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β-Turn exchanges in the α-synuclein segment 44-TKEG-47 reveal high sequence fidelity requirements of amyloid fibril elongation

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ABSTRACT

The folding of turns and β-hairpins has been implicated in amyloid formation, with diverse potential consequences such as promotion or inhibition of fibril nucleation, fibril elongation, or off-pathway oligomer formation. In the Parkinson’s disease-associated protein α-synuclein (αS), a β-hairpin comprised of residues 36–56 was detected in complex with an engineered binding protein, with a turn formed by the αS sequence segment 44-TKEG-47. Molecular dynamics simulations revealed extensive populations of transient β-hairpin conformations in this region in free, monomeric αS. Here, we investigated potential effects of turn formation on αS fibril formation by studying the aggregation kinetics of an extensive set of αS variants with between two and four amino acid exchanges in the 44-TKEG-47 segment. The exchanges were chosen to specifically promote formation of β1-, β1'-, or β2'-turns. All variants assembled into amyloid fibrils, with increased β1'- or β2'-turn propensity associated with faster aggregation and increased β1-turn propensity with slower aggregation compared to wild-type (WT) αS. Atomic force microscopy demonstrated that β-turn exchanges altered fibril morphology. In cross-elongation experiments, the turn variants showed a low ability to elongate WT fibril seeds, and, vice versa, WT monomer did not efficiently elongate turn variant fibril seeds. This demonstrates that sequence identity in the turn region is crucial for efficient αS fibril elongation. Elongation experiments of WT fibril seeds in the presence of both WT and turn variant monomers suggest that the turn variants can bind and block WT fibril ends to different degrees, but cannot efficiently convert into the WT fibril structure. Our results indicate that modifications in the 44-TKEG-47 segment strongly affect amyloid assembly by driving αS into alternative fibril morphologies, whose elongation requires high sequence fidelity.

1. Introduction

The formation of cross-β structured amyloid fibrils is an important factor in many, especially neurodegenerative, diseases, but also results in functional assemblies with diverse physiological roles [1,2]. Disease-related amyloid fibrils are constructed from in-register, parallel β-sheets. The individual protein molecules contribute a few β-strands to the fibril core, which are connected by kinks, turns, loops, or flexible segments [3].

A protein structural motif related to the fibril core architecture is the β-hairpin, in which two β-strands are connected by a turn [4,5]. In contrast to typical amyloid fibril cores, β-hairpins contain an anti-parallel β-sheet with intramolecular backbone hydrogen bonding. However, the amino acid sequence requirements for amyloid and for β-hairpin formation, e.g., segments with high β-sheet propensity separated by segments with turn propensity, are similar. Consequently, transient formation of β-hairpins in amyloidogenic protein monomers has frequently been detected in molecular dynamics (MD) simulations and also observed experimentally [6–19]. The folding of turns and β-hairpins may have diverse consequences, including promotion or inhibition of fibril nucleation [6,7,10,16,17,19–21], fibril elongation [19,22], or off-pathway oligomer formation [21,23–31]. Turns and β-hairpins can be targeted by inhibitors of amyloid fibril and oligomer formation [25,31–35], or can be designed to themselves act as aggregation modulators or inhibitors [22,36–43].

The 140 amino acid protein α-synuclein (αS) is intrinsically
disordered in its monomeric state [44], but assembles into cross-
β-structured amyloid fibrils that are associated with several neurode-
genegrative disorders (synucleinopathies), including Parkinson’s disease,
dementia with Lewy bodies, and multiple system atrophy [45]. Similar
to other disease-related amyloids, αS fibrils are polymorphic, and it is
hypothesized that specific αS fibril structures are linked to specific
synucleinopathies [46]. We have previously detected a β-hairpin in αS
that is established upon binding of monomeric αS to the engineered
aggregation inhibitor β-wrapin AS69. Binding leads to local folding of
the β-hairpin motif comprising residues 36–56, in which two β-strands
are connected by a turn around the αS sequence segment 44-TKEG-47
(Fig. 1, top left) [33]. MD simulations revealed extensive populations
of transient β-hairpin conformations in this region in free, monomeric
αS, which is also supported by experimental restraints [15,47]. The
β-hairpin region is critically involved in αS assembly: Binding of the
β-hairpin with β-wrapin AS69 results in aggregation inhibition [33,35];
stabilization of a hairpin by introduction of a disulfide yields an αS
variant that inhibits αS fibril elongation [22,48]; deletions in this region

