Steric Crowding of the Turn Region Alters the Tertiary Fold of Amyloid-β_{18–35} and Makes It Soluble*

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Aβ self-assembles into parallel cross-β fibrillar aggregates, which is associated with Alzheimer’s disease pathology. A central hairpin turn around residues 23–29 is a defining characteristic of Aβ in its aggregated state. Major biophysical properties of Aβ, including this turn, remain unaltered in the central fragment Aβ_{18–35}. Here, we synthesize a single deletion mutant, ΔG25, with the aim of sterically hindering the hairpin turn in Aβ_{18–35}. We find that the solubility of the peptide goes up by more than 20-fold. Although some oligomeric structures do form, solution state NMR spectroscopy shows that they have mostly random coil conformations. Fibrils ultimately form at a much higher concentration but have widths approximately twice that of Aβ_{18–35}, suggesting an opening of the hairpin bend. Surprisingly, two-dimensional solid state NMR shows that the contact between Phe19 and Leu34 residues, observed in full-length Aβ, is critically dependent on the hairpin turn and on the contact between the Phe19 and Leu24 regions, making them potentially sensitive targets for Alzheimer’s therapeutics. Our results show the importance of specific conformations in an aggregation process thought to be primarily driven by nonspecific hydrophobic interactions.

Alzheimer’s disease pathology has been associated with the aggregation of amyloid β (Aβ), a 39–43-amino acid-long peptide (1, 2). In this process, the unstructured monomers of Aβ get converted into amyloid fibrils composed of hairpin-shaped monomeric units assembled in a parallel β sheet arrangement (3, 4). The central part of this hairpin structure consists of a hydrophilic region flanked by hydrophobic β-sheet-forming segments at both ends (5–10). This particular shape is believed to be dictated by the specific pattern of hydrophobic and charged residues in the Aβ sequence and has been identified even in soluble Aβ aggregates (7, 11–17), including the very early stage oligomers of Aβ (18). Chemically constraining the side chains of Asp23 and Lys28 accelerates the kinetics of Aβ aggregation by stabilizing the hairpin structure (5). On the other hand, introducing a negative charge at Ser26 disrupts the Asp23–Lys28 salt bridge, reduces the plasticity of the turn region (Gly25–Gly29), and stabilizes the soluble monomeric and oligomeric assemblies of Aβ (19). This hairpin turn thus seems to be a critical factor dictating the self-assembly of Aβ under physiological conditions. Studying the influence of the turn region on the properties of Aβ, separately from that of the distal terminal regions and without changing the electrostatics, can yield valuable information on the logic of Aβ assembly.

Aβ aggregation appears to be primarily hydrophobic in nature. In fact a contact between hydrophobic regions containing Phe19 and Leu24 is one of the earliest contacts formed during the aggregation of amyloid β (18). In a generic hydrophobic aggregate, the burial of the hydrophobic surface can in principle proceed in an intermolecular fashion, which would appear to render the turn unnecessary. However, such “straight chain” hydrophobic interactions would end up sandwiching the charged midsection between the two hydrophobic flanking regions (type II; Fig. 1), which would be energetically costly. A hairpin-shaped aggregate on the other hand relieves the hydrophilic region from such constraints by exposing the turn region (type I; Fig. 1). It is likely that the glycine residue in the turn region, Gly25, facilitates the hairpin bend, because it allows the peptide to have enough space to avoid steric hindrance. If Gly25 is removed altogether, the peptide will not have significantly

*S.S.G25, amyloid β(18–35) minus the residue Gly25; S.G25G25, amyloid β(18–35) with P instead of Gly25; Aβ_{18–35}, amyloid β(40) minus the residue Gly25; TEM, transmission electron microscopy; MAS, magic angle spinning; PARIS, phase-alternated recoupling irradiation scheme; FCS, fluorescence correlation spectroscopy; ssNMR, solid state NMR; thio-T, thioflavin-T; ROESY, rotating frame Overhauser effect spectroscopy; COSY, correlation spectroscopy.
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![Diagram of Aβ turn with types I, IIA, and IIB conformations](image)

