the individual samples. Some typical parameters were as follows: set point, 660 mV; driving amplitude, 38 mV; driving frequency, 333.874 kHz.
3. Results

3.1 Aggregation and fibrillation of WT and mutant α-synucleins

The isoelectric points (pI) of α-synuclein increased by the introduction of Lys in the imperfect repeat sequences in place of Glu or Gln. The pI values of E13K, E35K, E46K, E61K and E83K (pI=4.81), as well as that of Q24K (pI=4.75), were slightly greater than that of WT (pI=4.67). However, all mutants showed electrostatic properties indistinguishable from those of WT, as identical purification procedures achieved similar purity levels and yields for all mutants.

To investigate the effect of amino acid substitution on the secondary structure in solution, far-UV CD analysis was carried out. Figure 2 shows that WT and all mutants share a random coil structure, which is characterized by minima in the vicinity of 198 nm. This shows that the amino acid substitutions had no impact on the native state of α-synuclein.
We investigated the effect of amino acid substitutions in the imperfect repeat sequences on the fibril formation ability using the fluorescence dye TfT, which interacts specifically with amyloid fibrils such that their long axes align in a parallel fashion. The fluorescence intensity in the vicinity of 482 nm increased when TfT bound to the amyloid fibrils. The kinetics of fibrillation usually shows a sigmoidal pattern, from which the lag time can be measured to indicate the time of nucleation.

Figure 3 shows that the E46K mutant has a shorter lag time and a higher maximal fluorescence intensity than WT, which is consistent with previous reports [17, 18, 22]. The E13K, Q24K and E35K mutations, located in the N-terminal region, greatly reduced the lag time compared with that for WT, while E61K and E83K mutations only slightly decreased the lag time. However, all mutants except E46K had a much lower maximal fluorescence intensity (< 40%) compared to WT.
As the TfT fluorescence assay is specific for amyloid fibrils, it cannot assess other types of aggregates such as oligomers and amorphous aggregates. To compare the total aggregation of WT α-synuclein and mutant α-synuclein, a light scattering assay was performed. Figure 4 shows that all mutants were more prone to aggregate than WT. Although the ΔOD_{330} values of most of the E/K mutants were more than twice that of WT, only that of Q24K was approximately 1.5 times that of WT.

3.2 α-Helix formation of α-synuclein wild type and mutants in the presence of HFIP

HFIP was used to induce the conversion of α-synucleins to α-helical structure as model system of membrane surface. Far-UV CD spectra of the seven proteins were recorded in 0% to 10% HFIP. All α-synucleins assumed spectra characteristic of fully α-helical protein at 10% HFIP (data not shown), with the minima at 208 and 222 nm.
However, in the presence of 2.5% HFIP (Fig. 5), the spectra of E(Q)/K mutants decreased around 216 nm, representing β-sheet structure, despite the that of WT was almost same in the absence of HFIP, showing random structure. These results suggested that E(Q)/K mutants were prone to form partially folded intermediates rather than WT. Furthermore, to examine the transitions, we produced phase diagrams (Fig. 6) showing the relationship between $\theta_{\lambda_{198}}$ and $\theta_{\lambda_{222}}$. A linear relationship reflects an all-or-none transition between two different conformations, while nonlinear relation represents multiple sequential transformations. For WT, E13K, Q24K (Fig. 6) and the other E(Q)/K mutants (data not shown), the structural changes with increasing HFIP clearly proceeded through two transitions: (I) from natively unfolded to partially folded, (II) from a partially folded intermediates to α-helical conformation. These observations are also consistent with earlier works [11, 13]. However, the breaking
points of E(Q)/K mutants between two transitions varied (Fig. 6), which suggests that E(Q)/K mutants can form into the distinct partially folded intermediates conformations.