![Fig. 1. The β-hairpin region comprising αS residues 36–56 in the context of the αS complex with β-wrapin AS69 (top left) and of different αS fibril polymorphs (pdb entries indicated in brackets). When bound to AS69 (grey ribbon structure) αS locally adopts a β-hairpin conformation with a turn around the 44-TKEG-47 segment (yellow) [33]. The turn segment forms kinks in αS fibrils [46,50–56]. Residues Gly-41 and His-50, which are in direct contact in the AS69-stabilized β-hairpin, are displayed in sphere representation for orientation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
strongly affect the aggregation properties [49]. In line with these observations, the αS region 36–56 is, at least partially, integrated into the core of all αS fibril polymorphs reported to date (Fig. 1) [46,50–56].

To further elucidate structural factors that are involved in αS fibril formation, we tested here if and how changes in turn propensity of the αS sequence segment 44-TKEG-47, which connects the two β-strands of the β-hairpin motif, affect amyloid formation. We generated a set of αS variants carrying between two and four amino acid exchanges in 44-TKEG-47, and characterized de novo amyloid formation as well as cross-elongation with wild-type (WT) αS. We find that specific β-turn propensities are associated with either faster or slower fibril formation. In cross-elongation experiments, turn mutant monomers exhibit a low ability to elongate WT fibril seeds and WT monomers exhibit a low ability to elongate turn mutant seeds. The results indicate that alterations in the turn region guide αS into alternative fibril polymorphs which require high molecular conformity in the turn region for efficient elongation.

2. Materials and methods

2.1. Design and cloning of αS variants

Sequences with high turn propensity were selected using the software BEAHIPRED [4], which employs the positional potentials of residues for formation of the different turn types determined by Hutchinson and Thornton [57]. Turn variants were cloned by In-Fusion site-directed mutagenesis (Takara Bio) of codon-optimized WT αS gene in pT7-7 vector. In Fusion-site-directed mutagenesis allows for the exchange of the entire tetrapeptide turn with a single pair of mutagenesis primers. For generation of the random double mutants an error-prone polymerase chain reaction adapted from McCullum et al. [58] was used on a vector carrying the αS gene to generate point mutations in the αS-coding sequence. The αS gene was cloned into the pT7-7 vector using NdeI and HindIII restriction sites.

2.2. Purification of proteins

Expression and purification of WT αS and variants was carried out as described previously, yielding N-terminally acetylated proteins [35].

2.3. De novo aggregation assay

All variants were prepared at a protein concentration of 25 μM in Reaction Buffer (RB) containing 20 mM MOPS pH 7.4, 50 mM NaCl, 0.04% NaN₃, 20 μM Thioflavin T (UltraPure Grade, Anaspec Inc.). Independent triplicates of each variant were prepared along with three samples containing only RB. A single glass bead with diameter of 2.85–3.45 mm (Carl Roth, Art. Nr. AS57.1) was placed in the relevant wells of 96-well half area assay plates, non-binding surface, black with clear bottom, polystyrene (REF 3881, Corning). The outermost wells were never used for samples and water was added to the empty wells in the immediate vicinity of samples, in order to prevent sample evaporation. The plate was sealed with sealing tape, clear polyelefin (232701) from Thermo Scientific. Assays were performed on a FLUOstar Omega (BMG Labtech) using 12-point orbital averaging with at diameter of 3 mm using bottom optics and a Thioflavin T filter with excitation at 448 nm (10 nm bandwidth) and emission at 452 nm (10 nm bandwidth) with a gain of 800. Measurements were performed every 6th minute for 100 h with a settling time of 1.0 s between measuring each well. The instrument had been preheated to 37 °C and shaking was done in orbital mode at 300 RPM. The reported lag-time of each sample was obtained by locating the first point where a five-point sliding average exceeded the maximum value of the samples only containing RB by a factor of 2.5.