**Figure 1.** Aβ<sub>18–35</sub> fragment with (S) and without (S<sub>G25</sub>) the Gly<sup>25</sup> amino acid. The fragment consists of a largely hydrophilic stretch (in blue) flanked by two hydrophobic stretches (in orange and red) on either side of Gly<sup>25</sup>. Gly<sup>25</sup> (black dot) is located in the middle part of this fragment. Under physiological buffer conditions, S readily self-assembles forming hairpin shaped fibrillar aggregates. The formation of oligomeric aggregates in the solution were assessed with the help of IR and solid state NMR (ssNMR) spectroscopy. Conformations of the aggregates were assessed with the help of IR and solid state NMR (ssNMR) spectroscopy. The intermolecular arrangements were specifically probed by cross-linking neighboring tyrosine residues, which assays in-register parallel β-sheet arrangements. The results yielded by these studies help us determine the role played by the hairpin turn in general, and the Phe<sup>19</sup>–Leu<sup>34</sup> interaction in particular (21, 22), in the self-assembly of Aβ under physiological conditions.

**Experimental Procedures**

**Materials**—Rink amide MBHA resin LL, Fmoc (N-(9-fluorenylmethoxycarbonyl) protected amino acids, O-benzotriazolene-N, N,N,N′,N′-tetramethyluroniumhexafluoro phosphate, and trisopropysilane were purchased from Merck. 1-Hydroxybenzotriazole, N-methyl morpholine, N,N,N-trimethylformamide, piperidine, colidine, TFA, tert-butyl methyl ether, acetonitrile, and isopropyl alcohol are obtained from S.D. Fine Chemicals Ltd. (Mumbai, India). 1.8-Diazabicyclo[5.4.0]undec-7-ene, hexafluoroisopropanol, thio-T, DMSO, 5(6)-carboxytetramethylrhodamine N-succinimidyl ester and the buffer salts are purchased from Sigma-Aldrich. Phenol, N,N′-diisopropylcarbodiimide, ethane dithiol, thioanisole, and trifluoroethanol are purchased from Fluka. The isotopically labeled amino acids are purchased from Cambridge Isotopes Laboratories (Tewksbury, MA).

**Synthesis and Purification of Peptides**—The peptides S<sub>G25</sub>, S<sub>G25P</sub>, S<sub>G25P</sub> and Aβ<sub>G25</sub> were synthesized, labeled with 5(6)-carboxytetramethyl rhodamine, purified, and characterized by mass spectroscopy as described previously (20, 23). The isotopically labeled peptides for ssNMR studies were synthesized by the same procedure but with selective introduction of uniformly <sup>13</sup>C and <sup>15</sup>N isotopically enriched Val<sup>18</sup>, Phe<sup>19</sup>, Ala<sup>21</sup>, Gly<sup>23</sup>, and Leu<sup>34</sup> amino acids.

**Aggregation Kinetics and Solubility**—The peptides were dissolved and adjusted to pH 12 with NaOH at different concentrations ranging from 1 to 2 mM and incubated for 30 min. The peptide is mostly in the monomeric or small oligomeric form in basic conditions (24). Then it was mixed with 20 mM phosphate buffer (containing 146 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, and 20 mM Na<sub>2</sub>HPO<sub>4</sub>) maintained at pH 7.4. Sodium azide (anti-bacterial) concentration in buffer is kept at 0.5 mM. The dilution factor was usually 10, and the final pH of the solution was verified to be within 0.1 pH unit of 7.4. The time of mixing was considered as the zero time of aggregation, and concentration was measured using the absorbance at 230 nm using the peptide backbone as a probe of concentration and also by tryrosine fluorescence in case of Aβ and Aβ<sub>G25</sub>. The solutions were constantly rotated at 20 rpm at room temperature (298 K). The initial reading was recorded without any centrifugation immediately after mixing. Later time points are measured from the supernatant of sample cen-
trifuged at 2000 × g for 20 min. The supernatant was mixed back with the precipitate after each measurement and rotated in a centrifuge tube with rpm of 200 for efficient mixing. The aggregation was studied for 7 days. Jasco double beam absorption spectrophotometer (model no. V-530; Jasco) was used for all measurements with necessary day to day blank corrections. To assess the effect of possible cross-seeding, aggregation kinetics of 100 μM Aβ40 in presence of 100 μM S or SAG25 were monitored using the fluorescence of tyrosine remaining in the supernatant with time.

