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Toward A Soluble Model System for the Amyloid State

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ABSTRACT: The formation and deposition of amyloids is associated with many diseases. β-Sheet secondary structure is a common feature of amyloids, but the packing of sheets against one another is distinctive relative to soluble proteins. Standard methods that rely on perturbing a polypeptide's sequence and evaluating impact on folding can be problematic for amyloid aggregates because a single sequence can adopt multiple conformations and diverse packing arrangements. We describe initial steps toward a minimum-sized, soluble model system for the amyloid state that supports comparisons among sequence variants. Critical to this goal is development of a new linking strategy to enable inter-sheet association mediated by side chain interactions, which is characteristic of the amyloid state. The linker design we identified should ultimately support exploration of relationships between sequence and amyloid state stability for specific strand-association modes.

Self-assembly of polypeptides to form amyloid deposits is thought to underlie many human diseases.[1] Low-resolution methods such as IR and x-ray fiber diffraction suggest that β-sheet secondary structure is a common motif in disease-relevant aggregated states, despite the variety of sequences and diversity of folding patterns among polypeptides known to form amyloids.[2] Recent solid-state NMR studies have provided atomic-level structures for several amyloids, including those formed by the peptides Aβ(1-40) and Aβ(1-42), which are associated with Alzheimer's Disease.[3,4] These structural models display three common features. (1) Strand-forming segments associate intermolecularly via in-register parallel β-sheet H-bonding networks.[5] The H-bonds run along the fibril axis, and the peptide assemblies are therefore unbounded in this dimension. (2) At least one segment of each polypeptide forms a loop that causes reversal of backbone direction. (3) β-Sheet layers pack against one another via side chain-to-side chain contacts; the side chains are mostly nonpolar, which suggests a hydrophobic drive for amyloid assembly. The term "amyloid state" has been used to describe this specific combination of secondary and quaternary structure,[6] which differs from the folding and assembly patterns observed among soluble proteins.

Understanding of relationships between sequence and protein structural stability has emerged from the ability to disrupt natively folded states, typically via heating or chemical denaturation of proteins that are soluble and monomeric.[7] This experimental approach allows one to evaluate how amino acid changes at individual sites affect conformational stability, so long as the change does not substantially alter the folded structure. The amyloid state, particularly as manifested by pathogenic examples,[8] is not ideally suited for this structure-disruption approach because a single sequence, such as Aβ(1-40), can adopt multiple amyloid forms with different backbone conformations and side chain interaction patterns.[3,4,9] Therefore, it is not clear that a particular amyloid structure will be maintained when the identity of a residue is altered in a polypeptide that forms pathogenic amyloids. (In contrast, functional amyloids,[8] which are subject to evolutionary selection pressure, are amenable to the residue-variation approach, because the backbone conformation and assembly pattern persist in mutants.[10])

Here we describe an important step toward a strategy for creating a minimum-sized, soluble model system for the amyloid state.[11] This approach may ultimately support sequence-stability measurements relevant to the amyloid state. Critical to our long-term goal is the ability to specify which portions of the design form β-strand segments, and which portions form inter-strand linkers. Our approach builds on previous development of small, parallel β-sheets in aqueous solution,[12] which required a diamine reverse-turn unit to link peptide strands via their C-termini[13] and a diacid to link peptide strands via their N-termini.[14] To build toward a minimal amyloid motif, we envisioned face-to-face packing of an N-linked parallel β-sheet against a C-linked parallel β-sheet (Figure 1A). Critical to achieving this goal is identifying a way to link parallel β-sheets that promotes the side chain-mediated association characteristic of the amyloid state, rather than the H-bond-mediated association characteristic of a multi-strand β-sheet.

