Protein Aggregation

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Sequestration of a β-Hairpin for Control of α-Synuclein Aggregation**

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Abstract: *The misfolding and aggregation of the protein α-synuclein (α-syn), which results in the formation of amyloid fibrils, is involved in the pathogenesis of Parkinson's disease and other synucleinopathies. The emergence of amyloid toxicity is associated with the formation of partially folded aggregation intermediates. Here, we engineered a class of binding proteins termed β-wrapins (β-wrap proteins) with affinity for α-synuclein (α-syn). The NMR structure of an α-syn:β-wrapin complex reveals a β-hairpin of α-syn comprising the sequence region α-syn(37–54). The β-wrapin inhibits α-syn aggregation and toxicity at substoichiometric concentrations, demonstrating that it interferes with the nucleation of aggregation.*

The conversion of specific peptides and proteins into amyloid fibrils has been identified as a causative mechanism underlying several neurodegenerative conditions. Monomeric and oligomeric misfolding intermediates are key species on the aggregation pathway;[1] thus, the stabilization of partially folded states of amyloid proteins has been suggested as an approach for drug development.[1d]

The protein α-synuclein (α-syn) is the major protein component of the intracellular neuronal deposits observed in Parkinson's disease and related synucleinopathies.[2] A role of α-syn aggregation in the pathogenesis is supported by various genetic studies showing the enhanced propensity of α-syn to aggregate as a result of disease-related mutation or multiplication of the α-syn gene. Structurally, α-syn is characterized by high conformational flexibility. Free monomeric α-syn is intrinsically disordered; however, some regions of the protein show intramolecular long-range interactions.[3] When bound to synthetic or biological membranes, α-syn can readily adopt α-helical conformation.[4] In the fibrillar form, the central region of α-syn forms several β-strands which assemble into in-register parallel β-sheets in the fibril core, whereas the N-terminal part of the protein is less ordered and the C-terminus remains unfolded.[5] In contrast to the parallel arrangement of α-syn in fibrils,[5b,6] α-syn oligomers have been shown to adopt an antiparallel β-sheet structure.[7]

To stabilize partially structured α-syn, we generated binding ligands to α-syn using $ZA\beta_3$, a binding protein to the amyloid-β peptide (Aβ), as a scaffold. $ZA\beta_3$ is a disulfide-linked homodimer derived from the Z domain of protein A.[8] According to isothermal titration calorimetry (ITC), $ZA\beta_3$ binds Aβ with a dissociation constant of 20 nM but shows no affinity to α-syn (Figure S1). To generate binding affinity to α-syn, we created a new phage display library through random mutagenesis of the gene encoding $ZA\beta_3$. The binding ligands selected from this library are referred to as β-wrapins (β-wrap proteins). The β-wrapin clone AS69 harboring four amino acid substitutions in each subunit, that is, G13D, V17F, I31F, and L34V, was found to bind α-syn with a K_d value of 240 nM (Figure S1). In comparison to the original $ZA\beta_3$, the affinity of AS69 towards Aβ was 400-fold reduced. ITC indicated that the AS69:α-syn interaction follows a 1:1 stoichiometry. The binding of AS69 to α-syn was confirmed by (^{1}H-^{15}N) HSQC NMR spectroscopy. The spectra of free [U-^{15}N]-α-syn obtained at 30 °C showed only a few resonance signals stemming from the C-terminus, whereas cross-peaks from the N-terminal and central regions were not detected due to intermediate exchange.[9] However, upon addition of [NA]-AS69 to [U-^{15}N]-α-syn, several well-dispersed resonance signals appeared, indicating partial folding of α-syn (Figure 1a). The new resonances were assigned to the region α-syn(35–56).

To identify the conformation of α-syn(35–56), we determined the structure of full-length α-syn in complex with β-wrapin AS69 by high-resolution liquid-state NMR spectroscopy (Figure 1b,d, Tables S1 and S2). α-syn(35–56) folds into a β-hairpin comprising residues ^{37}VLYVGSK^{43} (β1) and ^{48}VVHGVAT^{54} (β2), connected by a β-turn formed by amino acids ^{44}TKEG^{47} (Figure 1b). The aromatic amino acids Tyr-39 and His-50 are located at the center of one β-hairpin face with their side chains hydrogen-bonded by the hydroxy proton of Tyr-39 and the N^{δ}-nitrogen of His-50 (Figure 1b). The H^{η} of Tyr-39 and $H^{\varepsilon 2}$ of His-50 are protected from exchange with solvent as inferred from their detectability in the NMR spectra.