3.3 Morphology of α-synuclein fibrils

We investigated the morphology of α-synuclein fibrils and aggregation by AFM imaging to help explain why the maximal fluorescence intensity decreased while the light scattering increased for the E(Q)/K mutants. The results of AFM imaging demonstrate that the effect of these mutations on the morphology varies according to their location. WT typically forms amyloid fibrils, having a diameter of 8 nm and a length of 1 μm. Among the E(Q)/K mutants, mature fibrils could be visualized only in the Q24K and E46K mutants (Fig. 7). Instead of mature fibrils, the E35K, E61K and
E83K mutants formed short fibrils (6-8 nm in height and less than 0.5 µm in length).

The E13K mutant formed spherical shapes like small oligomers with a mean height of 8 nm. On the other hand, the Q24K mutant fibrils, having a diameter of 8 nm and a length of 1 µm, were twisted in form.
4. Discussion

The PD/DLB-causing mutation E46K has been reported on the fourth imperfect repeat of α-synuclein [16]. The N-terminal KTKEGV hexameric repeats of α-synuclein can adopt an α-helical secondary structure in the presence of lipid vesicles [8, 9]. These repeat sequences of α-synuclein may play the same roles, such as conferring a local propensity for helical conformation and its stabilization. In this study, we set out to determine whether or not the mutations in the corresponding position in the other imperfect repeats cause similar effects on fibrillation and aggregation.

All E(Q)/K mutations in the position which corresponds to E46K decreased the lag time of fibrillation compared with that of WT. The first critical step of the aggregation and fibrillation of α-synuclein is considered to be the folding from a natively unfolded
state to a partially folded intermediate; then, the intermediate becomes oligomerized, which finally leads to the formation of fibrils via a critical nucleus [23]. The decrease in lag time indicates that substituting the Glu or Gln residue in the imperfect repeat sequences with a Lys residue readily prompts the conformational change of α-synuclein to a partially folded intermediate. Monomeric α-synuclein assumes conformations that are stabilized by long-range interactions between the C-terminal tail and the N-terminal and NAC region and act to inhibit oligomerization and aggregation [24]. Recently, C. C. Rospigliosi et al. have suggested that E46K enhances C-terminal-to-N-terminal contacts in α-synuclein and any reduction in net negative charge of the protein such as truncation of C-terminal tail, sequences changes with disease (e.g. E46K in PD/DLB), and with divergent evolution (e.g. D121G in mouse α-synuclein), leads to enhance aggregation [25]. Since all E/K mutations
decrease the net negative charge of the protein, they are expected to enhance aggregation, as observed in our experiments. In contrast, the aggregation of Q24K mutation, which does not reduce the net negative charge of the protein, was lower than that of all E/K mutations. These results were consistent with their suggestion.

The reduction of the TfT fluorescence intensity in all mutants except E46K could be due to the morphology. The AFM imaging shows that the fibrils of all mutants were different from those of WT, and that mature twisted fibrils can be observed in the Q24K mutant. Interestingly, total aggregation of the Q24K mutant was lower than that of the other E/K mutants in the light scattering assay. These data suggest that the aggregation ability of the Q24K mutant may be lower than that of the other E/K mutants, even though Q24K mutant can form amyloid fibrils.

Several studies using hydrogen-deuterium (H/D) exchange and solid-state nuclear
magnetic resonance (ssNMR) spectroscopy and electron paramagnetic resonance (EPR) spectroscopy [26-28] have suggested that the cross-β region is located within residues 30-110, pinpointing residues 35, 61 and 83 in the β-sheet region. Thus, it is likely that the E35K, E61K and E83K mutants do not form mature fibrils due to the direct effects of the mutations on the formation of cross β-structures. Interestingly, residue 46 is not present in the β-sheet consisting of the cross-β region and located next to the hydrophobic residues V47 and V48. The E46K mutation may thereby promote the formation of new β-sheets without disrupting the endogenous β-sheets.

H. Heise et al. have reported that the well-ordered β-sheet region extends in disease-related A53T-mutant α-synuclein compared with that of WT [29]. It is interesting to note that the double mutant K45V/E46V showed a decreased lag time and formed twisted fibrils [30]. In addition, the E13K and Q24K mutants formed spherical shapes.
and twisted fibrils, respectively. Thus, our data suggest that the N-terminal region may be important in modulating the morphology, as well as aggregation and fibrillation.

