Protein Aggregation

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Contact between the β1 and β2 Segments of α-Synuclein that Inhibits Amyloid Formation**

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Abstract: *Conversion of the intrinsically disordered protein α-synuclein (α-syn) into amyloid aggregates is a key process in Parkinson's disease. The sequence region 35–59 contains β-strand segments β1 and β2 of α-syn amyloid fibril models and most disease-related mutations. β1 and β2 frequently engage in transient interactions in monomeric α-syn. The consequences of β1–β2 contacts are evaluated by disulfide engineering, biophysical techniques, and cell viability assays. The double-cysteine mutant α-synCC, with a disulfide linking β1 and β2, is aggregation-incompetent and inhibits aggregation and toxicity of wild-type α-syn. We show that α-syn delays the aggregation of amyloid-β peptide and islet amyloid polypeptide involved in Alzheimer's disease and type 2 diabetes, an effect enhanced in the α-synCC mutant. Tertiary interactions in the β1–β2 region of α-syn interfere with the nucleation of amyloid formation, suggesting promotion of such interactions as a potential therapeutic approach.*

Protein aggregation and the toxicity of the resulting aggregates are fundamental to the pathogenesis of several human degenerative diseases. For example, aggregates consisting of α-synuclein (α-syn), the amyloid-β peptide (Aβ), or islet amyloid polypeptide (IAPP) are pathological features of Parkinson's disease (PD), Alzheimer's disease (AD), and type 2 diabetes, respectively.[1] α-Syn is a cytoplasmic protein of 140 amino acids, which predominantly exists as an intrinsically disordered protein (IDP) in the cell.[2] The conformational ensemble of the IDP contains a substantial fraction of conformers that exhibit long-range intramolecular interactions, which may promote or inhibit aggregation.[3]

According to paramagnetic relaxation-enhancement NMR spectroscopy and molecular simulations, contacts between the β1 and β2 sequence segments are among the most prevalent tertiary interactions in monomeric α-syn.[3c] The designations β1 and β2 refer to two of the approximately five β-strands of α-syn molecules in the amyloid fibril state.[4] The β1–β2 region comprises amino acids 35–59, lies outside of the hydrophobic, fibrillation-triggering NAC region,[5] and is the most N-terminal sequence region of α-syn incorporated in the fibril core of most of the fibril polymorphs described to date.[4,6] Several lines of evidence support a critical role of the β1–β2 region for α-syn aggregation and pathogenesis: First, it harbors most of the disease-related mutations, which alter the oligomerization and fibrillation propensity of α-syn.[7] Second, it is part of the core of α-syn oligomers, exhibiting particularly high resistance to H/D exchange.[8] Third, it regulates α-syn strain type and seeding efficiency.[9] Fourth, we have recently shown that sequestration of the β1–β2 region by the engineered binding protein β-wrapin AS69 potently inhibits α-syn aggregation.[10] In complex with AS69, α-syn locally adopts a β-hairpin conformation with β-strands comprising residues 37–43 and 48–54, reminiscent of the β1 and β2 strands of fibrillar α-syn.[10] The tertiary contacts between the β-strands of the AS69-bound β-hairpin agree well with the β1–β2 contact map of free monomeric α-syn.[3c]

Considering the importance of the β1–β2 region for α-syn aggregation, β1–β2 tertiary contacts might be crucial regulators of aggregation. Herein we investigate the effect of contact between β1 and β2 on amyloid formation. A stable contact was established by introduction of an intramolecular disulfide bond in the double cysteine mutant G41C/V48C, called α-synCC. The C41–C48 disulfide bond is compatible with the β-hairpin conformation of AS69-bound α-syn (Figure 1a). The two exchanges G41C and V48C are located in the β1 and β2 strand, respectively, diagonally opposite of each other. The Cα–Cα distance of G41 and V48 in the α-syn:AS69 complex is 6.1 Å, which is within the Cα–Cα distance range of cysteine disulfide bonds in X-ray structures (average: 5.6 Å).[11] The steric demands of two disulfide-bonded cysteine residues (sum of residue volumes 207 $Å^3$) are similar to those of the original glycine–valine combination (sum of residue volumes 203 $Å^3$).[12] To analyze the conformation of α-synCC, the (^1H–^{15}N) HSQC NMR spectrum of [U-^{15}N]-α-synCC was compared to that of [U-^{15}N]-wt α-syn (Figure 1b). The limited resonance dispersion of wt α-syn was retained for α-synCC, demonstrating that the engineered