**Fluorescence Correlation Spectroscopy—FCS measurements** were performed on a home-built instrument and following the methods established earlier (25). Tetramethylrhodamine-labeled peptides were used at a concentration ratio of 1:1000 (labeled:unlabeled) as fluorescent markers for the peptide aggregates. MEMFCS, a fitting routine developed specifically for such measurements (26), was used to obtain a size distribution from the FCS data in a model-free manner. The instrument was calibrated using the dye rhodamine B.

**Circular Dichroism—CD measurements** were performed on 100 μM peptide solution incubated in 2 mM phosphate buffer at pH 7.4 for 6 and 16 h. The solutions were centrifuged at 2000 × g for 20 min. The measurements were performed with the solutions both before centrifugation and after centrifugation (with the supernatant). Far UV CD spectra for the peptides were measured over a range of 195–260 nm in a cuvette of 1-mm path length in a J-810 spectropolarimeter (Jasco).

**Thioflavin-T Binding Studies—Peptide samples** (100 μM each) were prepared in phosphate buffer (pH ~7.4) containing 5 μM thio-T. The peptide samples were diluted from pH 11 aqueous solution. Thio-T fluorescence from the samples were recorded in a FluoroMax 3 spectrophotometer (Horiba Scientific) using 444-nm excitation as a function of time after preparation of the sample in pH 7.4 buffer.

**Electron Microscopy—Solutions containing 1 mM SAG25 or 100 μM Aβ40 at pH 7.4 phosphate buffer were incubated at room temperature for 72 h. 10 μl of these solutions were placed on carbon-coated 100 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA) and allowed to be adsorbed for 2–3 min. The grids were blotted and mildly washed with MilliQ water three or four times. Then the samples were stained by 0.1% of uranyl acetate followed by drying under an infrared lamp. The samples were examined with a transmission electron microscope (LIBRA 120, EFTEM; Carl Zeiss). The fibril widths were analyzed with ImageJ (open source software).

**Nuclear Magnetic Resonance Spectroscopy—A 400 μM solution of SAG25 peptide incubated overnight was subjected to solution state NMR measurements. All measurements were performed on 500-MHz Bucker AVIII spectrometer using a 5-mm double channel direct probe equipped with Z-gradients.**

For ssNMR measurements, selective isotopically enriched peptide was allowed to aggregate from a starting concentration of 1–2 mM in the similar buffer system used for solubility measurements. The aggregates were isolated from the soluble peptide by centrifugation for 1 h. The pellet thus collected was washed with deionized water twice by resuspending it in water and centrifuging for 1 h each time. The final pellet so obtained was rapidly frozen using liquid nitrogen, lyophilized, and packed in a 2.5-mm magic angle spinning (MAS) rotor. All ssNMR measurements were performed on 700-MHz Bruker AVIII NMR spectrometer at MAS frequency (νz) of 12 and 17 kHz using a 2.5-mm triple resonance MAS probe. 1H dipolar decoupling was accomplished using swept frequency, two-pulse phase modulation (ν = 15°) decoupling scheme (27). Two-dimensional 13C,15N through-space NMR spectra were recorded using second order dipolar recoupling schemes of PARIS-xy (m = 1, n = 0.5) (28) and mixing periods of 20 (at νz = 17 kHz) and 320 (at νz = 12 and 17 kHz) ms, respectively.

All one-dimensional data were processed and analyzed using TopSpin 3.2. All two-dimensional spectra were processed with TopSpin 3.2 and analyzed using Analysis 2.3.1 (29). Secondary chemical shifts were calculated by subtracting the temperature and pH adjusted sequence-corrected random coil chemical shifts from the observed chemical shifts for each amino acid (30, 31).

