Kinetic Intermediates in Amyloid Assembly

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ABSTRACT: In contrast to an expected Ostwald-like ripening of amyloid assemblies, the nucleating core of the Dutch mutant of the Aβ peptide of Alzheimer’s disease assembles through a series of conformational transitions. Structural characterization of the intermediate assemblies by isotope-edited IR and solid-state NMR reveal unexpected strand orientation intermediates and suggest new nucleation mechanisms in a progressive assembly pathway.

The self-assembly of proteins into amyloid is an initiating step in misfolding1,2 and infectious prion3 diseases. The process is traditionally described as a thermodynamically-driven “ripening” of accessible conformations and the range of phases that exist under a given set of conditions is the result of amyloid polymorphism. Recently, secondary nucleation sites4,5 have suggested the possibility of a more progressive pathway contributing to different assembled phases. Here we experimentally demonstrate the minimal nucleating core6,7 of the Dutch mutant8 of the Aβ peptide of Alzheimer’s disease (AD), Aβ(16-22)E22Q or Ac16KLVFFA22Q-NH2, assembles as anti-parallel β-strands that later transition completely into parallel arrays. These studies define a progressive pathway for amyloid assembly, even for simple model peptides, and reveal new mechanisms for achieving polymorphic diversity in disease etiology.

Figure 1. Strand conformations of Aβ(16-22)E22L peptide showing positions of charged lysine (blue) residues. Electrostatic repulsion is attenuated in anti-parallel peptide orientation. Out-of-register strands place the bulky valine packed with the less bulky alanine. Arrows indicate valine (red)-alanine (brown) cross-strand pairing.

Simulation9-14 and emperical15-22 analyses of Aβ(16-22) assembly are consistent with an initial solvation free energy-driven oligomerization to a particle phase. We reasoned secondary structure formation within the less hydrated peptide particle phase22-24 may explain the observation that Aβ(16-22)E22L, Ac16KLVFFA22L-NH2, matures with antiparallel strand orientations19. Electrostatic repulsion between lysine side chains would select against charged N-terminal lysine residue proximity in parallel strands (Fig. 1) during nucleation.

Given that anti-parallel out-of-register alignment in Aβ(16-22)E22L is directed by the preference of the bulky valine side chain at position 18 being across (cross-strand pairing) from the less bulky alanine19,20, we hypothesized that uncharged substitutions would stabilize different strand arrangements. Structural25,26 and thermodynamic27 investigations have identified ordered glutamine side chains in cross-strand stabilization of parallel registries, and the Dutch mutant of Aβ8 manifested as the Aβ(16-22)E22Q congener which conservatively swaps a side chain -OH for an -NH2, appeared suitable to change the energy balance. Quite distinct from previous cytosine substitution28 or metal ion binding elements which stabilize sheet stacking interactions29, the E22Q substitution could stabilize parallel strand registry through cross-strand pairing via amide side-chain H-bonding.

Fig. 2A shows particles and short twisted ribbons that appear in electron micrographs early after Aβ(16-22)E22Q peptide dissolution and the FT-IR amide-I stretch centered at 1625 ± 1 cm⁻¹ indicates β-sheet assembly (Fig. S1). In contrast with the design for glutamine addition, isotope-edited IR analysis with 13C=O enrichment at the central F19 residue [1-13C]F19Aβ(16-22)E22Q, where 12C/13C coupling is most diagnostic of β-strand registry29,30,31, shows a band splitting29,31,32,34 of almost 40 cm⁻¹ and a 12C/13C band intensity ratio of less than 1 (Fig 2C, black, S2). These assignments are consistent with previously characterized assemblies30,31,33-37, and define one-residue out-of-register antiparallel stranded β-sheets (Fig 1).
Figure 2. Time dependence of the assembly of 1 mM Aβ(16-22)E22Q at acidic pH in 20% CH₃CN containing 0.1% TFA monitored by TEM (A,B) and isotope-edited IR analysis (C,D) using [1-13C]F19 Aβ(16-22)E22Q assemblies. (A) After 1 hour, wide ribbons (up to 40 nm) are observed, in contrast to (B) the 11.6 ± 1.2 nm fibers present after 20 days. (C) Dashed lines indicate positions of glutamine side chain, 12C and 13C amide-I band positions in mature fibers. (D) 12C/13C splitting (black) and relative peak height (red) for assemblies collected at multiples of 24hr as indicated on the time axis. Scale bars are 200 nm.