2.4. Seed preparation

Seeds were prepared similar to what was described in Agerschou et al. [35]. Briefly, 1 ml 25 μM αS variant in RB was placed at 37 °C with shaking at 1000 RPM for at least four days (seven days for the β-turn variants as these aggregated slower) to induce and complete fibril formation. Immediately before being used, the fibrils were sonicated twice using a UP200St sonicator with a VialTweeter (Hielscher Ultrasound Technology) for 20 s at 70% maximum amplitude.

2.5. Elongation assays

Fibril elongation was measured in seeded experiments as described previously [35]. Briefly, instrument settings, plate type, and preparation were similar to what is described above for the de novo aggregation assay with a few important differences. These differences include lack of shaking and the absence of glass beads. Furthermore, due to the faster kinetics, the first 100 measurements were done every 100 s and subsequent measurements every 360 s. Independent quadruplicates or triplicates of each sample were prepared at room temperature without seeds to a volume of 90 μl and placed into the wells of the plate. The plate was then sealed and incubated at 37 °C inside the plate reader for 10–20 min for temperature equilibration. The seal was removed and seeds injected by manually pipetting 10 μl seed solution (see seed preparation above) into the relevant wells. The final seed concentration was 2.5 μM in monomer equivalents. The plate was resealed and placed in the plate reader again for measurements. The initial rates were obtained by fitting linear functions to the first 25 data points, except β-turn mutant seeded experiments where 75 data points were used as these showed very slow elongation even in the case of self-seeding. The analysis of the initial slopes, rather than exponential fits to the full time course of the strongly seeded aggregation reactions [59] is particularly suitable under conditions where some variants display elongation rates that are too slow to reach equilibrium within the time scale of the experiment. However, this type of analysis either requires normalized fluorescence intensity values, or else relies on the final fluorescence values of the reactions to be very similar. The latter is the case here, as is discussed in the Results and Discussion section. The detection limits for β-turn mutant elongation was defined similar to how the lag-time was defined. In particular, samples that failed to reach 2.5 times the highest measured value in the absence of seeds were defined to be below detection limit. For the random mutants, a ratio of initial rates of 0.002 or smaller was set to be below the detection level.

2.6. Atomic force microscopy

Endpoint samples of several elongation assays were prepared for atomic force microscopy (AFM) by transferring the content of the wells into clean 1.5 ml Eppendorf tubes. From these tubes, 10 μl (25 μM in monomer equivalents in reaction buffer) was placed on freshly cleaved mica and incubated for 10–20 min. Samples were then washed by careful addition of 100 μl dH₂O that was immediately removed, a procedure that was repeated five times. Samples were then dried under a gentle stream of nitrogen. AFM was performed on a Bruker Multimode 8 using ScanAsyst-Air cantilevers using the ScanAsyst PeakForce tapping mode in air. flattening of the data was done using the manufacturer’s software with defaults settings. Extraction of profiles was carried out along the long axis of fibrils that were sufficiently long and straight enough to yield at least 0.3 μm profiles. Extraction was done using the software Gwyddion.

2.7. Quantification of fibril yield and remaining monomer

Samples of 80 μl were spun at 16100 g for 30 min using a tabletop centrifuge. 70 μl was removed without disturbing the pellet and the remaining 10 μl was mixed with 70 μl 9 M urea to dissolve the pellet. The
pellet samples were then diluted twofold by additional 9 M urea. 10 µl sample (diluted pellet or undiluted supernatant) was mixed with reducing SDS loading buffer (2 mM dithiothreitol, 2% SDS, 8% glycerol, 0.05% bromophenol blue, 0.05 M Tris:Cl pH 6.8), and incubated for 1 h at room temperature. Samples were loaded onto a Tris/glycine/SDS buffer (Bio-Rad) 15% polyacrylamide gel made from Rothphorese 30 (37.5:1) (Carl Roth). Gel electrophoresis was performed on a Mini-PROTEAN Tetra System (Bio-Rad), and the gels were stained using SERVA Blue R (equivalent to Coomassie brilliant blue R-250) (SERVA). Transilluminated images of the gels were recorded on a ChemiDoc MP* (Bio-Rad), using the standard coomassie filter.

