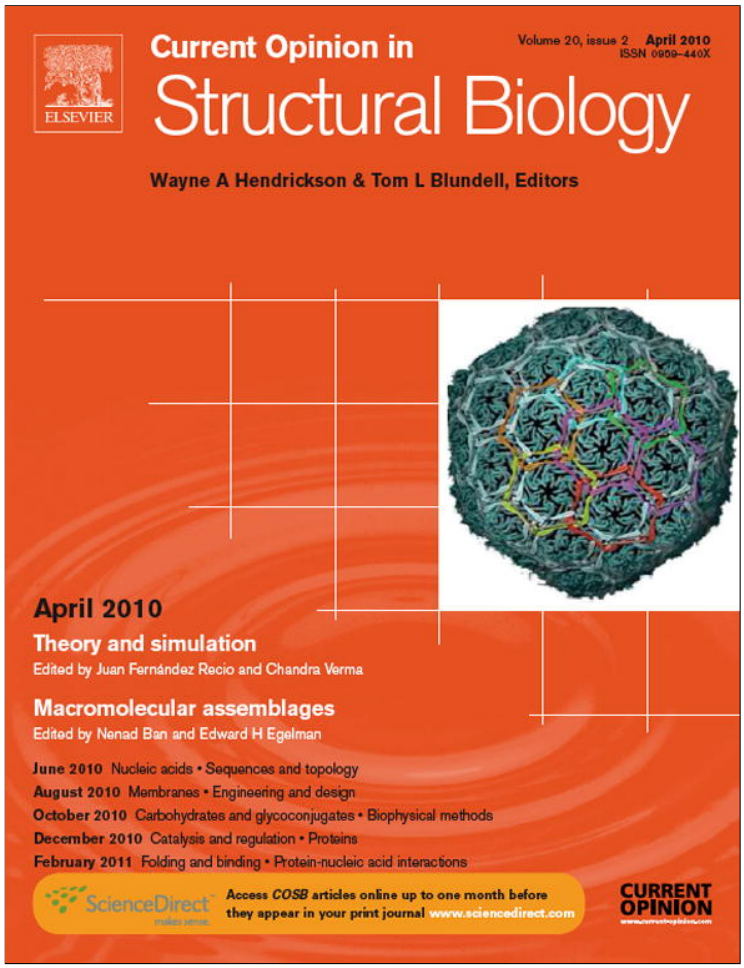

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Principles governing oligomer formation in amyloidogenic peptides

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Identifying the principles that describe the formation of protein oligomers and fibrils with distinct morphologies is a daunting problem. Here we summarize general principles of oligomer formation gleaned from molecular dynamics simulations of Aβ-peptides. The spectra of high free energy structures sampled by the monomer provide insights into the plausible fibril structures, providing a rationale for the 'strain phenomenon.' Heterogeneous growth dynamics of small oligomers of $A\beta_{16-22}$, whose lowest free energy structures are like nematic droplets, can be broadly described using a two-stage dock-lock mechanism. In the growth process, water is found to play various roles depending on the oligomer size, and peptide length, and sequence. Water may be an explicit element of fibril structure linked to various fibril morphologies.

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Introduction

According to the 'amyloid hypothesis' [1••], amyloid disease (AD) is caused by the accumulation of the Aβ-peptide which is a normal byproduct of the metabolism of amyloid precursor protein (APP). The cleavage of APP resulting in Aβ-peptide is achieved through the action of secretases [2]. The primary component of Alzheimer's related amyloid plaques is the Aβ-peptide, a 38-to-43 amino acid polypeptide of known sequence [3,4]. A variety of natural mutations that occur close to the secretase cleavage sites associated with variable AD pathology also produce large variations in the fibril growth rates [5–8]. The potential link between oligomer formation and amyloidogenic diseases has made it necessary to understand the energetics and dynamics of transitions from monomers to oligomers and beyond. Delineating the factors that contribute to the thermodynamics and kinetics of oligomer formation, which is an essential step in the cascade of events that turn the disordered 'collapsed coil' form of Aβ-peptide monomers [9,10] into fibrils with the characteristic cross β structure, is an important step in the discovery of methods that prevent their formation.