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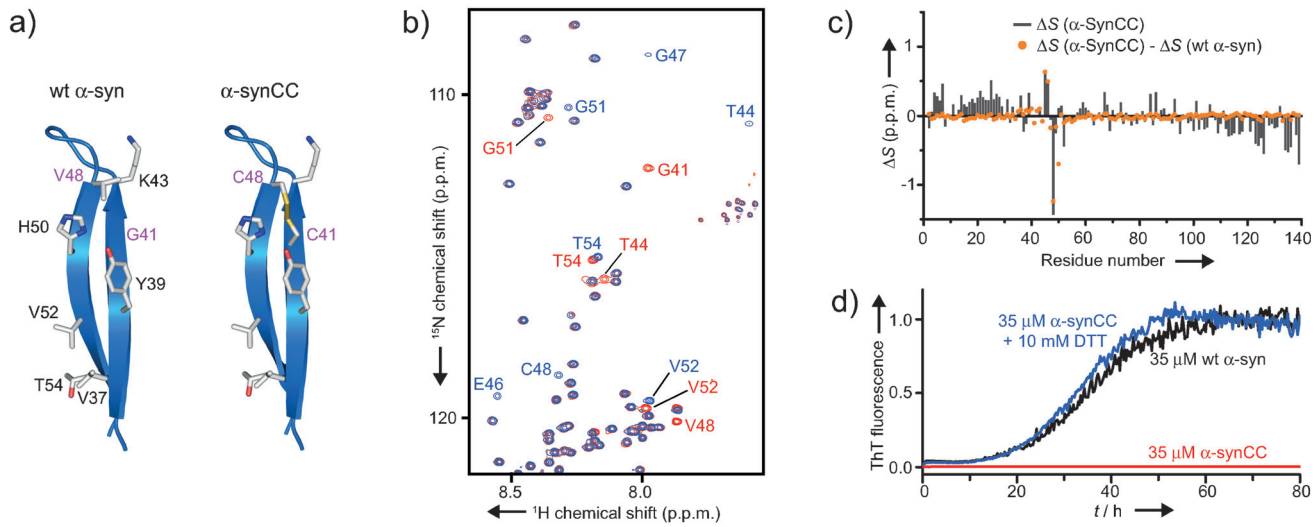

Figure 1. Protein engineering and characterization of α-synCC. a) Left: β-Hairpin conformation of the β1–β2 region of α-syn bound to β-wrapin AS69 (PDB: 4bxl). The side chains at the front face are shown as sticks. Right: Model of a β-hairpin conformation of α-synCC, in which residues 41 and 48 of wt α-syn have been exchanged to cysteines. b) Section of overlaid (^{1}H–^{15}N)-HSQC NMR spectra of [U-^{15}N]-wt α-syn (red) and [U-^{15}N]-α-synCC (blue) at 10 °C. Assignments of peaks affected by the double cysteine exchange are indicated. c) Averaged Cα and C′ secondary chemical shifts of α-synCC and difference in secondary chemical shifts between α-synCC and wt α-syn. d) ThT time course of fibrillation of oxidized and reduced α-synCC compared to wt α-syn.

disulfide does not induce folding into a stable conformation. α-synCC showed insignificant secondary chemical shifts, further indicating that it is an IDP like wt α-syn (Figure 1c). Noticeable differences in chemical shifts of α-synCC and wt α-syn were only observed for the amino acid residues adjacent to the two mutation sites; however, secondary chemical shifts did not support formation of any stable secondary structure in this region (Figure 1c). Oxidized α-synCC did not form fibrils after prolonged incubation in a fibrillation assay monitored by thioflavin T (ThT) fluorescence (Figure 1d). Breakage of the β1–β2 disulfide bond with the reducing agent dithiothreitol (DTT), however, resulted in aggregation kinetics similar to those of wt α-syn (Figure 1d). Formation of the β1–β2 disulfide bond thus renders α-synCC non-fibrillogenic, indicating that β1–β2 contacts entail conformations belonging to the pool of autoinhibitory conformations.[3a] Size-exclusion chromatography (SEC) confirmed that oxidized α-synCC did not form stable oligomers upon incubation but remained monomeric (Supporting Information, Figure S1). This is in contrast to AβCC, a double cysteine mutant of Aβ engineered following an analogous strategy (Figure S2), which was previously shown to form stable, neurotoxic oligomers.[13] This difference might be a consequence of the lower hydrophobicity of the hairpin region of α-synCC compared to the hairpin region of AβCC, with GRAVY (grand average of hydropathy)[14] values of 0.48 for α-synCC(37–54) and 1.27 for AβCC(17–36).