**Infrared Studies—200 μM of Aβ40, 200 μM Aβ40, and 1 mM of SAG25 were incubated separately for 72 h in phosphate buffer (pH 7.4). All the solutions were subsequently centrifuged at 16,000 × g for 15 min, and the supernatants were discarded. Precipitates so obtained were lyophilized and subjected to IR studies. The FTIR spectra of all the samples were recorded in a Nicolet 6700 FTIR instrument (Thermo Electron Corporation).**

**Tyrosine Cross-linking Experiments—We prepared separate peptides with one extra tyrosine residue at the N terminus of S and SAG25 for these experiments. 200 μM of Aβ1–40, 200 μM of tyr-S, and 1 mm of tyr-SAG25 were incubated for 14 days in pH 7.4 phosphate buffer (20 mM Na2HPO4, 150 mM NaCl, 5 mM KCl, and 2 mM NaN3; pH adjusted by adding HCl). Aggregates so obtained were separated by mild centrifugation (2000 × g for 10 min), discarding the supernatant. Aggregates were then resuspended in appropriate amount of phosphate buffer, so that all vortexed solutions had similar tyrosine fluorescence (which ensures matching final peptide concentrations). 10 μl of ice-cold H2O2 and 6 μl of freshly prepared FeSO4 were added simultaneously to 600 μl of these peptide solutions. These solutions were then vortexed for 60 s. Fluorescence spectra were obtained immediately by exciting at 260 nm. Although tyrosine has a fluorescence peak at ~305 nm, the cross-linked tyrosine peak is in the visible region. A similar experiment was performed with a similar concentration of free tyrosine solution to estimate the extent of random cross-linking in solution. The spectra were normalized by dividing them by the fluorescence intensity obtained at 305 nm before the cross-linking step. To remove the contribution from random cross-linking, the normalized spectra obtained from free tyrosine were subtracted from these normalized spectra.

**Results and Discussion**

Aggregation kinetics of the different peptides were compared by monitoring the concentration remaining in solution after gentle centrifugation (2000 × g for 20 min; Fig. 2A). The concentration is measured by recording the absorbance of the supernatant at 230 nm. The saturation concentration (Csat) is the limiting value of the concentration remaining in the solution after the solution is incubated for a sufficiently long time...
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FIGURE 2. A, saturation concentration \( C_{sat} \) value of Aβ40, Aβ40G25, S\_G25, S\_G25P, and S\_G25B, B, hydrodynamic radius \( R_h \) distribution of the soluble fractions of S and S\_G25 peptides after 6 h as determined by FCS measurements. C, hydrodynamic radii \( R_h \) of the putative monomers of S and S\_G25.

(32). S\_G25 was found to reach a \( C_{sat} \) of 566 ± 150 \( \mu M \) in 7 days. S on the other hand attains a \( C_{sat} \) of ~18 ± 2 \( \mu M \) over a similar period, which is close to the full-length Aβ40 (~12 ± 4 \( \mu M \)). Thus, deletion of a single residue Gly\(^{25}\) caused the solubility of Aβ\(_{18-35}\) fragment to increase by more than 20 times under physiological buffer conditions. The same deletion in the case of the full-length peptide also leads to a 3-fold increase in solubility (Aβ\(_{40}\) solubility = 36 ± 12 \( \mu M \); Fig. 2A). This increase is less than that of S\_G25 but still very substantial. We also examined two more peptide variants: S\_G25 and S\_G25P mutants of S. They were found to reach saturation concentrations of 9 ± 1 and 20 ± 4 \( \mu M \) (Fig. 2A), respectively. Therefore, both the nature and the location of a glycine residue are critical for peptide aggregation. If the turn favors aggregation, then we expect that initially the aggregation kinetics of S\_G25P may be slow (for the \textit{trans} population), but with time the \textit{trans} form may isomerize to the \textit{cis} form (the kinetics of such a \textit{cis-trans} isomerization in an ordered aggregate may be slow), and the solubility may finally become similar to that of the wild type. This is indeed corroborated by the data.