Significant advances have been made in the determination of high resolution crystal structures of a number of peptides that form amyloid-like fibrils [11,12••], and in the description of molecular events in the transition from disordered monomers to oligomers [13,14,15••,16•]. While the dynamics and phase diagram of full length aggregating proteins are expected to be considerably richer, the aggregation tendencies of smaller peptides are excellent model systems, which can be used to gain quantitative biophysical insights into oligomer formation. In recent years, a combination of experimental [11,17••,18–20] and computational studies [13,21••,22,23,24••,25•] has led to a microscopic picture of the oligomerization process of peptides including the role water of in their self-assembly. In this perspective, we focus on the role molecular dynamic simulations have played in elucidating some of the general principles that govern the process of protein aggregation with particular emphasis on the initial events in the assembly of Aβ-peptides and their implications for the growth into mature amyloid fibrils.

General considerations in peptide and protein aggregation

The routes to protein aggregation are intimately related to the folding landscape of proteins [26] as a function of several external factors protecting and denaturing osmolytes, presence of crowding agents, and protein concentration. The hallmark of amyloid forming peptides and proteins is that they access one or more 'assembly-competent' structures induced by denaturation, stress or thermal fluctuations with lifetimes sufficient to allow for interprotein interactions to occur. The molecular details of the steps leading to the formation of amyloid fibrils remain unknown because the species along the aggregation pathways are highly dynamic and are likely to be metastable. The overall growth process exhibits the characteristics of a nucleation growth process [27]. Once the critical nucleus (whose characteristics depend on sequence as well as external conditions) forms, the fibril formation process is essentially downhill in free energy. From this perspective, the qualitative scenarios for

explaining aggregation kinetics are in place. However, the details of the process, including the dependence of oligomer formation on the specifics of the sequence and the structural features of the intermediates in the multiple stages leading to the nucleus, are not understood.

Despite the complexity of the aggregation process several theoretical studies [21••,22,28•] show that the spectra of the states sampled by the monomers can provide insight into the tendency of specific sequences to form amyloid structures. Two extreme scenarios, which follow from the energy landscape perspective of aggregation [8], can be envisioned in the description of the early events in protein aggregation [29]. According to Scenario I, which applies to Aβ-peptides, fibril formation requires partial unfolding of the native state [30] or partial folding of the unfolded state. Both events, which are likely to involve crossing free energy barriers lead to the transient population of an ensemble of assembly-competent structures $\mathbf{N}^*$. According to Scenario II, which describes aggregation of PrP^{SCS} [31], the ensemble of $\mathbf{N}^*$ structures has a lower free energy than the structures in the native state ensemble thus making the folded (functional state) state metastable.

The scenarios based on the energy landscape perspective provide a plausible connection to the strain phenotypes that have been extensively studied especially in yeast prion biology [32,33••]. Originally found in the context of wasting diseases [34] and mammalian prions, strain phenotypes, which grow from the same protein but lead to different heritable states, are found even in peptide fibrils [35••] and amyloids grown from Aβ-peptides [17••,36••]. At what stage of the growth of fibrils is a particular strain 'encoded' in the structure? The suggestion that the $\mathbf{N}^*$ structures are aggregation prone implies that the strain phenotypes may be encoded in the monomer structures themselves. We speculate that the various $\mathbf{N}^*$ structures can form oligomers with different structures, which can subsequently lead to fibrils with structurally distinct fibrils. Certainly, it is unlikely that that information for polymorphism in amyloid fibrils is found in post-nucleus structures, which makes monomers or low order oligomers the likely candidates.

