Invited Paper

**Amyloid Structure: Models and Theoretical Considerations in Fibrous Aggregates**†

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In celebration of the many contributions to peptide chemistry from K.-T. Wang’s laboratory, this manuscript explores the structure of the Aβ(10-35) fibril. This central segment of the Aβ peptide of Alzheimer’s Disease self-assembles into well-ordered paracrystalline arrays that seem to contradict many of the accepted paradigms established for soluble, globular proteins. Here we exploit initial molecular modeling efforts to assist in understanding these apparent contradictions. The emerging structure is one of a large self-assembly of β-strands, whose stability is dependent on large spatial and temporal fluctuation about a central core, giving rise to what can be best described as a dynamic and fluid tube-like micelle. This fibril structure maintains features that are distinctly different from those of either synthetic or biological fibrils, and these differences have profound implications for biomedical intervention in amyloid diseases as well as for the design of self-assembling nanoscale fibrils.

INTRODUCTION

As our methods of analysis continue to improve, unusual secondary structures within proteins are recognized. These structures are often viewed as derivatives of the recognized α-helical and β-sheet structures, but they range from the rare to the very common within known proteins. Increasingly, these structures are proving to be critical to our understanding of protein structure and function. For example, the controversial γ-helix, whose existence and stability have been debated for decades, now appears to exist on average once in every protein and may be specifically associated with active sites or conformational switch regions. Amyloid fibril diseases, including Alzheimer’s disease, the spongiform encephalopathies, various systemic amyloidoses, and diabetes, are based on another unusual structure that has generated enormous interest. The contributions of many scientists, most notably those of Professor K.-T. Wang, to peptide chemistry and protein synthesis, has made it possible to synthesize these structures and study their properties. In this paper we discuss the combined use of spectroscopic and computational methods to further understand the self-association of peptides and proteins into amyloid fibrils.

“Amyloid” is a clinical term describing unusual proteinaceous tissue deposits. The properties of these deposits include insolubility, large molecular weight aggregates, resistance to proteolysis, and resistance to chemical denaturation even under strong conditions such as boiling detergents. Along with these properties, a defining characteristic of amyloid is association with the hydrophobic dye Congo Red. These stained aggregates display a characteristic apple green birefringence when viewed through crossed polarizing filters.

In Alzheimer’s disease, a series of cleavage events of the amyloid precursor protein (APP) gives rise to specific short peptide fragments, most notably Aβ(1-40) and Aβ(1-42). These fragments have the potential to aggregate and form amyloid deposits in brain tissue. At least two lines of evidence strongly suggest that the initiation of Alzheimer’s disease is associated with the aggregation of these peptides. First, mutations within the APP that affect the length of the peptides, or that increase the rate of production of the peptides, correlate with the severity of the disease. Second, synthetic fibrilized Aβ peptides are toxic to cultured neurons. As a result of these implications, a structure of the Alzheimer’s fibril has been sought for several decades.

FIBRIL STRUCTURAL MODEL

The large molecular weight, insolubility, and non-crystallinity of the aggregated state of the Aβ peptides have

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complicated the gathering of high-resolution structural information. X-ray fiber diffraction analyses\(^9\) give a characteristic cross-beta pattern with reflections corresponding to \(\sim 5\) and \(\sim 10\) Å. These reflections have been assigned to the spacing between the strands in \(\beta\)-sheets and to the mean distance between stacked \(\beta\)-sheets, respectively. The cross-beta pattern suggests that the long dimension of the fiber is composed of \(\beta\)-strands aggregated into \(\beta\)-sheets and that the perpendicular dimension is composed of stacked \(\beta\)-sheets. These X-ray results have also been interpreted to suggest macroscopic organization of the fibers into bundles, and some evidence has been produced for such an organization.\(^9\)

Based on FTIR and circular dichroism measurements,\(^10\) it was generally believed that the fibers were composed of anti-parallel \(\beta\)-strands. Indeed, some authors have argued that the reflection at \(\sim 10\) Å in fiber diffraction experiments arises from the repeat distance between \(\beta\)-strands that run in the same direction within \(\beta\)-sheets in the fibril (parallel to one another), but which are separated by \(\sim 10\) Å in an anti-parallel \(\beta\)-sheet.\(^{9a,e}\)