3. Results and discussion

3.1. Design of turn variants

To investigate the impact of turn formation on αS fibril formation, we generated a set of αS variants with increased propensity for turn formation by exchanging two to four amino acid residues in the 44-TKEG-47 segment of WT αS (Table 1). The mutations were chosen to specifically promote formation of β1-, β1’, or β2-turns, which are the β-turn types most frequently found in β-hairpins [4] (Fig. 2, Table 2). The different β-turn types support β-hairpin conformations with different registries: β1'- and β2'-turns support 2:2 β-hairpins, whereas β1-turns are frequently associated with 3:5 and 4:4 β-hairpins (X:Y nomenclature: X is the number of residues in the turn region; Y = X if the CO and NH groups of the two residues that precede and follow the turn form two hydrogen bonds; Y = X + 2 if these residues form only one hydrogen bond) [4]. Sequences with high turn propensity were selected using the software BEHAIRPRED [4], which employs the positional potentials of residues for formation of the different turn types determined by Hutchinson and Thornton [57]. Due to a lack of experimental methods for direct detection of transient β-hairpin formation in monomeric IDPs or in intermediates on the fibril formation pathway, the β-turn propensities of the αS variants studied here were purely inferred from these positional potentials derived from deposited protein structures. Care was taken that not all exchanges towards propensity for a certain β-turn type simultaneously altered charge or hydrophobicity in a common direction (Table 1). We also created two variants (labeled S-β1' and S-β2') whose exchanges in the 44-TKEG-47 segment resulted in maximal turn propensity for either a β1'- or a β2'-turn shifted by one residue position, i.e., in the 45-KEGV-48 segment. Competition by such alternative, shifted β-hairpin conformations has been observed in MD simulations and experimentally [5,61].

3.2. De novo fibril formation kinetics and fibril morphology are affected by β-turn exchanges

We first performed de novo aggregation assays by incubating under regular agitation 25 µM WT αS or turn variants in microwell plates equipped with one glass bead per well. All experiments in this study were performed with N-terminally acetylated αS. For all turn variants, Thioflavin T (ThT) fluorescence followed a sigmoidal time course typical for amyloid fibril formation (Fig. 3 and S1). The aggregation lag-times differed between ~3 and ~75 h (Fig. 3D). The lag-time correlated with the β-turn type intended to be promoted by the chosen exchanges. Exchanges towards a β1-turn slowed down aggregation compared to WT (Fig. 3A,D), whereas exchanges towards β1' or β2-turns accelerated aggregation (Fig. 3B-D). β1’- and β2'-turns support a common β-hairpin registry, i.e., 2:2 β-hairpins, whereas the β1-turn conformation is incompatible with 2:2 β-hairpins and instead associated with 3:5 and 4:4 β-hairpins [4]. This suggests that the differences in de novo aggregation

![Fig. 2. The β-turn types most common in β-hairpins. Backbone structures of examples of the β-turn classes β1 (transcription factor Mbp1, pdb: 1BM8, residues 20–23), β1’ (CdnL protein from Thermus thermophilus, pdb: 2LQK, residues 31–34), and β2’ (protein TA0095 from Thermoplasma acidophilum, pdb: 2JOI, residues 53–56) [1].](image)

Table 1

| αS variant | Sequence as 44–47 (as 45–48) | β1 potential | β1’ potential | β2 potential | Charge | Hydrophobicity |
|------------|-------------------------------|--------------|---------------|--------------|--------|---------------|
| WT         | TKEG (KEGV)                   | 1.69 (1.04)  | 0.28 (1.37)   | 0.44 (0.57)  | 0      | -8.5          |
| β1-TPDG    | TPDG                         | 2.73         | 0.23          | 0.50         | -1     | -6.2          |
| β1-NPDG    | NPDG                         | 3.05         | 0.15          | 0.51         | -1     | -9.0          |
| β1-SPOT    | SPOT                         | 2.48         | 0.27          | 0.62         | -1     | -6.6          |
| β1-SPNG    | SPNG                         | 2.77         | 0.25          | 0.63         | 0      | -6.3          |
| β1’-VNGK   | VNGK                         | 0.80         | 2.18          | 0.79         | +1     | -3.6          |
| β1’-YNGQ   | YNGQ                         | 0.72         | 2.08          | 0.83         | 0      | -8.7          |
| β1’-TNGG   | TNGG                         | 1.37         | 1.77          | 0.76         | 0      | -5.0          |
| β1’-YNGK   | YNGK                         | 0.87         | 2.20          | 0.88         | +1     | -9.1          |
| S-β1’-TNG  | TNG (TNGV)                   | 1.82 (0.78)  | 0.30 (2.05)   | 0.56 (0.82)  | 0      | -5.9          |
| β2-2GNT    | 2GNT                         | 1.52         | 0.51          | 1.68         | 0      | -5.4          |
| β2-2TNG    | TNG (TNGV)                   | 1.76         | 0.47          | 1.56         | 0      | -5.0          |
| β2-2GDS    | GDS                          | 1.57         | 0.48          | 1.51         | -1     | -5.5          |
| S-β2’-TNG  | TNG (TNGV)                   | 0.98 (1.17)  | 1.37 (0.74)   | 0.70 (1.62)  | 0      | -5.9          |