The sequence positions of the β-strands are in good agreement with those of the β1 and β2 strands (designated according to Vilar et al.)[5d] of fibrillar α-syn (Figure 1c). Long-range interactions between the side chains Tyr-39 in β1 and His-50 in β2 have also been detected in α-syn amyloid

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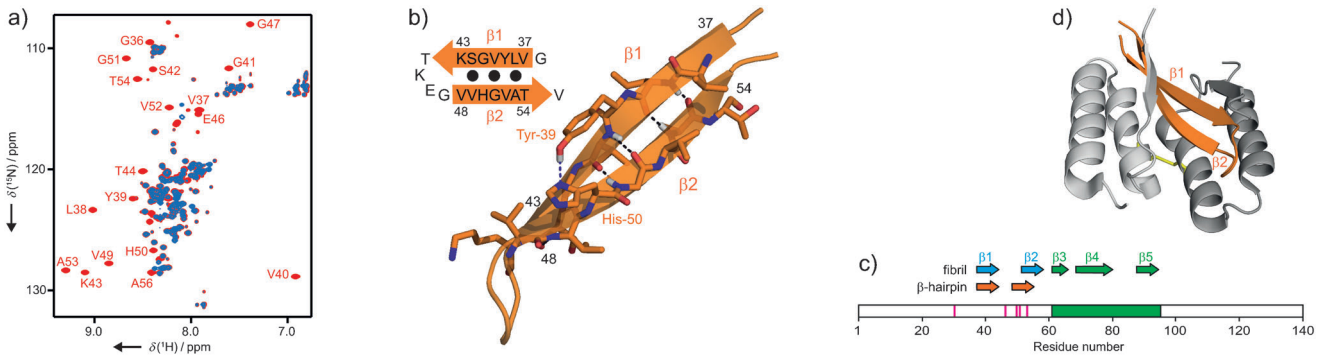

Figure 1. β-hairpin structure of α-syn in complex with β-wrapin AS69. a) (^{1}H-^{15}N) HSQC NMR spectra of [U-^{15}N]-α-syn recorded at 30 °C in the absence (blue) and presence (red) of an excess of [NA]-AS69. Assignments of peaks appearing upon complex formation are indicated. b) Ribbon drawing illustrating the β-hairpin of α-syn (orange). Amino acids forming the β1 and β2 strands are shown as sticks. The hydrogen bond between the hydroxy proton of Tyr-39 and the N$^{\delta}$-nitrogen of His-50 is indicated as a dashed blue line. Backbone hydrogen bonds are marked as dashed black lines. The corresponding amino acid sequence is shown and backbone hydrogen bonding across the strands is indicated with black dots. c) Features of the α-syn primary structure. The central NAC region is shown in green. The positions of disease-related mutations are given in magenta. The location of β-strands in α-syn within fibrils as identified by EPR and solid-state NMR analysis[5] is approximated by blue and green arrows. The location of the β-strands within the β-hairpin of α-syn is given by orange arrows. d) The α-syn:AS69 complex illustrated by ribbon drawing. Residues 13–58 of the two AS69 subunits are shown in light and dark gray, respectively. The disulfide bond is shown in yellow. The β-hairpin of α-syn is shown in orange.

fibrils.[5d] The NAC region comprising β-strands β3 to β5, including the aggregation-prone sequence stretches with the highest hydrophobicity and β-sheet propensity,[10] is unaffected by AS69 binding (Figure S2). This demonstrates the specificity of the interaction of AS69 with the β1–β2 region.