Next we probed the effect of Gly\(^{25}\) deletion on the soluble oligomeric species of the S. The size of this species was determined by performing FCS measurements using tetramethylrhodamine-labeled peptides as probes. FCS, in different forms, has proved to be an effective tool for following the size of evolving protein aggregates (32–34). 100 \( \mu M \) of both peptides (at 1000:1 unlabeled:tetramethylrhodamine-labeled peptide ratio) were incubated for 6 h under physiological buffer conditions. The soluble aggregates were obtained from the supernatant of these solutions by mild centrifugation at 2000 \( \times g \) at 6-h time points. S\_G25 is subsaturated under this condition and does not form insoluble aggregates. For S\_G25P, FCS data were recorded both before and after centrifugation at the 6- and 16-h time points. The FCS data were analyzed with the model-free MEM-FCS software (26) to obtain the distribution of size (specifically, the hydrodynamic radius, \( R_h \)) in the solution (Fig. 2B). The size distribution at 6 h has peaks at 0.96 and 1.5 nm for S and S\_G25P, respectively. Hydrodynamic radii of S and S\_G25 solution incubated for a longer time period at very low concentrations (500 \( \text{nm} \)), which is expected to allow the oligomers to dissociate to the smallest possible state at such concentrations (possibly monomeric), were found to be 0.66 ± 0.01 and 0.93 ± 0.02 nm, respectively (Fig. 2C). Therefore, the species observed in solution phase after 6 h of incubation are most likely dominated by small oligomers and not the monomers, of S and S\_G25. This tells us that both peptides form metastable oligomeric species at the early stages, with S\_G25 forming somewhat larger ones. However, these larger S\_G25 oligomers do not further grow into fibrillar aggregates, until a very high concentration is reached. On the other hand, the size of the putative monomer of S is consistent with the monomeric form of full-length Aβ after taking their masses into account (the \( R_h \) for a homogeneous spherical particle varies as the cube root of the mass). However, S\_G25 remains comparatively bigger in size. This implies that either this is a monomeric species, which is more open (\textit{i.e.} stiffer) than Aβ, or, even at such concentrations (500 \( \text{nm} \)) and such long incubation times (21 days), S\_G25 remains in a small oligomeric state.

If the conformation of the soluble S and S\_G25 species is similar to that of Aβ, then they may be expected to interact with it and affect its aggregation kinetics. This is examined by a cross-seeding study. The aggregation kinetics of 100 \( \mu M \) Aβ in presence of 100 \( \mu M \) S and S\_G25 respectively was monitored and compared with the aggregation kinetics of 100 \( \mu M \) Aβ by itself. The saturation concentration of Aβ\(_{40}\) is not significantly different in the presence of either S or S\_G25 (Fig. 3). However, S leads to a somewhat faster initial aggregation phase. This suggests that the soluble species of S have conformations similar to
that of Aβ40 (20) and is able to perturb the aggregation kinetics. However, because both Aβ and S have very similar saturation concentrations, the saturation concentration of the mixture is not altered. On the hand, SΔG25 does not interfere with the aggregation of Aβ40, likely because its own solubility is very high and its conformation is rather different from that of Aβ40.

These results suggest that the small oligomeric species formed by SΔG25 differ at the molecular level from S and Aβ. To study this aspect, the secondary structures of these small oligomeric species (initial concentration = 100 μM) were obtained by circular dichroism measurements on the supernatant solutions (Fig. 4A). The CD data show that secondary structure of the soluble oligomers is mostly random coil in both the cases. However, S oligomers contain considerable amount of β-sheet, whereas SΔG25 oligomers contain some amount of α-helix, as determined by Reed’s method (S, 77% random coil and 23% β-sheet; SΔG25, 82% random coil and 17.3% α-helix) (35). The CD spectrum of SΔG25 does not change with time, as shown by a spectrum recorded 16 h after preparation (Fig. 4B). As may be expected, thio-T binding studies revealed that a considerably richer β-sheet character develops in the larger aggregates of S compared with that of SΔG25 as the aggregation proceeds (Fig. 4C), starting with an initial concentration of 100 μM. Of course, only S forms these larger aggregates at these concentrations.

It is interesting to ask exactly what kind of secondary structure SΔG25 forms. The high solubility of SΔG25 gives us an opportunity to directly probe the structure of these oligomeric species with solution state proton NMR. Fig. 5 shows amide proton region of 1H⁻¹H ROESY (mixing time = 300 ms) spectrum of a day-old 400 μM SΔG25 solution recorded at 298 K (Fig. 5A) and 283 K (Fig. 5B). Most of the Hα-Hα cross-peaks could only be observed at the lower temperature, indicating the flexible nature of SΔG25 units in these soluble oligomeric states. 1H chemical shift assignment could be achieved for 13 of 17 amino acids in SΔG25 using 1H⁻¹H COSY, total correlation spectroscopy, and ROESY spectra recorded at 283 K. Chemical shifts so obtained are listed down in Table 1 along with the Hα chemical shift values for each amino acid. Because only two amino acids, Asn27 and Met35, showed Δδ values greater than 0.1 ppm, it is evident that the SΔG25 peptide is mostly devoid of well ordered secondary structure in its soluble aggregated states (36).

