

A Four-Amino Acid Linker between Repeats in the α -Synuclein Sequence Is Important for Fibril Formation

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Supporting Information

ABSTRACT: α -Synuclein is a 140-amino acid protein that can switch conformation among intrinsically disordered in solution, helical on a membrane, and β -sheet in amyloid fibrils. Using the fluorescence of single-tryptophan mutants, we determined the immersion of different regions of the protein into lipid membranes. Our results suggest the presence of a flexible break close to residues 52–55 between two helical domains. The four-amino acid linker is not necessary for membrane binding but is important for fibril formation. A deletion mutant lacking this linker aggregates extremely slowly and slightly inhibits wild-type aggregation, possibly by blocking the growing ends of fibrils.

α -Synuclein (α Syn) is a 140-amino acid intrinsically disordered protein strongly expressed in the brain and localized at neural terminals.^{1,2} Its physiological functions are yet unclear but are believed to be connected to the interaction with synaptic vesicles.^{1,3,4} α Syn amyloid fibrils are the main component of Lewy bodies, intracellular inclusions in the brain of patients affected by Parkinson's disease.⁵

α Syn consists of a hydrophobic region (residues 60–90) that serves as the amyloid fibril core,^{3,6} an amphiphilic basic N-terminal part, and a negatively charged unstructured C-terminus. The protein sequence incorporates seven imperfect 11-residue xxKTEGVxxx repeats (Figure 1A).

α Syn shows remarkable conformational plasticity. It has no distinct secondary structure in solution but readily forms β -sheet rich amyloid fibrils upon aggregation and adopts α -helical structure upon membrane binding.⁷ The presence of amphiphilic repeats in the N-terminal part inhibits fibrillization of α Syn⁸ and determines membrane binding affinity. α Syn binds anionic and rigid neutral membranes^{9–11} and forms helices oriented parallel to the membrane surface,^{9,12,13} making three turns per 11-amino acid repeat.¹³ Nuclear magnetic resonance (NMR) structural studies of α Syn bound to sodium dodecyl sulfate (SDS) micelles show two antiparallel α -helix fragments (residues 3–37 and 45–92).^{12,14} The conformation of α Syn on lipid membranes was variously reported to be extended¹³ or broken^{15,16} α -helix depending on specific conditions.^{9,17} The position of the break is widely assumed to be the same as on micelles¹⁴ but has not yet been conclusively experimentally demonstrated. The conformation of α Syn on membranes was studied by electron paramagnetic resonance¹³ and fluorescence,^{18–21} showing continuous periodic changes in the extent of immersion of residues 18–

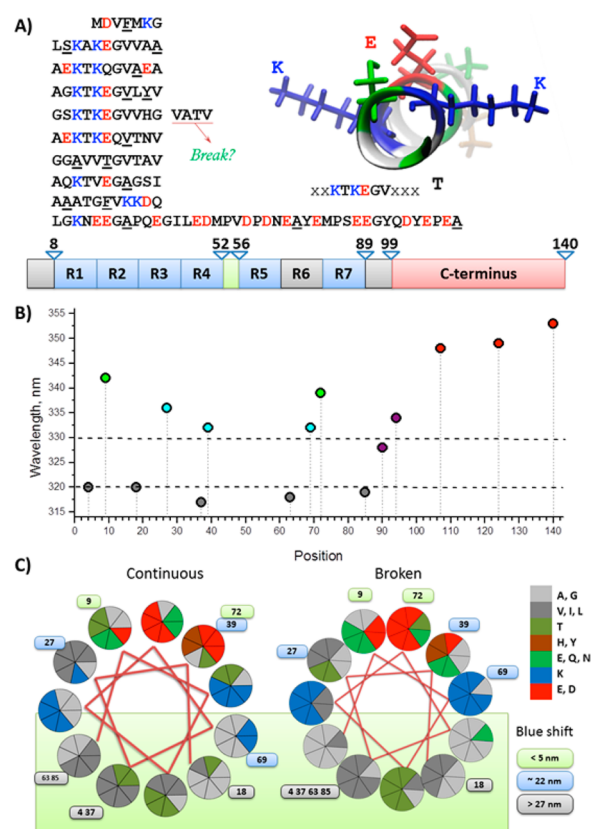


Figure 1. (A) Amino acid repeats in the α Syn sequence. Residues mutated to Trp are underlined. The inset shows the structure of one 11-amino acid repeat bound to SDS micelles.¹⁴ (B) Emission maxima of single-Trp α Syn variants bound to dioleoylphosphatidylglycerol (DOPG) vesicles. The size of the symbol approximately corresponds to the experimental error (± 1.5 nm). (C) Wheel diagram of the α Syn repeat regions (residues 8–89) for continuous and broken helix models.