Contacts between β1 and β2 are among the most prevalent transient tertiary interactions in monomeric α-syn according to paramagnetic relaxation enhancement data.[3c] Moreover, nascent β-structure was detected in the β1–β2 region of α-syn monomers.[1c] These findings suggest that structural features of the AS69-bound β-hairpin are present within a subset of the conformational ensemble sampled by free α-syn. This is in line with the observation that the binding mechanism of intrinsically disordered proteins is not solely of the induced-fit type, but also involves conformational selection.[11]

A comparison of the structures of the α-syn:AS69 and Aβ:ZAβ$_3$ complexes confirmed that the introduced mutations did not affect the overall structure of the β-wrapin protein scaffold in the bound state. The AS69 molecule is a dimer of two identical subunits covalently linked by a disulfide bond involving the Cys-28 residues of both subunits. The folding topology of AS69 comprises two β-strands and four α-helices forming a large hydrophobic tunnel-like cavity in which the β-hairpin of α-syn is buried (Figure 1d). Most of the exchanged amino acids are in direct contact with the β-hairpin (Figure S3). For example, the Phe-31 residues of both AS69 subunits are involved in aromatic–aromatic interactions with Tyr-39 and His-50 of α-syn.

The potency of AS69 to inhibit α-syn aggregation was evaluated by a Thioflavin T fluorescence assay. In the absence of AS69, a 35 μM solution of α-syn aggregated after a lag phase of roughly 10 h. However, the aggregation of α-syn was completely inhibited in the presence of an equivalent concentration of AS69 within an 8 day experiment (Figure 2a). Addition of an equimolar amount of AS69 to α-syn aggregation reactions at different time points prevented further fibrillation of α-syn (Figure 2b). These observations indicate that sequestration of the β-hairpin renders α-syn monomers incompetent to aggregate. In addition, AS69 inhibited fibrillation of α-syn at substoichiometric concentrations. For AS69:α-syn molar ratios of 1:10, 1:100, and 1:1000, AS69 prolonged the lag time of α-syn fibrillation 9-fold, 6-fold, and 2-fold, respectively (Figure 2d and Figure S4). Size-exclusion chromatography confirmed that the binding of β-wrapin AS69 to α-syn delayed fibrillation at substoichiometric ratios and furthermore revealed that during the lag time of the aggregation experiment stable oligomeric species were not formed (Figure 2c). The substoichiometric inhibition cannot be explained by monomer sequestration. Thus, a second inhibitory mechanism must be operative which interferes with the nucleation of aggregation. This mechanism might involve the binding of AS69 to the β1–β2 region of α-syn within early aggregates. However, other α-syn epitopes may also be crucial for the substoichiometric inhibition. In this context, it is of note that α-syn oligomers exhibit antiparallel β-structure.[7] With the present data the mechanism of substoichiometric inhibition cannot be elucidated. Possible mechanisms include: 1) a small fraction of AS69-bound α-syn molecules within oligomers precludes the concerted conformational conversion to ordered amyloid fibrils; 2) AS69 binds with high affinity to fibril ends, thereby blocking fibril growth.

To evaluate the effect of AS69 on α-syn toxicity, the viability of human SH-SY5Y neuroblastoma cells was analyzed upon addition of α-syn samples aged in the absence and presence of β-wrapin AS69. For fibrillar α-syn samples, we observed a concentration-dependent decrease in the cellular viability as assessed by an MTT assay (Figure 2e). The viability of SH-SY5Y cells was rescued when α-syn samples were incubated in the presence of AS69. The viability rescue was dependent on the concentration of AS69, with complete