These studies reveal that S (and not SΔG25) acquires β-sheet secondary structural elements at an early stage of aggregation, which are further propagated as the aggregates grow in size. Even though deletion of Gly25 from this fragment does not prevent the formation of small oligomers in the solution phase, it completely alters the conformation of these oligomers. It is interesting to speculate what holds these oligomers together despite the lack of secondary structures. This is likely driven by the nonspecific intermolecular interaction of the hydrophobic residues, but our experiments do not probe this aspect.

Next we ask whether this peptide is capable of forming amyloid fibrils and whether these fibrils bear the same characteristics as that formed by S. For this, 1 mM solution of SΔG25 was incubated for 72 h. The precipitated fraction of this solution was probed by using TEM, and the results are shown in Fig. 6. The TEM images showed amyloid-like fibrillar structures, very similar in appearance to the fibrils obtained from S (20). However, a closer look at the TEM images revealed a very significant difference between the S and SΔG25 fibrils. Although for SΔG25 the average fibril width is 7.2 ± 0.2 nm (average of 30 positions recorded from 5 different images obtained from duplicate sample preparations), it was found to be only 3.0 ± 0.7 nm for the SΔG25 fibrils.
fibrils (20). These results strongly favor the hypothesis that S\textsubscript{AG25} forms wider fibrils by opening up the hairpin (type II; Fig. 1). We note that this does not rule out the presence of a minor population of precipitates, which do not form proper fibrils and may have a hairpin turn. For straight and wider fibrils, the peptides may assemble in a parallel (type IIA; Fig. 1) or in an anti-parallel (type IIB; Fig. 1) orientation. Of course, this assumes that the fibrils are still made up of \( \beta \)-sheets. To resolve this issue, we further investigated the secondary and tertiary structural contents of the S\textsubscript{AG25} fibrils by ssNMR and IR studies.

ssNMR studies were performed on aggregates of 7S\textsubscript{AG25} peptide containing isotopically enriched Val\textsuperscript{18}, Phe\textsuperscript{19}, Ala\textsuperscript{21}, Gly\textsuperscript{29}, Asp\textsuperscript{23}, Val\textsuperscript{24}, Ser\textsuperscript{26}, Gly\textsuperscript{29}, Ala\textsuperscript{21}, Phe\textsuperscript{20}, Ile\textsuperscript{12}, Gly\textsuperscript{23}, and Leu\textsuperscript{14} residues. These studies revealed that S\textsubscript{AG25} fibrils are also rich in \( \beta \)-sheet conformations, similar to the amyloid fibrils formed by S and full-length A\textsubscript{\beta} (20). Fig. 7A shows selected regions of two-dimensional \( ^{13}\text{C}-^{13}\text{C} \) PARIS-xy (m = 1, \( n = 0.5 \)) through space correlation spectrum recorded at 17-kHz MAS using a mixing time of 20 ms. Also shown in the figure is the sequence assignment for each isotopically enriched amino acid. Two structural conformers were identified for all but the Phe\textsuperscript{19} amino acid. Weakly populated conformations, as adjudged from the peak intensities, are marked with a prime symbol. This shows the presence of structural heterogeneity at molecular level in S\textsubscript{AG25} fibrils, a prevalent feature of amyloid aggregates (37–39). Chemical shifts so obtained (Table 2) were used to calculate the secondary chemical shifts (\( \Delta \delta \)) of \( \alpha \), \( \beta \), and carbonyl carbons for each conformer and are shown in Fig. 7B. A positive value for \( \beta \) and negative values for \( \alpha \) and carbonyl carbons are indicative of \( \beta \)-sheet propensity of an amino acid. A comparison with S shows that although the terminal residue Val\textsuperscript{18} is unstructured, Phe\textsuperscript{19} is in a \( \beta \)-sheet conformation in both the peptide fibrils. Ala\textsuperscript{21} has its major population in \( \beta \)-sheet conformation in both cases, but the weaker one is structured only in the case of S\textsubscript{AG25}. Leu\textsuperscript{14} conformers show \( \beta \)-sheet propensities in both cases. The only difference was observed in the case of Gly\textsuperscript{23}, which shows enhancement in heterogeneity, as well as nonstructured propensities in S\textsubscript{AG25}. Overall, we can say that S and S\textsubscript{AG25} fibrils share strong similarities at the secondary structure level.