α-synCC inhibited the aggregation of wt α-syn both at equivalent and at substoichiometric concentrations (Figure 2a). The inhibitory effect at substoichiometric ratios indicates that α-synCC interferes with nucleation and/or elongation of wt α-syn fibrils. The inhibitory effect is a consequence of the β1–β2 disulfide linkage as it was abolished by disulfide reduction by DTT (Figure 2a). To test if α-synCC inhibits elongation of wt α-syn fibrils, seeded fibrillation reactions of wt α-syn were performed in the absence and presence of α-synCC and monitored either in a fluorescence microplate reader (Figure S3) or by total internal reflection fluorescence microscopy (TIRFM) (Figure 2d). Ultrasonicated wt α-syn fibrils were used as seeds. Addition of α-synCC entailed a concentration-dependent inhibition of seeded wt α-syn aggregation (Figure S3). Wt α-syn fibril seeds were imaged by TIRFM as particles with several fibrillation sites, resulting in a fibril network after quiescent incubation with wt α-syn monomers (Figure 2d).[15] In contrast, fibril networks were not formed when α-synCC was incubated with wt α-syn fibril seeds, in agreement with the finding that α-synCC is non-fibrillogenic (Figure 2d). Incubation of wt α-syn monomers with wt α-syn fibril seeds did not lead to the formation of fibril networks when α-synCC was present (Figure 2d). The inhibitory effect of α-synCC on wt α-syn fibril elongation was dependent on the β1–β2 disulfide linkage as it was abrogated by disulfide reduction by DTT (Figure 2d). Thus, the seeded fibrillation experiments indicate that α-synCC interacts with fibril ends. Wt α-syn samples that were aged under aggregation-promoting conditions reduced the viability of human SH-SY5Y neuroblastoma cells, as assessed by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 2e). However, when ageing of wt α-syn was performed in the presence of α-synCC, the cell viability was rescued.

Different protein aggregation disorders, associated with amyloidogenic proteins of non-homologous sequences, frequently overlap clinically and pathologically, suggesting a mutual interference of the aggregation reactions of the involved proteins.[9,16] While Aβ plaques are often found in PD patients, α-syn Lewy bodies are found in most of the AD cases.[17] Similarly, IAPP oligomers and plaques were identified in the brains of diabetic AD patients.[18] We investigated potential heterotypic interactions of α-synCC with other