Recently, however, a comprehensive and experimentally constrained model for the amyloid fiber formed by A\(\beta(10-35)\) has been proposed to be that shown in Fig. 1.\(^{11}\) According to the model, the fibers formed by A\(\beta(10-35)\) peptides consist of parallel \(\beta\)-strands aggregated into parallel \(\beta\)-sheets along the long axis of the fiber. In the perpendicular dimension, the fiber is composed of six such parallel \(\beta\)-sheets, stacked one on top of the other, each sheet apparently also parallel to the others. As the fiber lengthens, i.e., as the number of parallel \(\beta\)-strands added to the \(\beta\)-sheets grows, the arrangement slowly spirals around the fiber axis, with a helical half-repeat distance on the order of \(1200\) Å. One half turn of this slowly twisting fibril would be a rectangular species of approximately \(60\) Å \(\times 80\) Å \(\times 1200\) Å, containing 1440 peptides, 6 sheets \(\times 240\) peptides \(\times 5\) Å/peptide, each having a molecular mass of 2855 Da.

The most well determined feature of this model is its parallel arrangement of \(\beta\)-strands established by solid-state NMR studies. In each experiment, a single \(^{13}\)C label was synthetically incorporated at the carbonyl position of one amino acid within the primary sequence of A\(\beta(10-35)\), and the peptide was allowed to form fibrils. When fibrillization was complete, the resulting intermolecular distance between the \(^{13}\)C enriched carbonyls was analyzed using the DRAWS solid-state NMR pulse sequence, a sequence which recouples dipole-dipole interactions between like spins.\(^{12}\) These distance measurements were used to establish that the entire A\(\beta(10-35)\) peptide stacks in a parallel, in-register orientation throughout the length of the fibril as shown in Fig. 2.\(^{11,12a,13}\)

Recently, this feature of the model was independently verified for A\(\beta(1-40)\).\(^{14}\)

The motif of six, stacked \(\beta\)-sheets is also well determined. Small angle neutron scattering experiments permitted assignment of the mass per unit length of the fiber to be \(3569 \pm 340\) Da/Å. For a single A\(\beta(10-35)\) peptide separated from its neighbors by \(5\) Å, the corresponding mass per unit length would be \(572\) Da/Å. Thus, the fibril is composed of \(6.2 \pm 0.6\) stacked peptides, however at lower pH, the number of laminated sheets can be smaller.\(^{11}\)

Less well defined is the relative orientation of the individual sheets. Apart from the physical reasonableness of a parallel arrangement of the stacked \(\beta\)-sheets, which clusters and hence buries hydrophobic surface area, the structure is supported by a biochemical cross-linking experiment originally developed by Dudek et al.\(^{15}\) Cross-linking by tissue transglutaminase specifically condenses Q15 and K16 side chains of A\(\beta(10-35)\)\(^{13a,11}\) to give hexameric covalent aggreg-
gates. Because Q15 and K16 are adjacent to one another in primary sequence and project from opposite sides of an extended β-strand, the side chains are too far apart to be linked intramolecularly. Likewise, if such β-strands assemble into an in-register parallel β-sheet, all Q15 and K16 side chains are localized on opposite faces of the β-sheet. Therefore, cross-linking must occur between sheets, rather than within the same sheet. Although anti-parallel stacking of these sheets appears possible, parallel stacking would better sequester hydrophobic surface area, maximize contact by minimizing the vacancies between sheets, and place Q15 and K16 of adjacent sheets in proximity.