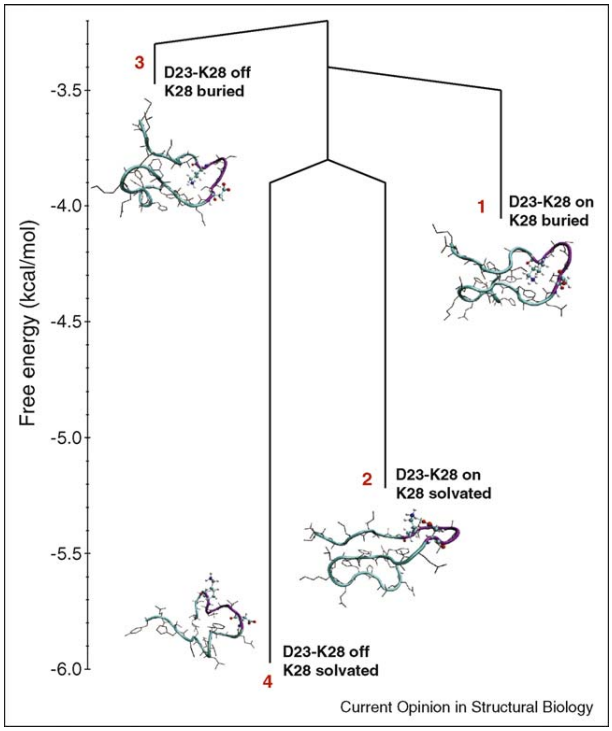
Folding spectra of Aβ monomers

The analysis of protein aggregation in terms of $\mathbf{N}^*$ leads to two key predictions. The first is that ordered aggregation starts in all likelihood from one of the structures that encompass the $\mathbf{N}^*$ ensemble. The second is that the ease of aggregation is related to the probability of populating the $\mathbf{N}^*$ species, which implies that the free energy barriers that separate the lowest free energy basin (unfolded **U** or folded **N** states) and $\mathbf{N}^*$ conformations should dictate the growth kinetics. A number of studies have focused on the characteristic structures that are sampled by the monomer in the hope of gleaning insights into their amyloidogenic tendencies [21••,22,28•,37•,38]. Before discussing the free energy spectra of monomers it is useful to describe the arrangements of Aβ in two well-known fibril models. The solid-state NMR-based structures [39,40] for the fibrils of $A\beta_{1-40}$ (Tycko model) include, as a key structural element, a bend involving residues $V^{24}GSN^{27}$. The structural motif with the $V^{24}GSN^{27}$ turn and intrapeptide salt-bridge between D23 and K28 ensures that isolated charges are not buried in the low-dielectric interior of the fibril. Such a structural motif when stacked in parallel leads to a fibril that satisfies the 'amyloid self-organization principle' that the stability of amyloid fibril arises by maximizing the number of hydrophobic and favorable electrostatic interactions (formation of salt-bridges and hydrogen bonds) [21••].

A different structural model (Luhrs model) for $A\beta_{1-42}$ fibril [41], which maintains the basic strand–bend–strand motif of the Tycko model, suggests that residues [17••,18–20,21••,22,23,24••,25•,26,27,28•,29–32,33••,34,35••,36••,37•,38–42] form in-register parallel β sheets formed from a minimum of two peptides. In this model the side chains of strand 1 (β 1 spanning residues (18–26)) from the n th peptide interdigitate with those of strand two (β 2 that runs from residues (31–42)) of the $(n-1)$ th peptide [41]. The arrangement of strands in this model is somewhat reminiscent of a domain-swap mechanism, which has been proposed as a generic way in which ordered structures can form [42]. A natural consequence of the Luhrs model is that the bend in the monomer involves residues $S^{26}NKGA^{30}$ with K28 being positioned in such a way that it can form a salt-bridge with D23 from β 1 of the neighboring peptide. The possibility of forming an interpeptide salt-bridge was also proposed by Tycko and coworkers [18].

Several experimental and simulation studies have examined the interactions that stabilize the 'folded' (lowest free energy states) of the $A\beta_{21-30}$ fragment [37•,43–45]. Detailed MD studies of $A\beta_{10-35}$ and $A\beta_{9-40}$ monomers validate a key prediction of the '$\mathbf{N}^*$ postulate,' namely, structural elements resembling those in the fibrils manifest themselves in soluble monomers [21••,38]. Using extensive simulations and novel analysis of the data, it was shown that the formation of a stable structure with an intact D23–K28 salt bridge and the VGSN turn is highly improbable in the monomer. Our results suggest that overcoming the large barrier to desolvation of D23 and K28, which can only occur at finite peptide concentration, must be an early event in the oligomer formation. The spectrum of conformational states of $A\beta_{10-35}$ (Figure 1) suggests that a variety of high free energy states are accessible to the monomer. Among them the structures that belong to Basin **4**, with the D23–K28 salt-bridge and $V^{24}GSN^{27}$ formed, resemble those found in the Tycko model for $A\beta_{1-40}$. Similar conclusions were reached in MD simulations of

Figure 1



Free energy spectrum of $A\beta_{10-35}$ monomer obtained from MD simulations. States with the disrupted salt-bridge are more favorable, and a large barrier makes the transition between the formed and disrupted substates improbable. Burying K28 in the peptide interior is an unfavorable process. The number of microstates associated with each of the four basins is indicated in parentheses. D23–K28 on stands for salt-bridge present, while D23–K28 off stands for salt-bridge broken. Reprinted from [21••].