* Residues differing from the WT sequence are underlined.
† β-Turn potentials were calculated with BEHAIRPRED [4], which employs the positional potentials of Hutchinson and Thornton [57]. For every variant, the β-turn type with the highest potential is underlined.
‡ Total side chain charge at neutral pH.
§ Hydrophobicity values represent the sum of the amino acid hydrophathy scores by Kyte and Doolittle [60].
kinetics are a consequence of β-hairpin formation around the 44–47 turn, with 2:2 β-hairpin formation promoting and/or 3:5 or 4:4 β-hairpin formation disfavoring fibril formation. Interestingly, the β1′- and S-β2′-variants, which promote a shifted β-sheet registry, show similar kinetics as the other β1- and β2-variants, suggesting that the promotion of these turn types is more important than formation of a specific β-registry. The data does not disclose which steps of fibril formation, particularly all β1-variants studied here.

Similar to the other β1-variants, all β2-variants have a K45N mutation, all β2′-variants a K45G mutation, and both S-β variants a K45Y mutation. Based on our kinetic data alone, we cannot exclude that these systematic mutations in each β-hairpin class exert their effects on the kinetics of fibril growth through effects in addition to their predicted change in β-hairpin propensity. Nevertheless, the data indicate that the 44-TKEG-47 segment has a strong impact on de novo αS fibril formation and that the formation of specific turns and transient β-hairpins may be implicated.

To investigate if the different β-turn exchanges result in different fibril morphologies, we imaged selected mutants using atomic force microscopy (AFM). Fibril samples were prepared by adding monomers under quiescent conditions to de novo first generation fibril seeds formed under mechanical agitation. The second generation fibrils showed an increased length, which facilitated analysis. Intriguingly, WT, β1-NPDG, β1′-VNGK, β2′-SGNT resulted in distinct fibril morphologies (Fig. 4A-D and Table 3). Particularly striking was the difference in appearance of WT and β2′-SGNT fibrils. WT formed uniform and twisted fibrils with a periodicity of ~100 nm (Fig. 4A and Table 3), as has been observed previously [62,63]. In contrast, β2′-SGNT fibrils were sheet-like with variable width and no apparent twist (Fig. 4D).

The final ThT fluorescence intensities of the de novo aggregation reactions differed widely between WT and the various turn mutants (Fig. 3A-C, Fig. S1). In order to test whether this difference in ThT quantum yield also reflects the differences in fibril structure and morphology, or whether it simply reflects differences in the degree of conversion of monomers into fibrils, we monitored the relative amounts of αS in the fibril pellet and in the soluble fraction after centrifugation of samples at the end of the aggregation assay (Fig. 5). For WT, β1′-VNGK, and β2′-SGNT, the largest fraction of the protein was found in the pellet, suggesting that observed differences in ThT fluorescence reflect fibril polymorphism. In contrast, β1-NPDG remained mostly in the soluble fraction, indicative of a lower thermodynamic stability of β1-NPDG fibrils.