MODEL EVALUATION

The model of the Aβ(10-35) fiber contains a number of features inconsistent with known globular protein secondary and tertiary structures. First, the size of the assembly is extremely large and requires a very favorable Gibbs Energy of self-assembly. Second, the model involves parallel β-sheets, which are generally agreed to be less stable than their anti-parallel counterparts. At least in the absence of compensating interactions, the parallel β-sheet implies angled hydrogen bond vectors, rather than the near co-linear arrangement that is possible in anti-parallel structures. Third, the model predicts that the twist within each parallel β-strand is small, whereas the twists observed in β-strands occurring in proteins can be quite large. In native proteins containing anti-parallel β-sheets, the twist associated with movement along a β-strand tends to separate adjacent strands from one another, limiting the number of constituent amino acids that can hydrogen bond. Likewise, parallel β-strands are usually formed by stretches of amino acids that are relatively distant from one another in primary sequence, and often the intervening sequence forms a secondary structure that stabilizes the parallel β-sheet, but limits its length. Such arrangements are readily apparent in the parallel β-barrels, for example, in triosephosphate isomerase. The Aβ(10-35) β-strands in amlyoid are composed of 26 amino acids, a much larger number than found in globular proteins. Finally, the model predicts that like charges assemble in space close to one another, an association that is expected to be energetically unfavorable.

In light of the above-mentioned energetic restrictions, the model has been evaluated computationally using a 6 × 6 block of the fibril - a six-stranded parallel β-sheet of the Aβ(10-35) in one dimension and six such sheets stacked in a parallel array in the perpendicular dimension-constructed in Sybyl6.7. The starting structure aligned all backbone amides in the same plane, and constrained distances between pairs of carbonyls to those distances presented above in Fig. 2. Side chains were accommodated within the 10 Å gap of the parallel laminated sheets. The system was solvated using TIP waters. An immobile boundary of solvent 8 Å wide was placed 30 Å from the surface of the assembly to provide a bounded solvation shell. Thus, an inner fluid shell of water (22 Å wide) surrounded the amyloid model. Since a description of the dynamics and flexibility of the amyloid fibril was sought, the use of an outer rigid wall of water surrounding a fluid inner bath was considered adequate to reflect experimental properties in the interior of the peptide fibril.

Molecular dynamics (MD) simulations with the Kollman all-atom force field and charges (NTV conditions) were run initially at 20 K, and then increased stepwise to 50 K, 100 K, 200 K, and 300 K. At each temperature, the system was allowed to reach equilibrium, as evinced by the appearance of harmonic motion among the vibrationally coupled side chains. Subsequently, the system was subjected to MD for an additional 65 ps at 300 K after which time the backbone OC-CO and CO-HN distances were measured. The four β-sheets located in the center of the system, that is, those not in direct contact with the nearest water shell, were used for analysis. Overall, the measured backbone OC-CO distances from the simulation corresponded to the experimental solid-state 13C-13C distance measurements. Residues at the terminal regions of any strand are located closer in space to adjacent strands than the residues in the middle region (residues 27 to 34) where longer distances were observed (Fig. 3). Specific
distortions were apparent in the vicinity of these central residues. For example, there appears to be a general expansion of the inter-peptide distances, consistent with, and possibly a result of, the longer distances observed by NMR and used initially to constrain this region. Moreover, these distortions are characterized by a loss in the regularity of backbone conformation of the kind observed in more terminal regions of the sequence. While other regions of the peptide strands within a sheet were seen to bend and buckle almost in tandem with one another, in the central region of the sheet the conformation of one strand seems to be uncoupled from the conformations of those that surrounded it. These distortions are particularly apparent along the GSNKG sequence, and may reflect the presence of a “glycine-channel” created by the absence of side chains at glycine, each of which occur on the same face of each sheet. In the simulation, this channel allows for solvent molecules to penetrate the protein layers over time to solvate the β-strands.

What is driving these distortions?

A search of the Protein Databank for proteins with parallel β-sheets that do not twist to form a β-barrel identified phosphoglycerate kinase (PhP). While the sheet is relatively planar, the lengths of the PhP β-strands stretch to only 4-5 residues. Silk is known to consist of stacks of relatively planar anti-parallel β-sheets, but again strand length is on the order of 5-6 residues. Therefore, the energetic cost of forming very long idealized β-strands appears to be prohibitive.