90 and the absence of membrane binding of the C-terminus.^{10,12} Tryptophan fluorescence reports moderate immersion for the central part of α Syn but relatively deep insertion for N-terminal and NAC regions.^{18,22}

To analyze the conformation of membrane-bound α Syn, we studied the immersion of 15 positions using fluorescence of single-tryptophan mutants. In lipophilic environments, the

Received: October 20, 2013

Revised: December 30, 2013

Published: January 3, 2014



emission maxima of tryptophan fluorescence shift to the blue, reflecting the extent of immersion into the membrane. All 15 mutants show similar affinity and final α -helix content with a K_d of $\sim 0.25 \pm 0.1 \mu\text{M}$ and a lipid/protein stoichiometry of 40 ± 10 upon binding to dioleoylphosphatidylglycerol (DOPG) vesicles (Figure S2 of the Supporting Information), in agreement with previously published data ($0.5 \mu\text{M}$ and 30 ± 10 , respectively).¹⁰ As expected, the changes in the Trp fluorescence emission upon membrane binding depend on the position. The strongest blue emission shifts ($\geq 25 \text{ nm}$), corresponding to the deepest immersion, were observed for Trp at positions 4, 18, 37, 63, and 85, while positions 9 and 72 exhibit minor shifts; labels on the flexible negatively charged C-terminal tail show virtually no changes (Figure 1B and Table S1 of the Supporting Information). The observed differences in the membrane immersion of residues close in sequence, for example, 4 and 9, are in good agreement with an αSyn orientation parallel to the water–membrane interface and the correspondingly different environments for lipophilic and hydrophilic faces of the helix. The membrane immersion of amino acids correlates with the position within the 11-amino acid repeats (Figure 1) in accordance with the 3/11 amino acid periodicity of the protein helix.¹³

Two proposed models for membrane-bound αSyn are the extended, continuous¹⁴ helix and the helix with a break.^{15,17} In both cases, the hydrophobic parts of the helices face the membrane, the negatively charged hydrophilic regions face water, and lysines are close to the membrane–water interface (Figure 1C). We studied 10 αSyn mutants containing Trp residues introduced into the repeat region (amino acids 8–89). For the continuous helix model (Figure 1C, left), one would expect fully water-exposed residue 9; strongly exposed residues 27, 39, and 72; and membrane immersion for the six other tested positions. In our experiments, the emission of tryptophan at positions 39 and 69 is similar and points to an environment more apolar than that of residues 9 and 72 but significantly less apolar than that of residue 18, 63, or 85 (Figure 1B and Table S1 of the Supporting Information). These data better correlate with the model of a helix containing a break close to the four-amino acid insert (residues 52–55) between the repeats (Figure 1C, right). In this assumption, residues 4, 37, 63, and 85 are on the most immersed part of the helix (opposite the acidic residues) while positions 39 and 69 are on the membrane–water interface. The broken helix conformation also shows a more optimal distribution of lipophilic and hydrophilic residues on a wheel diagram (Figure 1C), by removing membrane-immersed lysines and decreasing the number of water-exposed valines. The model with a break in the vicinity of amino acids 38–44, similar to the structure on SDS micelles, would also fit the data but would require a difference in structure for very similar third and fourth 11-amino acid repeats (Figure S1 of the Supporting Information). Prior reports have addressed the implications of such discontinuities in an otherwise extended helical topology.^{15,16}

We used mutants with Trp residues at positions 90, 94, 107, 124, and 140 to localize more precisely the end of the helical domain and the beginning of the C-terminal part of αSyn not involved in membrane binding. Tryptophan at positions 107, 124, and 140 shows no changes in fluorescence upon membrane binding, while αSyn -90W and -94W show significant blue shifts close to those observed for residues 39 and 69. Both continuous and broken helical conformations propose water-exposed positions for these two residues (Figure S3 of the Supporting

Information) that do not match the observed blue-shifted emission. We suggest that αSyn adopts a helical conformation only in the repeat region and that residues 89–97 located after the last 11-amino acid repeat are membrane-immersed but are not in the helical conformation because of the absence of proper periodicity in amino acid polarity. This observation is in line with the average α -helical content calculated for αSyn based on circular dichroism (CD) spectroscopy ($\sim 60\%$) that corresponds to an ~ 84 -amino acid α -helix. The behavior of αSyn was very similar in charged (DOPG) and neutral (DPPC) membranes but significantly different in SDS micelles (Figure S2 of the Supporting Information). In SDS micelles, the extent of immersion of residues 37 and 39 was significantly lower than in lipid bilayers, perhaps reflecting an increased level of solvent exposure in the break region reported in the NMR-based structure of SDS-bound αSyn .¹⁴

To validate our hypothesis of the break position (amino acids 52–55) of membrane-bound αSyn , we prepared a protein variant lacking these residues ($\alpha\text{Syn}\Delta$). In the case of the continuous α -helix or another break position, such a construct should show significantly lower membrane affinity than wild-type (WT) αSyn because of the shift in the α -helix hydrophobicity pattern. Indeed, deletion of four residues (residues 2–5) at the N-terminal α -helix was reported to significantly decrease the level of membrane binding.²³ Titration experiments show a moderate decrease in the binding affinity of $\alpha\text{Syn}\Delta$ compared to that of the WT protein (Figure S4 of the Supporting Information). Moreover, tryptophan mutants $\alpha\text{Syn}\Delta$ -W37 and $\alpha\text{Syn}\Delta$ -W63 show emission spectra in the membrane-bound form almost identical to those of αSyn -W37 and αSyn -W63, pointing to similar conformations on the membrane of protein regions flanking the repeat (Figure S4 of the Supporting Information). Taken together, these results allow us to conclude that the helix of membrane-bound αSyn contains a break close to residues 52–55.