3.3. Fibril cross-elongation is impeded by β-turn exchanges

To test if the turn variants can elongate αS WT fibrils, and if αS WT elongates turn variant fibrils, seeded fibril growth assays were performed starting from 25 μM of the monomeric protein in the presence of 10% (in monomer units) of the heterologous seed. Overall, a poor ability to cross-elongate became apparent in these experiments, both for the elongation of WT fibril seeds with turn variant monomers (Fig. 6A-C and S2–S4) and for the elongation of turn variant fibril seeds with WT monomers (Fig. 6B,D and S2–S4). In approximately half of the cases elongation was so slow that it could not be detected (horizontal lines in Fig. 6C-E indicate the detection limit). In the cases where elongation could be detected, the ThT fluorescence of the cross-seeding reactions typically approached the final ThT fluorescence intensities of the self-seeding reactions (Fig. 6A and S2–S4). For example, β2′-SGNT variant monomer elongating WT fibril seeds approached the same final ThT fluorescence as elongation of WT fibril seeds by WT monomers (Fig. 6A),
Fig. 4. Imaging of fibril morphologies by AFM. (A)-(D) Images obtained after self-seeding of (A) WT, (B) β1-NPDG, (C) β1'-VNGK, (D) β2'-SGNT. (E) Cross-seeding of WT seeds by β2'-SGNT monomers. Left and middle panels show two representative images per sample. Right panels show magnifications of the areas indicated by red boxes in the middle panels. The height is indicated by colour with the scale shown to the right of each image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
whereas elongation of β2'-SGNT variant fibril seeds by β2'-SGNT monomer resulted in substantially lower fluorescence intensity (Fig. 5).

**Table 3**

| Monomer type | Seed type | Height (nm) | Periodicity (nm) | Fibrils analyzed |
|--------------|-----------|-------------|------------------|------------------|
| WT           | WT        | 6.8 ± 0.4   | No twist         | 10               |
| β1-NPDG      | β1-NPDG   | 6.0 ± 0.6   | No twist         | 10               |
| β1'-VNGK     | β1'-VNGK  | 6.2 ± 0.8   | No twist         | 10               |
| β2'-SGNT     | β2'-SGNT  | 6.1 ± 0.8   | No twist         | 10               |
| β2'-WT       | WT        | 6.4 ± 0.8   | 106 ± 32 nm     | 10               |

**Fig. 5.** Quantification of fibrillar αS by SDS-PAGE. End-stage aggregation samples (25 μM αS) were fractionated by centrifugation and pellets (P) and supernatants (S) were analyzed. Pellet samples were diluted 2-fold compared to supernatant samples. Lane 11, molecular weight marker. Lane 12–15, concentration references of 0.5 μM, 2 μM, 8 μM, 20 μM αS monomer.

**Table 3** Fibril characteristics of selected turn variants determined by AFM.

3.4. β-Turn variants moderately inhibit WT αS fibril elongation

We have previously reported that stabilization of a hairpin in the αS region comprising residues 36–56 by introduction of disulfides through double exchanges to cysteine (CC48: G41C/V48C; CC50: G41C/H50C) yielded αS variants that inhibit elongation of WT fibrils by WT monomers [22,48]. These αS variants block the growing WT fibril ends, with the inhibitory potency dependent on the precise positions of the disulfide [22,48]. In the inhibitory variants CC48 and CC50, the positions of the disulfides are compatible with the β-sheet registry of αS bound to β-wrapping A669 (Fig. 1, top left). We tested if the β-turn variants exhibit a similar inhibitory effect on WT αS fibril elongation by WT monomers. All seven β-turn variants that did not themselves elongate WT fibrils to any detectable level were included in these experiments. At stoichiometric amounts of the turn variants and WT monomers, the rate of WT fibril elongation was reduced to 20–80% of its value in the absence of turn variant (Fig. 7 and S6). This effect is only moderate compared to that of CC48, for which the rate of WT fibril elongation drops to 50% at a ratio of 1:15 CC48:wt, but approaches that of CC50, for which this occurs at a ratio of 1:2 CC50:wt [22]. Thus, the inhibitory effect observed for αS variants with disulfide-stabilized hairpins seems to be recovered in the 44–47 turn mutants. Of note, we did not observe a correlation of the inhibitory activity with the promotion of a specific β-turn type.