However, these assemblies do not persist. Approximately one week after assembly is initiated the FT-IR spectra begins a cooperative transition into long smooth fibers that after 20 days have diameters of 11.6 ± 1.2 nm (Fig. 2B, S3). Changes in the frequency and amplitude of the 12C/13C amide-I bands (Fig. 2D, S4) track with the morphological transitions seen by EM. A CO stretch at 1677 cm⁻¹ assigned to ordered glutamine side chains grew with the transition. The final assemblies with circular dichroism ellipticity minimum at 217 nm (Fig S5) and X-ray powder diffraction (Fig. S6) d-spacing reflections at 4.76 Å and 10.1 Å are typical of cross-β assemblies.¹⁹

The orientation and specific registry of each Aβ(16-22)E22Q strand is defined via the rate of double-quantum coherence build-up through homonuclear dipolar coupling in 13C DQF-DRAWS solid-state NMR experiments. Using the infinite array approximation (Fig S7), and including the effects of double quantum relaxation, T₂DQ = 11.7ms (Fig S8), the build-up of [1-13C]-L17 intensity from enriched E22Q assemblies uniquely fits a parallel in-register strand arrangement (Fig. 3B, S9). These analyses do not support the glutamine/inter-sheet H-bonding (Q-tracks) prevalent in Huntington’s inserts, as they require laminate spacings of 8 Å for backbone to side chain H-bonding rather than the 10.1 Å spacing seen in these assemblies. These data, together with the glutamine side chain CO stretch at 1677 cm⁻¹ (Fig. 2C, red), are consistent with cross-strand pairing along the sheet surface through extended side chain H-bonding Q-tracks as shown in Fig. 3C.²⁵,²⁶
Figure 3. β-sheet registry in Aβ(16-22)E22Q assemblies. (A) Cartoons showing positions of $^{13}$C (green) enriched residues in various β-sheet registries. (B) Determination of peptide registry with $^{13}$C-$^{13}$C distance measurements between leucine backbone carbonyls of 1 mM [1-$^{13}$C]L17 Aβ(16-22)E22Q assembled as fibers with $^{13}$C DQF-DRAWS NMR pulse sequence. Data points are peak intensity for double-quantum buildup divided by total $^{13}$C signal intensity. Best fit to DQ buildup (black line) is with a 4.7 Å $^{13}$C-$^{13}$C distance. (C) Aβ(16-22)E22Q parallel β-sheet registry. For clarity, non-polar hydrogen atoms are not displayed but the lysine (blue) and glutamine (gold) tracts are highlighted.