The imperfect repeat sequences of α-synuclein are the regions that form the α-helical conformation when mixed with lipid vesicles, including negatively charged phospholipids, HFIP or SDS. E46K mutation enhanced the ability to bind phospholipids [17]. However, NMR studies revealed that the E46K mutation resulted in subtle changes in the conformation of the monomeric protein in the presence of SDS micelles [22]. Our data suggested that all E(Q)/K mutants formed α-helical conformation in the presence of 10% HFIP, but their propensities of secondary structure, partially folded intermediates, were different from that of WT. Therefore, the effect of these mutants on interaction with membrane phospholipids may vary because they have the distinct propensity to form α-helical conformation.
Our results clearly indicate that E(Q)/K mutants can form the distinct morphology of aggregates. It is now clear that many proteins misfold into a variety of aggregates in different conditions such as pH, temperature, and covalent changes (mutations or chemical modification) [31]. Recently, it has been reported that structural diversity of huntingtin-exon1 is involved in apparently different toxicity effects of the amyloids [32]. Therefore, the toxicity effects of E(Q)/K mutants might be different due to distinct conformations.

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References


108.


Figure Legends:

**Fig. 1 Amino acid substitutions in the imperfect repeat sequences.** The primary structure of α-synuclein is divided into three regions: the N-terminal region (upper), the NAC region (middle) and the C-terminal region (lower). The gray letters represent the amino acids in the characteristic hexamer motif (KTKEGV). The positions of three missense mutations (Ala53Thr, Ala30Pro, Glu46Lys) involved in the early onset of Parkinsonism-related disease are marked with asterisks (*). The positions of the α-synuclein mutations used in this study are shown under the letters.

**Fig. 2 CD spectra of WT and mutant α-synucleins.** The CD spectra were determined after purification: WT (black line), E13K (black dashed line), Q24K (black dotted line), E35K (gray line), E46K (gray dashed line), E61K (gray dotted line) and E83K (gray dashed and dotted line)
Fig. 3 Effect of amino acid substitution on the amyloid fibril formation of α-synuclein. Time course of fibril formation of α-synuclein and its mutants, as determined by TfT fluorescence assay: WT (black cycle), E13K (black square), Q24K (black triangle), E35K (black reverse triangle), E46K (white cycle), E61K (white square) and E83K (black diamond). The error bar indicates the SEM (n=3).

Fig. 4 Changes in α-synuclein light scattering. The OD_{330} values recorded after the incubation of the samples were subtracted from those before incubation to determine the increase in light scattering (ΔOD_{330}). The error bar indicates the SEM (n=3).

Fig. 5 CD spectra of WT and mutant α-synucleins in the presence of 2.5% HFIP.

The CD spectra were recorded in the presence of 2.5% HFIP: WT (black line), E13K
(black dashed line), Q24K (black dotted line), E35K (gray line), E46K (gray dashed line), E61K (gray dotted line) and E83K (gray dashed and dotted line).

**Fig. 6 Phase diagrams for the structural changes induced in α-synuclein WT, E13K, and Q24K by HFIP.** The effect of HFIP on the conformation of α-synucleins: WT (black circles and black solid lines), E13K (white circles and dashed lines), and Q24K (gray circles and gray solid lines). The roman numbers, I and II represent the phases of transitions: (I) from natively unfolded to partially folded intermediates, (II) from partially folded intermediates to α-helical structure. The arrows represent the breaking points.

**Fig. 7 AFM images of α-synuclein and its mutants.** The samples for AFM imaging were prepared by incubation for 5 days. (A) WT, (B) E13K, (C) Q24K, (D) E35K, (E)
E46K, (F) E61K, (G) E83K. Scale bar: 500 nm
$\theta_{\text{MCD}}$ (deg cm$^2$ dmol$^{-1}$)

wavelength (nm)