$A\beta_{9-40}$ monomer (see Figure 5 in [38]) and $A\beta_{21-30}$ monomer [43]. It is likely that in the interaction-driven aggregation process such a monomer structure will be accessed relatively early in the fibril formation process.

Interestingly, the salt-bridge is absent and K28 is in the interior in the ensemble of structures in Basin **3**. The burial of charged residue is compensated by additional electrostatic interactions between the ammonium group of K28 and the backbone carbonyl oxygen of F20 and E22 and the hydrophobic interactions between the aliphatic side chain of K28 and the side chains of V24 and I31. It appears that the burial of K28 necessarily distorts the $V^{24}GSN^{27}$ turn, and perhaps displaces the turn region to $S^{26}NKGA^{30}$, as in the Luhrs model [41]. The ensemble of structures in Basin **3** has a significant overlap with the fibril model for $A\beta_{1-42}$. We speculate that if the monomers in Basin **3** are packed to form fibrils, with intact $S^{26}NKGA^{30}$, then the uncompensated charge on K28 can only be accommodated by an intermolecular D23–K28 salt-bridge. The $\mathbf{N}^*$ postulate explains the plausible differences in the different fibril morphologies in terms of the monomer seeds from which they are likely to grow.

We should stress that the $\mathbf{N}^*$ ensemble alone cannot determine the diverse structures observed in the fibrils. The heterogeneous fibril morphologies with helical twists and striations, domain-swapped fibrils, and distinct symmetry arrangements can only be predicted using interactions between multiple chains. These intriguing variations even among fibrils grown from identical sequences cannot be anticipated by focusing on the conformational diversity of the monomers alone. After all, 'More is Different' [46]. Nevertheless, the structures in the different basins of a monomer suggest potential candidates whose packing might provide insights into some of the morphologies observed in amyloid fibrils.

Free energy landscape for $A\beta_{10-35}$ dimer formation

In order to characterize the early stages of Aβ-peptide aggregation pathway, formation of the $A\beta_{10-35}$-peptide dimer was studied in aqueous solution [47••]. Dimer structures were evaluated for stability relative to the separated monomeric peptides, using computed estimates of the desolvation and electrostatic interaction energies, in an effort to identify putative stable dimer structures. The potential of mean force associated with the dimerization of the peptides in aqueous solution was computed using umbrella sampling and classical molecular dynamics simulation at constant temperature and pressure.

Two extreme models for monomer association — one which supposes that the principal mechanism stabilizing the dimer structure is the burial of hydrophobic surface and the other that supposes that the electrostatic interaction is the primary associative stabilizing interaction — were examined. It was found that the former leads to more energetically favorable dimerization [47••]. It is more efficient to remove the entropically unfavorable structured water between the opposing hydrophobic regions of the two monomers than to stabilize the monomer solely through electrostatic interactions.

This finding agrees with the experimental observation that the mutation E22Q — where a charged glutamic acid residue is replaced by a polar glutamine residue — increases the propensity for amyloid formation [48,7] and our previous computational studies of solvation of the E22Q mutant and wild type (WT) peptides [49]. In more recent simulations of the $A\beta_{16-35}$ peptide monomers and dimers, no significant secondary structure formation was observed, while the key $E^{22}DVGSNK^{28}$ region is observed to form a 'loop' structure similar to that observed in shorter fragments and peptide fibrils. It would be profitable to carry out a more detailed energy landscape analysis as has been done for the aggregation of