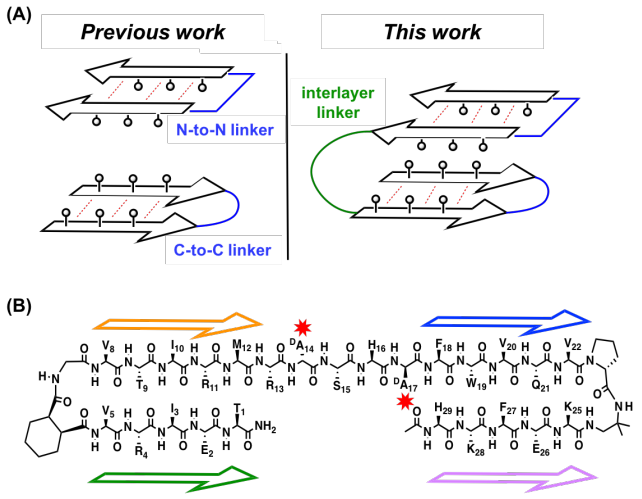

Figure 1. (A) Design of two-stranded parallel β-sheets based on linking strand segments via their C-termini (N-to-N linker), or via their N-termini (C-to-C linker) (left), and the use of parallel β-sheets to generate a minimal model for the amyloid state, which requires a new type of interlayer linker (right). (B) Peptide **1**. Intended strands indicated by colored arrows. The strand indicated in green is deleted from **2**. Red stars indicate the positions in the β-arc that contain D residues in **1** and are altered in **3-5**.

For guidance on the design of an inter-sheet linker, we turned to the multi-layered β-solenoid motif among soluble proteins.[15] Strand segments in neighboring β-solenoid layers interact via parallel β-sheet H-bonds. Within each layer, the strand segments are linked via short loop-forming segments. Strand segments in a given layer and in adjacent layers interact in a side chain-to-side chain manner that is reminiscent of amyloid-state packing. Kajava et al. have proposed the term "β-arch" to describe a β-solenoid strand-loop-strand motif in which the antiparallel strands interact exclusively via side chains;[15a] in contrast, strands in a "β-hairpin" interact via H-bonds.[16,17] The loop segment in a β-arch is designated a "β-arc".[15a] Bioinformatic analysis suggested that a β-arc must contain at least five residues to allow antiparallel orientation of attached strands with an interstrand separation sufficient for amyloid-like side chain-side chain contacts. Among five-residue β-arcs, the most common local conformation at positions 1, 3 and 4 displays standard β-sheet φ and ψ torsion angles (designated *b*). At positions 2 and 5, the φ and ψ torsion angles are consistent with a *left-handed* α-helix (designated *l*),[15a] a local conformation that is rare in proteins. The PDB has grown considerably since the original study,[15] and we therefore updated the analysis of five-residue β-arcs, confirming the prevalence of the *blbbl* conformation.[18]

Our mini-amyloid design hypothesis features D-Ala at β-arc residues 2 and 5, to favor *l* conformations at these sites (**1**, Figure 1B). The most common L-residues[18] were placed at β-arc positions 1 (Arg) and 4 (His), while Ser was placed at 3 rather than the more common Cys, to avoid redox complications. Statistical preferences guided our choice of the strand segments immediately adjacent to the β-arc, Ile-Arg-Met and Phe-Trp-Val. The remaining strand residues in **1** were chosen based on: (1) high β-sheet propensity, (2) net positive charge, to ensure solubility at acidic pH, and (3) maximal residue diversity, to promote ^{1}H NMR resonance dispersion.

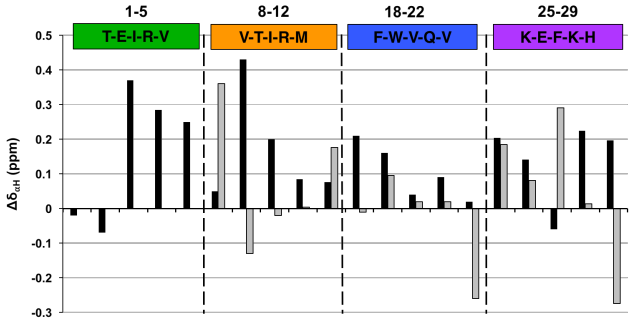

Figure 2. Secondary α-proton chemical shifts of peptides **1** (black bars) and **2** (gray bars). NMR spectra were obtained at 5° C, with 0.2 mM peptide, in 9:1 H_2O:D_2O, 2.5 mM NaOAc-d_3, pH 3.8. Random coil values were calculated using CSDb[2].