Surprisingly, long range contacts in S\textsubscript{AG25} were observed between amino acids Phe\textsuperscript{19} and Leu\textsuperscript{34} and between amino acids Phe\textsuperscript{19} and Gly\textsuperscript{23} in two-dimensional \( ^{13}\text{C}-^{13}\text{C} \) PARIS-xy (m = 1, \( n = 0.5 \)) through space correlation spectrum recorded at 12-kHz MAS using a long mixing time of 320 ms (Fig. 7C). Through space contact between Phe\textsuperscript{19} and Leu\textsuperscript{34} is a hallmark feature of the parallel hairpin structure typically observed for full-length A\textsubscript{\beta} fibrils (6–8, 40) and also for S (20). In fact the disruption of this contact completely changes the toxicity of A\textsubscript{\beta} (22). However, this appears to be at odds with the TEM results, which predict that the peptide remains in an open state in the fibril. However, the apparent contradiction can be resolved if S\textsubscript{AG25} monomers are arranged in an anti-parallel \( \beta \)-sheet, with the Phe\textsuperscript{19} residue of one monomer juxtaposed to the Leu\textsuperscript{34} residue of the other. Also, a long range contact between Phe\textsuperscript{19} and
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Gly33 was observed in S_{A2G25} fibrils, which is not observed for S and Aβ fibrils (20). The presence of this new contact is consistent with the presence of an atypical structural conformation in S_{A2G25} fibrils, possibly an open and anti-parallel arrangement as depicted in Fig. 1 (type IIB).

The hypothesis of an open conformation in an anti-parallel arrangement is further supported by the IR spectra. IR amide band frequencies are directly correlated to the secondary structure components. The amide I band (Fig. 8A) of S_{A2G25} (solid line) is wider than Aβ_{1–40} (dashed line). The peak at 1626 cm\(^{-1}\) indicates the presence of a β-sheet conformation in the fibrillar state, and the peak at 1680 cm\(^{-1}\) is characteristic of anti-parallel β-sheet strands (41).

A more direct proof for this intermolecular antiparallel β-sheet arrangement comes from tyrosine cross-linking experiments. Wild type Aβ has a tyrosine at the 10th position. The parallel in-register arrangement of Aβ peptides in the fibril form places their tyrosine residues next to each other, which can easily be cross linked by photo-induced cross-linking of unmodified proteins (42, 43) or simply by Fe (II)/H\(_2\)O\(_2\) treatment (44, 45). The formation of the cross-linked tyrosine can be quantitatively monitored by measuring the red shifted fluorescence of dityrosine (46, 47). Because S does not have any tyrosine residue in it, a tyrosine residue was added at the N terminus of both S (Tyr-S) and S_{A2G25} (Tyr-S_{A2G25}). Aggregates that possess a parallel in-register arrangement similar to that of full-length Aβ should have their tyrosine residues amenable to cross-linking. The results show a high amount of cross-linking in case of Tyr-S and Aβ_{1–40} and negligible cross-linking for Tyr-S_{A2G25} fibrils (Fig. 8B), clearly showing that the Tyr residues in S_{A2G25} are not close to each other. This provides a strong corroboration of our model of antiparallel β-sheet conformation for the A2G25 mutant, in contrast with the parallel, in register β-sheet conformation in the wild type S and Aβ. Interestingly, unlike S_{A2G25}, Aβ_{A2G25} fibrils were found to be folded (fibrillar width 7–10 nm; Fig. 9A), and the IR spectrum suggests that it contains a parallel β-sheet structure (Fig. 9B). These results indicate that absence of the glycine residue in the 25th position makes the fold costlier even for the full-length Aβ, but the additional 22 residues ultimately induce the hairpin fold in the final fibrillar aggregates.