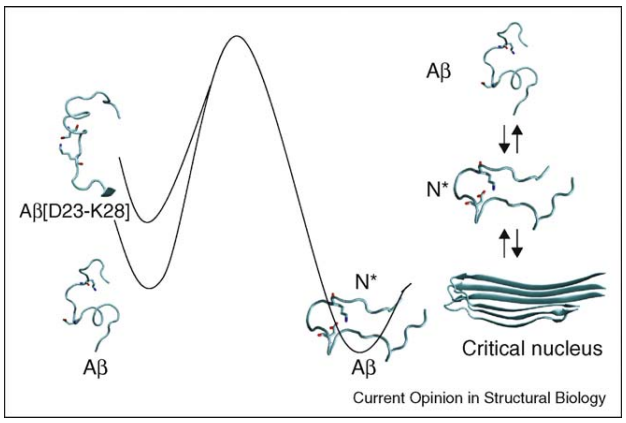
human transthyretin protein fragments [50] and KFFE tetrapeptide [51] to better evaluate the energetics of the aggregation ensemble.

Recent experiments have shown that the congener, $A\beta_{1-40}$ [D23–K28], in which the side chains of residues Asp23 and Lys28 are linked by a lactam bridge, forms amyloid fibrils that are structurally similar to the WT Aβ-peptide at a rate that is nearly one thousand times faster than the WT [52•]. All-atom molecular dynamic simulations of the WT dimer, as well as a monomer and dimers of $A\beta_{10-35}$ [D23–K28] with constrained D23–K28 salt-bridge in explicit solvent, have been used to explore the origin of the observed enhanced rate of fibril formation [38].

Those simulations show that the assembly-competent monomers (**N***), with strand conformations in the residues spanning the N and C termini and a bend involving residues $D^{23}VGSNKG^{29}$, are populated to a greater extent in $A\beta_{10-35}$ [D23–K28] than in the WT, which has negligible probability of forming **N***. The salt-bridge in **N***, whose topology is similar to that found in the fibril, is hydrated. The reduction in the free energy barrier to fibril formation in $A\beta_{10-35}$ [D23–K28], compared to the WT is attributed to entropic restrictions that arise from the salt-bridge constraint (see Figure 2). A decrease in the entropy of the unfolded state and the lesser penalty for conformational rearrangement, including the formation of the salt bridge in Aβ-peptides with D23–K28 constrained, results in a reduction in the kinetic barrier in the constrained $A\beta_{1-40}$ [D23–K28] compared to the WT peptide.

Although a number of factors determine the growth of fibrils, the decrease in the free energy barrier of formation of **N*** in the $A\beta_{1-40}$ [D23–K28] congener, relative to the WT peptide, is a major factor in the rate enhancement for fibril formation [52•]. Qualitatively similar results were obtained using simulations of $A\beta_{9-40}$ peptides. These results support the **N*** conjecture that mutations or other constraints that preferentially enhance the population of **N*** species would enhance aggregation rates.

Figure 2



Free energy landscape of $A\beta_{1-40}$ and the $A\beta_{1-40}$ [D23–K28] peptide congener. By constraining the D23 and K28 residues to be proximate, through a theoretical constraint or formation of a covalent β-lactam bond, the free energy barrier between the monomer and the aggregation competent **N*** is reduced. Reprinted from [38].

Probing the structural characteristics of oligomers of Aβ-peptides

The unstable nature of oligomers makes it difficult to determine their dynamics and structures. Simulations using coarse-grained (CG) models [25•,53,54,55••] have revealed that the formation of oligomers and subsequently fibrils involves a number of distinct stages during which the monomers, oligomers, and protofilaments undergo substantial conformational changes. Interestingly, it has been proposed the mutations that diminish β-strand propensity in the monomeric peptides may diminish fibril formation (by reducing the population of **N***) while enhancing the formation of potentially toxic oligomeric structures [55••]. Typically, it is found that disordered oligomers form readily and the peptides then adopt ordered conformations even when the size of the oligomer is less than the critical nucleus size [13,15••,47••]. While the results from the CG models establish the generic features of protein aggregation, detailed all-atom MD simulations are often necessary to identify the driving force for protein aggregation and the formation of ordered structures.