Peptide **1** appeared to be monomeric over the concentration range 0.02-0.2 mM in 3 mM aqueous sodium acetate buffer, pH 3.8, based on NMR diffusion measurements (DOSY) and analytical ultracentrifugation (AUC). 2D NMR measurements (COSY, TOCSY and NOESY) for 0.2 mM **1** in 9:1 H_2O:D_2O, 3 mM NaOAc-d_3, pH 3.8, 5° C, allowed assignment of almost all proton resonances. Figure 2 summarizes chemical shift data from the α-protons of the residues in **1** that are intended to adopt strand conformations; we plot the parameter ΔδαH = δαH(**1**) - δαH(random coil).[19] Residues involved in β-sheet secondary structure tend to display positive ΔδαH values, while residues in α-helices tend to display negative ΔδαH values.[20] The generally positive ΔδαH values for **1** are consistent with population of the mini-amyloid folding pattern. Peptide **2**, the analogue of **1** that lacks the T_1-V_5 strand, does not display a consistent positive trend among ΔδαH values, which suggests that a minimum degree of hydrophobic side chain burial is necessary to drive mini-amyloid folding.

To gain further insight on the folding of **1** in aqueous solution, we examined NOEs between protons on residues that are not adjacent in sequence, which provide strong qualitative evidence for the presence of compact conformations. Parallel β-sheet secondary structure is associated with a characteristic NOE pattern involving an amide N-H that serves as an H-bond donor and CαH of the residue aligned on the neighboring strand.[21] Two CαH--HN NOEs of this type are observed between the

strands connected by the N-to-N linker, and three are observed between the strands connected by the C-to-C linker (red arrows, Figure 3). Several longer-range NOEs suggest side chain-mediated packing of one parallel β-sheet against the other (blue arrows). These diagnostic NOEs are consistent with partial population of the mini-amyloid folding pattern. ^{1}H resonances of **1** become less dispersed as temperature is raised,[18] suggesting that the degree of mini-amyloid folding decreases at elevated temperature.

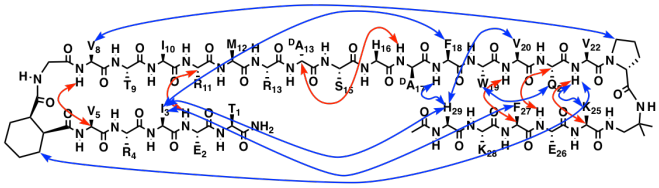

Figure 3. Backbone-backbone NOEs (red) and NOEs involving at least one side chain proton (blue) for peptide **1** (0.2 mM in 9:1 H_2O:D_2O, 2.5 mM NaOAc-d_3, pH 3.8, 5 ºC).

Circular dichroism (CD) in the far-UV region arises largely from backbone amide groups and therefore provides insight on secondary structure.[22] Peptide **1** (0.2 mM in 2.5 mM aqueous NaOAc, pH 3.8) displays a moderate minimum at ~220 nm (Figure 4), suggesting a modest amount of β-sheet secondary structure, as expected if the mini-amyloid state is partially populated. In contrast, truncated peptide **2** does not show a minimum in this region.

In order to test our β-arc design hypothesis, we used CD to compare **1** with full-length analogues varying only in the β-arc segment. Peptide **3**, the diastereomer of **1** with two D-Ala→L-Ala changes in the β-arc, lacks a minimum between 210 and 220 nm, suggesting little or no β-sheet. This observation is consistent with the bioinformatics-derived conclusion that amyloid-like folding requires left-handed α-helix torsion angles at positions 2 and 5 of the β-arc. In **4**, β-arc positions 2 and 5 are L-Asn, the residue we found to be most common in these positions of natural β-arcs.[18] The CD data for **4** suggest that L-Asn in these positions is not significantly better than L-Ala in promoting amyloid-like folding. The CD signature of **5**, which has Gly residues at β-arc positions 2 and 5, is very similar to that of **4**. 2D NMR analysis of peptides **3-5** indicated lower dispersion of resonances relative to **1**, which supports the conclusion that **3-5** do not adopt a specific folding pattern, in contrast to the mini-amyloid folding of **1**. The lower NMR dispersion precluded NOE assignment for **3-5**.