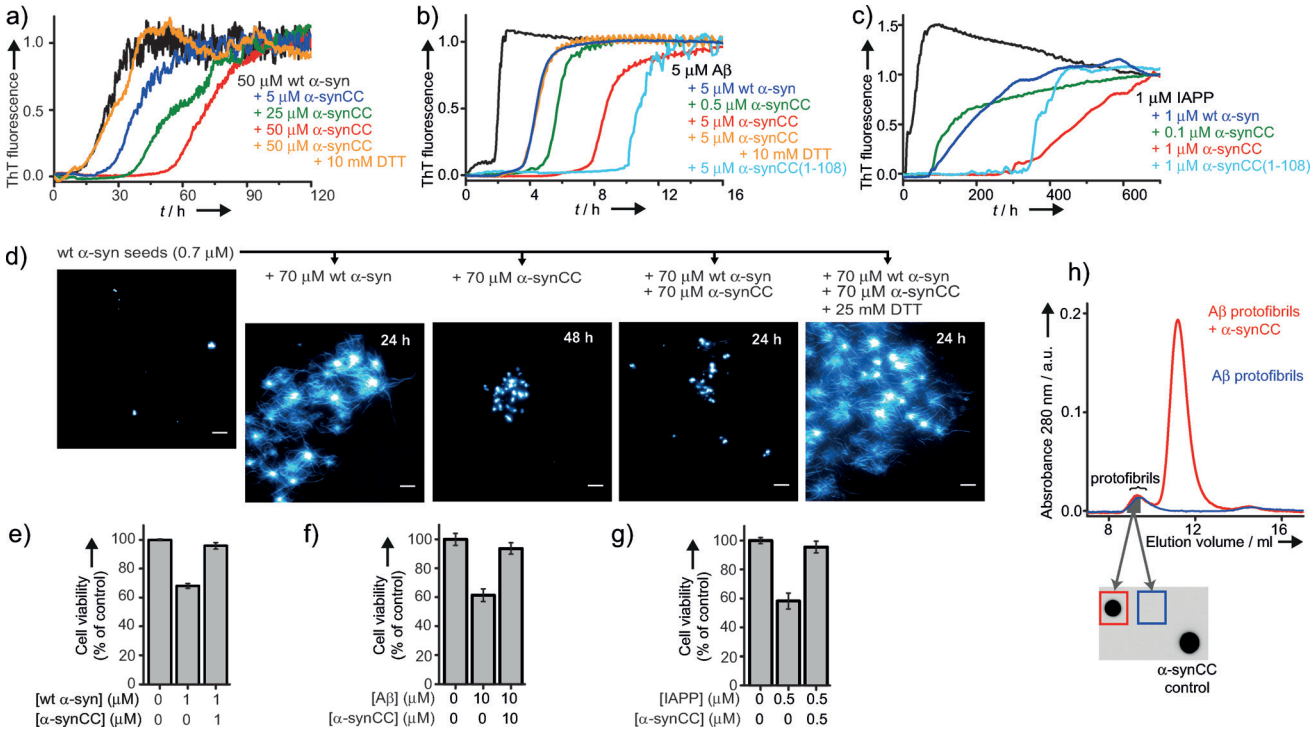

Figure 2. α-SynCC inhibits aggregation and toxicity of wt α-syn, Aβ and IAPP. a)–c) ThT time course of fibrillation of wt α-syn (a), Aβ (b), and IAPP (c) in the absence and presence of α-synCC. d) TIRF microscopy of fibrillation of α-synCC and wt α-syn in the presence of wt α-syn fibril seeds under quiescent condition. Scale bar: 5 μm. e)–g) MTT assays to evaluate the toxicity of wt α-syn (e), Aβ (f), and IAPP (g) aged in the absence and presence of α-synCC to SH-SY5Y neuroblastoma cells. The data are representative of experiments carried out in triplicate (mean ± s.d.), expressed as percentage relative to the untreated cells (control). h) α-SynCC interacts with Aβ protofibrils. SEC chromatograms of purified Aβ protofibril samples re-injected onto the column after 10 min incubation at room temperature in the absence (blue) or presence (red) of a twofold excess (calculated in monomer units) of α-synCC. Aβ protofibrils elute close to the void volume (ca. 8.5 mL), while the elution peak at circa 11.5 mL corresponds to α-synCC. Dot blot analysis of the protofibrils fractions using the anti-α-syn antibody 211 is shown below the chromatogram. Fresh α-synCC served as positive control. Aβ(1-40) (b,f) or Aβ(1-42) (h) with an N-terminal methionine[19] was used.