H-bond pairing is indirectly evaluated with N5-methylated, Aβ(16-22)E22QNHCH3 and N5,N5-dimethylated, Aβ(16-22)E22QN(CH3)$_2$, peptides. The N5,N5-dimethyl peptide (Fig. S10C) assemblies, as visualized with EM, appear morphologically indistinguishable from Aβ(16-22) nanotubes and ribbons$^{17,19,22}$. With [1-$^{13}$C]F19 enrichment, the $^{12}$C/$^{13}$C band splitting is 40 cm$^{-1}$ and the diagnostic antiparallel band at $\sim$1695 cm$^{-1}$ (Table S1 & Fig S10D) support the same one-residue out-of-register antiparallel β-strands. Unlike Aβ(16-22)E22Q, no distinct IR band at 1677 cm$^{-1}$ diagnostic for ordered glutamine sidechains is apparent in the N5,N5-dimethylated glutamine peptide assemblies. In contrast, the mono-methylated Aβ(16-22)E22QNHCH$_3$ peptide assemblies as fibril bundles (Fig S10B) with individual widths ranging from 7 nm in a single fiber to bundles containing up to five twisted fibers (Fig S11). Time-dependent IR spectra of assembling [1-$^{13}$C]F19 enriched monomethyl peptides (Fig S12) reveals a similar early antiparallel orientation that also transitions to parallel with growth of the glutamine side chain band at 1677 cm$^{-1}$, but the spectra are most consistent with the mature assemblies containing a mixture of parallel, antiparallel in-register, and antiparallel out-of-register assemblies (Fig S12, inset). Monomethylation appears to weaken the extended glutamine side chain H-bonding Q-track, and is expected to create a methylated surface. We predict that mixed fibers, or even mixed domains within individual fibers, are responsible for the bundling.
Figure 4. Time dependence of Aβ(16-22)E22Q assembly upon seeding with 1% preformed Aβ(16-22)E22Q fibers. FT-IR spectra of $^{13}$C amide-I band of 0.8 mM [1-$^{13}$C]F19 Aβ(16-22)E22Q seeded with mature [1-$^{13}$C]F19 Aβ(16-22)E22Q. $^{13}$C band positions for anti-parallel out-of-register (o) and parallel assemblies (p) are indicated with dashed lines.

These data argue that the nucleating core of the Aβ peptide of AD follows an obligatory hydrophobic collapse to intermolecular molten particles.22-24 The E22Q substitution provides an energetic constraint sufficient for a new transition, and one that does not manifest in the molten particle phase. The addition of 1% by weight pre-assembled fibers prepared from mature parallel E22Q assemblies complete the transition to parallel strands within hours (Fig 4, S13). Once initiated, parallel strand assembly propagates quickly, establishing the propagation of parallel strands is not limiting.

Figure 5. Model for the progressive transitions observed for Aβ(16-22). Paracrystalline forms emerge under these conditions as ribbons with antiparallel out-of-register β-strands. A subsequent transition to fibers is observed with parallel in-register strands. In cartoons, blue represents lysine residues and orange represents glutamine residues.

Simulations of propagation find that the growing fiber ends can accept strands with altered orientations,13 and in the aqueous environment on a template where lysine side-chain repulsion may be attenuated and cross-strand pairing to a pre-organized glutamine side-chain stabilizing (Fig. 3C), conformational “mutations” could accumulate as stabilized by extended glutamine side chain H-bonded Q-tracks. After a certain parallel concentration threshold is reached, fibril fragmentation of mutation-rich domains would generate new parallel ends and grow autocatalytically. This mechanism makes several predictions, which can now be explored through experimentation and simulation. Other mechanistic models, including the glutamine-rich C-termini exposed along the length of the fibril (Fig 5) serving as a secondary nucleation site,4,5 need also to be explored.

Like many materials,44 these minimal amyloid peptides experience the competing tensions of thermodynamically-controlled growth and kinetic nucleation, but amyloid assemblies have also been implicated in disease evolution.2,45-47 While the larger disease relevant peptides certainly have greater potential for kinetic and thermodynamic conformational diversity, their assembly is also expected to be even more diversified by the complex fluid48 environment of the eukaryotic
The conformational evolution seen in disease likely follows a progressive and irreversible path, and any of these kinetically accessible phases\(^\text{27}\) could be nucleated and stabilized by the cellular matrix for a Darwinian-like diversification and selection.\(^\text{24,45,50}\) Reconsideration of the confounding determinants required for a conformational evolution in the cell could open new strategies for defining and diverting disease-relevant assemblies for therapeutic intervention.

**ASSOCIATED CONTENT**

**Supporting Information**

Synthetic and experimental details, including peptide synthesis and purification, synthesis of methylated glutamine, NMR, TEM, CD and XRD. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

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