To try to approach physiological ionic strength, we analyzed 0.1 mM **1** in pH 3.8 buffer containing 100 mM NaCl by NMR, CD and AUC; the data indicated increased β-sheet content and self-association.[18] This associated form was completely soluble and could be reversibly disrupted by heating. Future efforts to approach physiological conditions will include studies at pH 7.

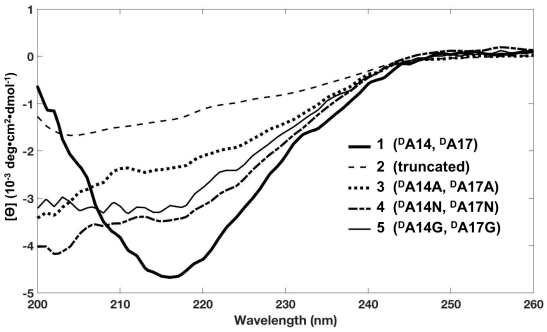

Figure 4. Per-residue CD of 0.2 mM **1-5** in aqueous 2.5 mM NaOAc, pH 3.8 (20º C).

The behavior of **1** represents a significant step toward modeling the unique structural properties of pathogenic amyloids in a soluble system. A combination of 2D NMR and CD data suggest that **1** can adopt a conformation in which the four strand residues associate in a manner reminiscent of the strand arrangement in pathogenic amyloids, with parallel β-sheet H-bonding between two pairs of strands, and side chain-to-side chain packing between the two parallel β-sheets. Our findings with linker variants **3-5** support the bioinformatics-derived hypothesis that a five-residue loop with *blbbl* conformation allows side chain-mediated packing of parallel β-sheets against one another, a feature that is characteristic of the amyloid state.

Our approach to amyloid mimicry is complementary to those of other groups. Eisenberg et al. have provided numerous crystal structures of very short peptides derived from amyloidogenic proteins.[23] These structures reveal at atomic resolution both antiparallel and parallel modes of side chain-mediated strand packing. Nowick et al. have described peptide macrocycles that enforce specific intramolecular antiparallel β-sheet H-bond registries.[24] NMR and crystallographic data for these macrocycles elucidate specific modes of side chain-mediated assembly that may also occur in toxic oligomers formed by amyloidogenic peptides. Collectively, these precedents and our new approach to generating an amyloid state model in aqueous solution should be useful for acquiring deeper insight on the factors that control amyloid stability.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Figures S1-S12 and Tables S1-S4 along with peptide preparation, NMR, AUC, and CD experimental set-ups can be found in the SI.

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ACKNOWLEDGMENTS

This research was supported in part by NIH grant R01GM061238 (SHG). DNW and GJB are supported by the ERC (340764), and DNW holds a Royal Society Wolfson Research Merit Award (WM140008). V. M. Kung was supported in part by the Integrated Training for Physician-Scientists program, T32 GM008692. AUC data were obtained at the UW-Madison Biophysical Instrumentation Facility, which was established with support from BIR-9512577 (NSF) and S10-RR13790 (NIH). The Bruker AVANCE 600 NMR spectrometer was supported by NIH grant S10-OD012245.

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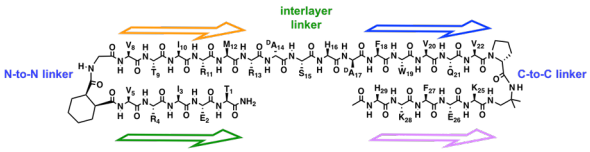

soluble “mini-amyloid” system