In order to better understand the conformational energies, the approximate cost of expanding the length of a parallel β-strand was modeled with short strands and minimized using MacroModel 6.5 with GB/SA solvation and the AMBER* force field. β-strands were built from fragments of 2, 3, 4, 5 and 6 residues from the core KLVFFA sequence (amino acids 16-22) of the amyloid peptide. First, the strands were constrained to adopt ideal parallel β-strand geometry, and minimized. Next, the constraint to maintain ideal parallel β-strand geometry was removed, and the system was re-optimized. The energy difference between the unconstrained and minimized peptide and the constrained and minimized peptide was determined as shown in Fig. 4. For peptide lengths of 2, 3, 4, 5, and 6 residues, these energy differences were 5.2, 6.0, 8.8, 11, and 13 kcal/mol, respectively, with the unconstrained system being more stable in all cases. Although the energy differences between the constrained and unconstrained geometries are a function of the specific amino acid residue, the observed energy required to maintain an idealized geometry across this sequence is approximately linear with chain length. The average value, representing the cost of lengthening a parallel β-strand with ideal geometry by one residue, was calculated to be 2.1 kcal/mol.

**How do these distortions affect the H-bonding along the strands?**

Estimates of the extent of backbone hydrogen bonding were made using the definition that only a CO-HN distance between 1.8-2.3 Å qualifies. While groups or blocks of H-bonds were identified, regions where more than a few hydrogen bonds appear consecutively along the backbone are rare. Further, the location of these backbone hydrogen bond blocks were found to vary with time in a seemingly random pattern. However, at any given time step in the simulation, the total number of hydrogen bonds appears constant. For example, after 50 ps of equilibration at 300 K, an average of 33 hydrogen bonds occurs between β-strands and that number remains constant over the next 6 ps for the four internal sheets (Table 1).

Fig. 5 shows a view along the length of two adjacent strands within a sheet. Where hydrogen bonds occur, they define an approximate plane, but a number of unsatisfied hydrogen bonding partners have arisen by out-of-plane twisting. Although the exact reason for the twisting distortion is unknown, one possibility is that such movement relieves the dihedral pressure of maintaining the parallel β-strand geometry.

![Fig. 4. Cost of ideal parallel β-strand geometry vs. peptide length.](image-url)

**Fig. 4.** Cost of ideal parallel β-strand geometry vs. peptide length. The length of the β-strand was varied from two residues to six within the core amyloid sequence KLVFFA (residues 16-21). Geometry optimizations (using AMBER* and GB/SA solvation) were performed on the small peptides initially with the backbone amide constrained to the MacroModel 6.5 idealized β-strand geometry and, then again with all constraints released. The apparent cost of maintaining the ideal β-strand geometry is given by the slope, 2.1 kcal/mol/residue (linear regression, R² = 0.9764).
try. Since motion within one sheet clearly affects adjacent sheets, via the sheet-to-sheet side chain packing, coupled motions within the fiber are expected. Such coupled motions may help to explain how, as presented in Table 1, the total number of hydrogen bonds predicted within the fiber remain constant, even though their positions change with time. As a result, strand energies appear to limit the number of residues possible in stretches of regular parallel β-strand structure, but it might be the movement of these blocks of short stretches of hydrogen bonding that simultaneously permits global fibril stability and the local flexibility required to form long regular structures.

In addition, it was found that a significant number of hydrogen bonds occur between peptide backbone and solvent molecules within the fiber. Additionally, these solvent molecules were observed to move within the structure. Thus, solvent not only serves to fill spaces between β-strands, as in the region from G₃₀ to G₃₄ discussed above as a glycine channel, but may also provide additional elements of stability by hydrogen bonding peptide side chains within the fiber. On the basis of this modeling, the amyloid fibril appears dynamic and flexible, participating in time-dependent breakage and formation of backbone hydrogen bonds and generating distinct regions of regular and irregular structure.

How can buried charges be accommodated?

As stated above, the parallel arrangement of β-strands within the amyloid fiber brings like charges close in space. However, ionic detergents and other ionic amphiphiles cluster like charges as their hydrophobic portions are desolvated, and the charged surfaces of cells or lipid vesicles are much larger and more highly charged than amyloid peptides. In this regard, how serious of an objection to the model is actually represented by the fact that it predicts the clustering of like charges?