Together, the TEM, ssNMR, IR, and tyrosine cross-linking data show that Aβ_{A2G25} fibrils consist of straight monomers assembled in an antiparallel intermolecular arrangement. We note that molecular dynamic simulations of the phosphorylated Ser-26 and the S26D mutants, which cannot form the salt bridge present in the wild type, also show a tendency to open up at the turn region (19). The “Iowa” mutant D23N, which does not form a salt bridge, also shows an antiparallel arrangement in the protofibrils (9). In summary, the major fraction of S_{A2G25} monomers seem to aggregate without adopting the most prominent structural feature of S and the full-length Aβ peptide, viz., the hairpin turn.

Conclusion

Our results show the central role that the turn region plays in determining the aggregation propensity of Aβ. A deletion of a glycine residue may in general be expected to have a relatively small effect on a hydrophobically driven aggregation process. However, it leads to a steric crowding of the turn region, prevents the peptide from bending, and makes the peptide at least 20 times more soluble. Interestingly, small oligomers still form, perhaps driven by the nonspecific intermolecular interactions of the hydrophobic residues. However, the oligomers have random coil architecture and cannot easily grow in size, whereas the oligomers of S and full-length Aβ have at least some β-sheet character, and they easily grow to form mature fibrils (7, 11, 18). The final fibrillar architecture of S_{A2G25} obtained only at very high concentrations, shows that β-sheets do form, but the hairpin opens up, and the β-sheet is anti-parallel in nature. Surprisingly, the distal contact between Phe\(^19\) and Leu\(^34\) is still present, suggesting that forming this contact provides considerable energy stabilization for aggregation. Candidate drug molecules with the ability to crowd the turn region or to disrupt the contact between Phe\(^19\) and Leu\(^34\) are expected to have a very strong effect on the aggregation of Aβ.

| Amino acids | δ_{13C} | δ_{15N} | δ_{R} |
|-------------|---------|---------|-------|
| Val\(^{31}\) | 179.84  | 68.53   | 34.05 |
| Val\(^{33}\) | 178.36 (175.79) | 62.98 (62.71) | 33.81 (32.56) |
| Phe\(^{19}\) | 174.01 (175.14) | 57.30 (57.54) | 41.12 (39.39) |
| Ala\(^{21}\) | 176.78   | 51.16   | 23.74 |
| Ala\(^{21}\) | 175.81 (177.49) | 50.98 (52.51) | 21.71 (19.06) |
| Gly\(^{21}\) | 171.38   | 48.96   |       |
| Gly\(^{21}\) | 169.56 (173.89) | 48.28 (44.91) |       |
| Leu\(^{34}\) | 175.49   | 54.76   | 45.95 |
| Leu\(^{34}\) | 174.59 (177.73) | 55.42 (55.28) | 43.64 (42.26) |

TABLE 2

13C chemical shift values of uniformly 13C- and 15N-labeled amino acids in S_{A2G25}

All values are in ppm are referenced with respect to DSS. The values in the parentheses are sequence-corrected random coil chemical shifts at 298 K (30, 31). Weakly populated conformations are marked with a prime symbol.

FIGURE 8. A, IR absorption spectra of S_{A2G25} (solid line) and Aβ_{1–40} (dashed line) fibrils in the region between 1480 and 1715 cm\(^{-1}\). The dashed vertical line corresponds to vibrational frequency of 1680 cm\(^{-1}\). B, normalized fluorescence spectra obtained in the tyrosine cross-linking experiment.

FIGURE 9. Characteristics of Aβ_{A2G25}. A, electron micrographs of the aggregates of Aβ_{A2G25}. Scale bar, 500 nm. B, IR absorption spectra of Aβ (dashed line) and Aβ_{A2G25} (solid line) fibrils in the region between 1480 and 1705 cm\(^{-1}\). The dashed vertical line corresponds to vibrational frequency of 1680 cm\(^{-1}\).
Steric Crowding of the Aβ Turn

Author Contributions—S. P. D., P. K. M., and S. M. designed and coordinated the study. M. C. and D. B. synthesized and purified the peptides. M. C., R. A., and D. B. performed the FCS measurements. Aggregation kinetics and CD measurements were performed by M. C., D. B., B. S., and R. A. Solid and solution state NMR experiments were designed by V. S. M. and P. K. M. and performed by H. S. and V. S. M. D. B. performed the TEM, FTIR, and tyrosine cross-linking studies. M. K. performed several of the fluorescence experiments, including the thio-T studies. M. C., V. S. M., and S. M. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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