It is interesting to reflect on the origin of the inhibitory effect on fibril elongation of the mutants that are themselves apparently unable to incorporate correctly into the WT seed fibrils. It has been shown that most amyloid fibrils, in particular also those of αS, grow through monomer addition [59,72]. Therefore, inhibition of elongation can stem either from interaction with the monomeric αS or with the fibril ends. In the present case, the former scenario is not impossible, but relatively unlikely, as αS is predominantly monomeric in solution [44] and the turn mutations would have to induce a significant affinity for WT monomer. On the other hand, inhibition by temporarily blocking the exchanges at critical positions in the fibril structure can suffice to strongly affect the fibril elongation rate, as has been observed for the SH3 domain of bovine phosphatidylinositol 3’-kinase: Both the reduction of hydrophobicity in the fibril core by isoleucine-to-alanine exchanges and the creation of charges in the hydrophobic core resulted in a dramatic decrease of elongation rate [70,71]. In order to benchmark the high sequence identity requirements for (cross-)elongation detected here in the 44–47 turn region with that of other αS sequence regions, we investigated a set of αS variants with random amino acid changes, that was obtained by error-prone PCR. We focused on αS variants with two amino acid exchanges, of which at least one is located in the sequence region 30–100, i.e., the core of the majority of fibril polymorphs (Fig. 1). The comparison of cross-elongation efficiencies of these mutants with those of the turn variants that also contain only two amino acid exchanges is shown in Fig. 6E. Five of the nine random variants elongated WT fibril seeds rather efficiently (Fig. 6E and S5). In contrast, no cross-elongation with WT fibril seeds was found for four other random variants, just as for the two residue exchanges towards β1-, β1'-, or β2'-turns in the 44–47 segment. Of the four random variants showing no cross-elongation with WT fibril seeds, one contained a T44P exchange in the turn region, and another one a charge in residue K80 that makes crucial long-range contacts in αS polymorphs (Fig. 1). These results demonstrate that the high sequence fidelity requirements of fibril elongation found for the 44–47 turn region also apply to certain other sequence positions in αS. However, despite the small sample size, it appears that the turn region is particularly vulnerable to sequence changes, compared to other regions of the protein.

In summary, the cross-elongation experiments indicate that alterations in the 44–47 turn segment have a pronounced effect on αS fibril formation, as they guide αS into different fibril morphologies, which require sequence identity in that particular region for efficient elongation.
fibril ends is a plausible scenario [22]. Before incorporating into a fibril end, monomeric \( \alpha \)S will loosely associate with the fibril end, as revealed by the overall sub-linear, Michaelis-Menten-like dependence of the elongation rate on the monomer concentration [59]. This initial, loose contact is likely to have less stringent sequence requirements as the adoption of the template structure of the seed fibril and therefore the fibril end can be expected to be occupied/blocked for some fraction of the time in the presence of the turn variants.

4. Conclusion

The data presented on a large set of \( \alpha \)S variants with amino acid exchanges in the 44-TKEG-47 sequence segment indicates that this region, which exhibits a propensity for turn formation, has a strong impact on \( \alpha \)S fibrillation. The AFM data, the ThT fluorescence intensity values, and the marginal or absent ability of WT and turn variants to cross-elongate indicate that different variants form different sets (of) fibril polymorphs. We find that specific \( \beta \)-turn propensities are associated with either faster (\( \beta \)-turn, \( \beta \)-turn) or slower (\( \beta \)-turn) fibril formation. The cross-elongation data reveals that all investigated turn exchanges result in \( \alpha \)S monomers that are impeded in their ability to incorporate into WT fibrils. Binding to WT fibril ends does not seem to be suppressed, as turn variants can block elongation of WT fibrils by WT monomers. This indicates that the conformational conversion of \( \alpha \)S monomers incorporating into the fibril end requires high sequence fidelity in the 44-TKEG-47 turn region. This study highlights the potential of kinetic assays to aid in the investigation of amyloid fibril polymorphism and expands the currently rather limited data on fibril cross-elongation.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author statement

E.D.A., M.P.S., A.K.B., and W.H. designed the experiments. E.D.A., M.P.S., N.R., M.M.W., and H.S. performed the experiments and analyzed the data. E.D.A., A.K.B., and W.H. wrote the manuscript. All authors commented on the manuscript.
Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2020.106519.

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