A defining characteristic of αSyn is its ability to switch conformations among α -helix on membranes, random coil in solution, and β -sheet in amyloid fibrils. Comparative analysis of mouse and human αSyn fibrils by solid state NMR suggests that residues 52–55 are in a β -sheet region.²⁴ We tested the influence of the deletion of the four-amino acid linker on the protein aggregation propensity. The deletion mutant showed almost no tendency to aggregate compared to WT (Figure 2). The aggregation of αSyn in the simplest model could be described as slow formation of oligomers that yield initial fibrils followed by

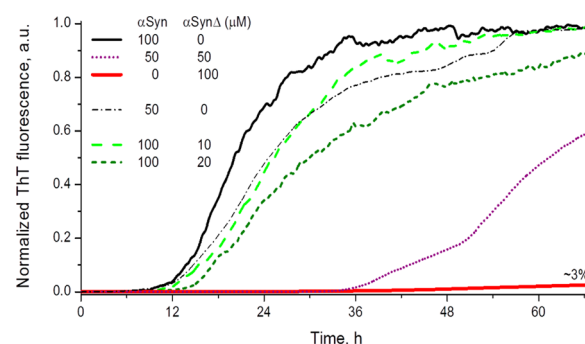


Figure 2. Aggregation of WT αSyn and $\alpha\text{Syn}\Delta$ monitored by thioflavin T (ThT) fluorescence. Averaged curves of eight samples, normalized by the maximal ThT fluorescence of $100 \mu\text{M}$ WT. Experiments performed at 37°C in pH 7.2 phosphate buffer containing 150 mM NaCl.

relatively fast fibril growth.²⁵ The slower aggregation of α Syn Δ compared to that of WT could be because of less efficient initiation of fibril formation or slower fibril extension. To understand which of the two mechanisms predominates, we checked if WT α Syn could initiate aggregation of the deletion mutant in a mixture of 50 μ M WT α Syn and 50 μ M α Syn Δ . Surprisingly, the aggregation rate of the mixture was significantly slower than that of 50 μ M WT α Syn alone, suggesting not only that WT α Syn cannot initiate aggregation of the deletion mutant but also that the mutant can inhibit aggregation of WT α Syn. The aggregation rate of WT also decreases upon addition of small amounts of α Syn Δ ; 10 and 20 mol % α Syn Δ slows the aggregation by ~25 and ~40%, respectively (Figure 2). This could happen if the mutant binds the growing ends of fibrils and hinders their further extension. To check this hypothesis, we estimated the relative content of α Syn Δ in the fibril by aggregating mixtures in which either WT α Syn or α Syn Δ incorporated a Trp residue and by measuring the relative amounts in fibrils and solution by absorbance and fluorescence. The experiments show that aggregation of the mixture containing 50 μ M WT and 50 μ M α Syn Δ leads to fibrils composed mostly of WT (>85%), leaving most of the deletion mutant in solution (Figure S6 of the Supporting Information). The small amount of α Syn Δ in fibrils and its ability to decrease the aggregation rate of WT could possibly be explained by binding of α Syn Δ to the fibrils ending with WT and subsequent inhibition of fibril growth due to the low affinity of both WT α Syn and α Syn Δ for fibrils ending in the deletion mutant (Figure S8 of the Supporting Information).

Bousset et al. recently reported²⁶ on the formation of two distinct polymorphs of α Syn fibrillar structures at different salt concentrations, termed “fibrils” and “ribbons”. Residues 52–55 are incorporated into a β -sheet in “fibrils” formed at physiological salt concentration but are suggested to be in a loop region in “ribbons” formed under low-salt conditions. We performed experiments at physiological salt concentrations. It is possible that α Syn Δ could bind to fibril ends only in an alternative conformation corresponding to the low-salt morphology that may decrease the affinity of the following α Syn monomer to be recruited to the fibril end, thus impairing fibril growth.

Our results suggest that α Syn bound to membranes consists of two 3/11-helical fragments, a short nonhelical membrane-immersed region (amino acids ~89–95) and a flexible C-terminus. The break between the two helical parts corresponds to the four-amino acid linker between 11-amino acid repeats and in the vicinity of disease-related mutation positions. The 11-amino acid repeat motif and four-amino acid insertion after the fourth repeat are common features of α -, β -, and γ -synucleins and appear to play an important role in the conformational flexibility of the protein. The flexible insert between repeats is one of the key factors determining α Syn fibril growth.

■ ASSOCIATED CONTENT

■ Supporting Information

Materials, methods, and supplementary figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding

We acknowledge NanoNextNL Project 3A.03 for support.

Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

We thank Nathalie Schilderink and Kirsten van Leijenhorst-Groener for protein purification and Dr. Thomas M. Jovin (MPI-BPC, Göttingen, Germany) for access to the CD spectrometer.

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