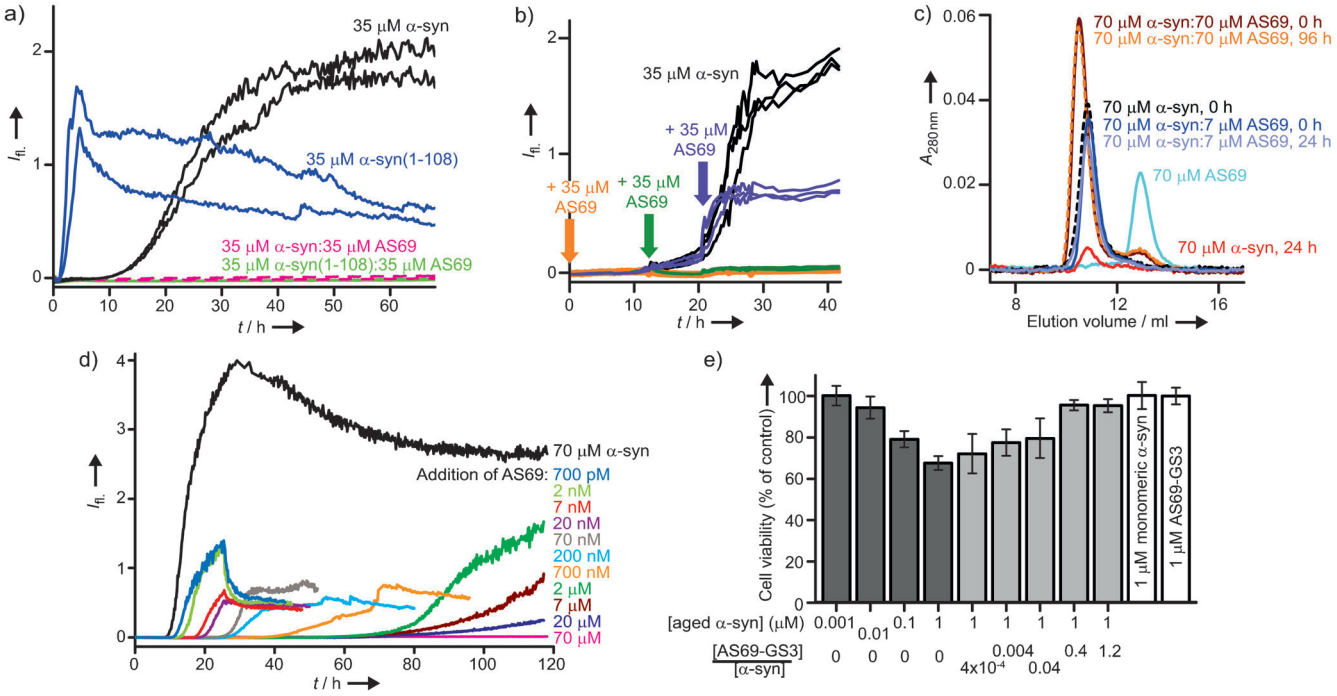

Figure 2. Inhibition of α-syn aggregation and toxicity by β-hairpin sequestration. a) Thioflavin T (ThT) time course of fibrillation of full-length α-syn and of the highly aggregation-prone, C-terminally truncated α-syn(1–108) in the absence and presence of AS69 in stoichiometric amounts (duplicate experiments). The extension of this experiment to 8 days did not lead to an increase in ThT fluorescence intensity for the samples containing AS69. b) Addition of stoichiometric amounts of AS69 at different time points, indicated by arrows, to fibrillation reactions of 35 μM α-syn monitored by ThT fluorescence (triplicate experiments). c) Size-exclusion chromatography analysis of α-syn aggregation in absence and presence of AS69. Protein samples at indicated concentrations were subjected to an aggregation assay for the indicated times and loaded on a Superdex 75 10/300 GL column (void volume ca. 8 mL). In the absence of AS69, the elution peak of α-syn was greatly reduced after 24 h incubation due to the formation of amyloid fibrils which do not enter the column bed. d) Fibrillation kinetics monitored by ThT versus the concentration of AS69. One example time trace for each AS69 concentration is displayed. The mean lag time determined from three experiments is shown in Figure S4. e) MTT assay to evaluate the toxicity of α-syn solutions aged in the absence (dark gray bars) and presence (light gray bars) of AS69-GS3 on SH-SY5Y cells. The data are the mean and standard deviation of 12 measurements performed in four wells for each of three independent experimental repeats.

rescue at equimolar ratio of AS69:α-syn and partial rescue at substoichiometric amounts.

This study shows that sequestration of a β-hairpin in the region α-syn(37–54) interferes with aggregation and toxicity. The importance of this region for the pathogenesis of Parkinson's disease is underscored by the fact that it contains most of the reported disease-related point mutations (Figure 1c). The H50Q, G51D, and A53T mutations are located within the β2 strand, whereas the E46K mutation resides in the turn connecting the β-strands. The A53T and E46K mutations promote oligomerization and fibril formation.[2a] Out of a set of charge-changing mutations, in particular those mutations immediately preceding (E35K) and succeeding (E57K) the β-hairpin region have been shown to accelerate oligomer formation.[1f] These results support a link between the β-hairpin region, oligomerization, and disease pathogenesis.

The data presented here provide detailed insight into the interaction of α-syn with the aggregation inhibitor β-wrapin AS69. The study shows that α-syn aggregation and toxicity can be controlled by sequestering a β-hairpin, offering perspectives for early interference with the pathogenesis of synucleinopathies.

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