Amyloid fibrils are less amphiphilic than the detergents and some of the charged side chains are internal. Amyloid formation is generally known to be pH dependent, to proceed relatively slowly under strongly acidic conditions, likely reflecting a requirement to deprotonate basic sites within the sequence before fibrilization can ensue. The Aβ(10-35) sequence certainly contains acidic and basic residues, and Burkoth showed that Aβ(10-35), with 3000 MW polyethylene glycol attached at the C-terminus, attains the greatest extent of β-character at pH 5.6. As the pH approaches the pI, peptide-peptide contacts and self-association become increasingly possible with Aβ(10-35). This observation is consistent with typical protein behavior, as native proteins are often found to aggregate or precipitate in the vicinity of their pIs.

Thus, protonation and deprotonation events may be major contributors to the processes by which amyloid stabilizes polar and charged species, and may also be important in the fibrilization pathway. In addition, the simulations reported here suggest another method by which polar and charged groups can be accommodated within the interior of the fiber. As noted above, solvent molecules were identified within the proposed glycine channel in the central region of the fiber, and observed to move throughout vacancies within the structure. The presence of interior solvent molecules could stabi-

Table 1. Variation of the Number of Backbone Hydrogen Bonds in the Internal β-Sheets as a Function of Time

<table>
<thead>
<tr>
<th>Laminated Sheet*</th>
<th>Time (picoseconds)</th>
<th>Average (per sheet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 51 52 53 54 55</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 13 10 11 13 19</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>5 8 7 8 4 2 6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9 6 10 8 9 9</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>9 9 4 5 5 6</td>
<td>6</td>
</tr>
<tr>
<td>Total (per ps)</td>
<td>33 26 31 32 31 35</td>
<td></td>
</tr>
</tbody>
</table>

* Sheets 1 and 6 excluded due to possible solvent effects.

Fig. 5. The hydrogen bond plane as viewed down two adjacent β-strands. The yellow lines indicate the position of the H-bonds and show the variation of the relative position of the amide plane of one strand with respect its intra-sheet partner. The variable position of the side chains is most apparent in this view above and below the indicated H-bonding plane.
lize the buried polar and charged groups, partially screening them from the energetic consequences of their sequestration. Specific hydrogen bonding can both encapsulate the charged groups or bridge among them by virtue of H-bonded solvent networks. Furthermore, although the simulations did not explicitly involve salts, ionic species are certainly present physiologically and in experimental amyloid preparations, and these could be readily carried into the fiber as it incorporates solvent.

**DISCUSSION**

As we move into the next phase of protein structure and functional analysis, an understanding of unusual conformations and assemblies takes on increasing importance. Spectroscopic and computational methods have now advanced to the point where large nanoscale self-assemblies can be analyzed and structurally defined. In this paper, the model for the Aβ(1-35) fibril, developed from spectroscopic analyses, has been presented, criticisms of its unusual features outlined, and questions raised by these features addressed with molecular dynamics simulations. The solid state NMR and neutron scattering data, made possible by the homogeneous assembly of the Aβ(1-35) peptide, represent global averages of the inter-atomic positions. Ironically, the NMR reveals that N-terminal and C-terminal portions of the sequence aligned approximately 5 Å from one another, but the central portion maintains longer distances, approaching 6 Å. The NMR experiments have not yet resolved whether these longer distances arise from greater heterogeneity, greater flexibility, or both, within the central regions of the peptide.

In contrast to the spectroscopic and scattering measurements, the simulations analyze the local order in energetic terms and help to address this heterogeneity in a number of ways. First, they indicate that the central portions of the sequence are substantially more flexible, and able to adopt many different conformations. Second, these simulations have demonstrated that β-strand character occurs within local domains, and specifically that the central portion of the peptide sequence deviates significantly from a continuous β-strand conformation. Further, examination of Fig. 4 reveals that local order does occur within the sequence, namely the core hydrophobic sequence of KLVFFA is well defined as a parallel β-strand from one sheet to the next, but those residues preceding it exhibit greater irregularity. Finally, the simulations demonstrate continuous formation and deformation of β-sheet hydrogen bonds within the fiber. Just as structure is irregular within the fiber from one strand to the next, it now appears to be irregular from one time point to the next. Fiber diffraction experiments have indicated that the reflections visible for amyloid peptides tend to be those of the grossest structural details of the fiber; that is, a 5 Å reflection corresponding to the distance between β-strands, and a 10 Å reflection corresponding to the distance between stacked β-sheets. An implication of these observations is the existence of disorder elsewhere within the fiber, suggesting that its interior is not well-packed like that of a folded protein, but rather of indefinite conformation and more “molten-globule” like. The transient nature of the hydrogen bonds observed in the simulations supports the idea of a molten-like structure applying to the backbone conformations as well. As greater organization within a structure decreases its entropy, increased crystallinity within the fibril would be energetically unfavorable. The amyloid fibers are notoriously stable, and these dynamics simulations showing significant temporal and spatial disorder suggest that some of the stability of the fiber, and even its favorable Gibbs energy of self-assembly, may be due to its flexible regular structure and its continuous reorganization with time.