amyloidogenic proteins by testing its effects on the fibrillation of Aβ and IAPP. The effects of α-synCC were compared to those exerted by wt α-syn. Wt α-syn and α-synCC both inhibited fibrillation of Aβ and IAPP, with more potent inhibition exhibited by α-synCC (Figure 2b,c). The lag-time of fibrillation of 5 μM Aβ increased 2- and 4-fold upon addition of an equimolar amount of wt α-syn and α-synCC, respectively (Figure 2b). Reduction of the disulfide bond in α-synCC decreased the inhibitory effect of α-synCC to the level of wt α-syn, demonstrating that the β1–β2 disulfide linkage is responsible for the higher inhibitory potential of α-synCC (Figure 2b).The lag-time of fibrillation of a 1 μM solution of IAPP increased by 10- and 40-fold in the presence of an equimolar amount of wt α-syn and α-synCC, respectively (Figure 2c). A 1:10 ratio of α-synCC:Aβ or α-synCC:IAPP was sufficient to achieve a significant prolongation of the fibrillation lag time, supporting an impact of α-synCC on the nucleation and/or elongation of Aβ and IAPP fibrils (Figure 2b,c). A chaperone-like activity of α-syn was observed before in thermally induced and chemically induced protein aggregation assays.[20] The acidic C-terminal tail was critical for this activity by serving as a solubilizing domain. A C-terminally truncated variant of α-synCC, α-synCC(1-108), however, caused similar increases in the fibrillation lag times of Aβ and IAPP as full-length α-synCC (Figure 2b,c). β1–β2-mediated aggregation inhibition does therefore not depend on the acidic C-terminal of α-syn and must act through a different mechanism than the previously reported chaperone-like function. To complement the ThT fluorescence data, Aβ aggregation was analyzed by SEC and transmission electron microscopy (TEM), demonstrating that α-synCC inhibited the formation of oligomers and fibrils (Figure S4). Ageing of solutions of Aβ and IAPP under aggregation-promoting conditions resulted in cytotoxicity in an MTT assay on SH-SY5Y neuroblastoma cells (Figure 2f,g). However, when the cells were treated with Aβ and IAPP samples aged in the presence of α-synCC they displayed a viability similar to that of untreated cells (Figure 2f,g).

To identify the molecular species interacting with α-synCC, binding of α-synCC to monomers of wt α-syn, Aβ, or IAPP, and to Aβ protofibrils, metastable neurotoxic oligomers, was tested. Biotinylated monomers of the target proteins were coated on streptavidin SA sensor chips. No response indicative of binding was detected for any of the three target proteins when α-synCC was passed as analyte over the sensor surfaces (data not shown). Freshly prepared Aβ protofibrils were incubated for 10 min with or without α-synCC. The incubated samples were analyzed by reinjection onto the SEC column, isolation of the protofibril fraction, and dot blot using the anti-α-syn antibody 211 (Figure 2h). The Aβ protofibrils sample pre-incubated with α-synCC showed immunoreactivity, indicating binding of α-synCC to Aβ protofibrils.

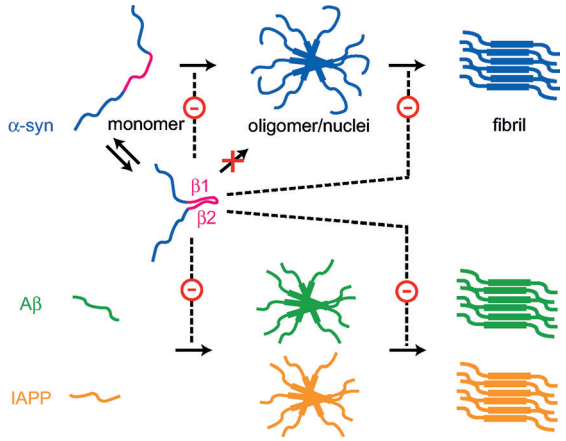

Figure 3. Representation of the inhibitory effect of tertiary contacts in the β1–β2 region of α-syn on the aggregation of amyloidogenic IDPs. Conformations of α-syn featuring β1–β2 contacts are incompetent to form oligomers and fibrils. Furthermore, they inhibit fibril nucleation and elongation of α-syn, Aβ, and IAPP.

The present study shows that α-synCC with established disulfide bond actively interferes with the aggregation of amyloidogenic proteins (Figure 3). How does the β1–β2 contact lead to inhibition of protein aggregation? Considering the amino acid sequence, formation of β1–β2 contacts is likely accompanied by population of conformers with clusters of hydrophobic and aromatic amino acids, including, for example, Tyr-39 and His-50. These conformers might not be compatible with the fibrillation reaction, but prone to interact with other species on the aggregation pathway that also exhibit hydrophobic patches, such as oligomeric nuclei and fibril ends, eventually precluding further assembly of these species.

This work supports the view that the β1–β2 region is an important regulatory element of α-syn aggregation. Tertiary interactions between β1 and β2 interfere with aggregation and steer the capacity of α-syn to engage in heterointeractions with other amyloidogenic IDPs. In the search for inhibitors of amyloid formation,[21] promotion of β1–β2 contacts therefore constitutes a potential approach.

Keywords: aggregation · intrinsically disordered proteins · protein engineering · protein folding · protein–protein interactions

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