As suggested above, the region of the structure that is expected to be the most dynamic and flexible is the side chain packing in the sheet-sheet interaction. Thus far, information about this feature of amyloid structure has been the most difficult to obtain largely as a result of the inherent symmetry of the assembly. Recently, however, a theoretical treatment of fiber formation has provided groundwork for investigating the issue of fiber thickness. Nyrkova et al. argue that the thickness of a self-assembled peptide fiber will be determined by the interplay of two factors: (i) the interaction energy between β-sheets, and (ii) the elastic deformation energy caused as sheet-sheet interactions which distort the equilibrium structures of the component sheets. They argue that a fiber will increase in thickness until the total elastic deformation energy approximately equals the total sheet-sheet interaction energy. While this theory doesn’t provide specific structural predictions for amyloid, it does allow speculation about the relationship between fiber dimensions and the characteristics of the peptides that make up the fiber. Among these characteristics are charge, amphiphilicity, and primary sequence length. Indeed, unpublished results with other truncation peptides of Aβ have suggested that the primary sequence length does partially determine the overall dimensions of the fiber, specifically by altering its lamination thickness.

To the extent that Nyrkova et al.’s theoretical framework applies, which seems likely since the number and character of amino acids in a self-assembling peptide are ex-
pected to determine the interaction energies between self-assembling sheets, then the theory addresses another published criticism of the Aβ(10-35) model. Serpell’s primary objection to the model was its inability to address the electron microscopic observation that the fibers formed by Aβ(1-40) and Aβ(10-35) peptides have approximately the same dimensions. In an electron micrograph, the observed width of a fiber arises both from the inter-sheet and sheet-width terms. If the shorter peptide forms thicker fibers and the longer peptide forms thinner fibers, then the two fibers might well show the same dimensions, even though reasoning based on the length of the constituent peptides alone would predict very different dimensions.

CONCLUSIONS

Aside from consistency with solid state NMR and neutron scattering data, an appealing aspect of the presented model is its success in explaining previously described characteristics of amyloid fibrils in general. For example, the model requires large scale clustering of hydrophobic surface area, specifically by bringing together the hydrophobic C-termini, consistent with the micelle-like behavior of Aβ peptides. Given that paired Aβ(10-35) fibrils are conceptually similar to long, rod-like micelles, the model takes the detergent-like character of the peptide one step further, by suggesting that the peptide’s amphiphilicity both defines some of the gross structural features of the fibril as well as the pathway for its formation. As the model involves parallel β-strands, like residues are clustered within the fiber, among them, the potential metal binding residues H13 and H14, which are predicted to form multiple metal binding sites within the assembled fibril. The model therefore, also helps to explain the binding of multivalent metal ions to amyloid peptides.

When taken together, the Aβ(10-35) fibrils and possibly amyloid fibrils more generally, can now be understood globally as a large arrangement of self-assembled β-strands whose stability is dependent on large spatial and temporal fluctuation about a central core, giving rise to a structure that can be best described as a dynamic and fluid tube-like micelle. These fibers are functionally different from synthetic fibers, at least in part by their ability to self-assemble, and structurally distinct from biological fibrils whose function is to both stabilize large-scale biological order and mediate mechano-chemical movement. These differences have profound implications for biomedical intervention in amyloid diseases as well as for the design of self-assembling nano-scale functional fibrils. Efforts to further extend the molecular modeling in conjunction with and as a guide to physical measurements in the design and analysis of other fibrils are in progress.

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Key Words

Molecular dynamics; Parallel β-sheets; Amyloid structure; H-bonds.

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