Oligomer growth mechanism of $A\beta_{16-22}$ fragments

The fragment $A\beta_{16-22}$ ($K^{16}LVFFAE^{22}$) that encompasses the central hydrophobic cluster (CHC) $L^{17}VFFA^{21}$ is predominantly a random coil in isolation [13] with some tendency for the hydrophobic residues (especially V18) to adopt β conformation [56]. In the fibril state the peptides are arranged in an antiparallel manner [57], which results in salt-bridge formation and maximization of hydrophobic interaction between the residues in the CHC. Trimers of $A\beta_{16-22}$ coalesce to rapidly form disordered aggregates driven primarily by hydrophobic interactions between the CHC residues. In the process, the peptide transiently adopts α-helical conformations even though there is no evidence for the isolated monomer to be found in the α-helical basin [13]. At longer times the peptides are arranged in an antiparallel fashion as in the fibril with substantial excursions to other basins of attraction in which the peptides adopt alternate structures [15••,58–60]. The mutants $G^{16}LVFFAG^{22}$ and $K^{16}SVSSAE^{22}$ are unstable [13], thus establishing the importance of both the hydrophobic and electrostatic interactions in stabilizing the oligomer and presumably the fibrils [61].

The growth mechanism of $(A\beta_{16-22})_n$ for $n > 3$ probed by monitoring the reaction $(A\beta_{16-22})_{n-1} + A\beta \rightarrow (A\beta_{16-22})_n$

for n ranging from 4 to 6 has provided a detailed picture of the growth dynamics of oligomers [15••]. In this reaction the unstructured monomer is added to a preformed 'fluid-like' template composed of $(n-1)$ monomers. The lowest free energy structures of the oligomers $(A\beta_{16-22})_n$ resemble nematic droplets with the β-strands aligned along a director resulting in a value of ~ 0.9 for the liquid crystal order parameter. The process of adding a monomer to a preformed nematic droplet can be globally described by a two-stage dock-lock (DL) mechanism, which was first suggested to describe the addition of a monomer to a growing fibril [62].

According to the DL mechanism[62,8], in the first stage the monomer rapidly and nonspecifically docks onto the preformed nematic droplet. In the locking stage the monomer adopts the β-strand conformation of the template nematic droplet. The qualitative aspects of the DL mechanism capture essential features of the time-dependent changes in the β-strand content of the peptides in the nematic droplet and the monomer for the reaction $(A\beta_{16-22})_5 + A\beta \rightarrow (A\beta_{16-22})_6$ [15••]. In the absence of interaction with the nematic droplet the probability of the monomer adopting β-strand is negligible. Interaction with the nematic droplet first results in an increase in the end-to-end distance with a concomitant increase in the β-strand content. The high initial β-strand content of the nematic droplet is maintained during the course of the simulation.

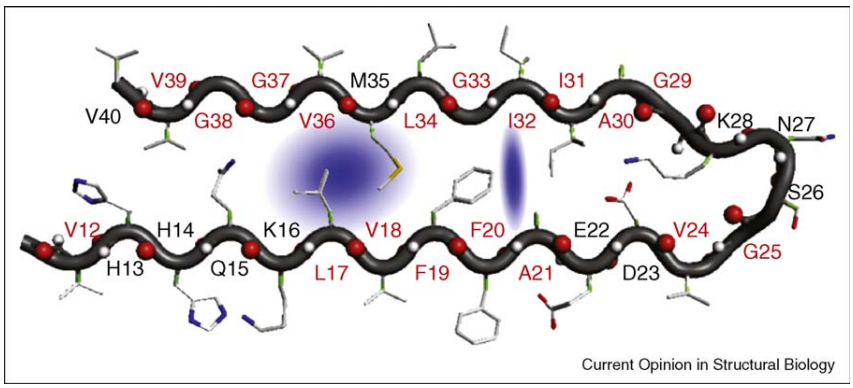
The β-strand content of the added monomer grows in two stages. In the first phase, the β-strand content increases substantially from its initial low value, which shows that most of the growth occurs immediately upon docking. The extent of strand formation continues to increase over a period of tens of *ns* during which there are large changes in the structure of the nascent monomer. In the second stage the monomer adopts a β-strand conformation on a very long time scale.

A few comments about the DL mechanism are in order. First, although discussed in the context of addition of $A\beta_{16-22}$ to a preformed nematic droplet the global description of the growth process in terms of a broad two-stage dynamics is applicable to other systems as well [63••]. The locking time scale, which increases as the number of peptides increases, can be approximately described using the Lifschitz–Slyazov growth mechanism, that is, $\tau_{\text{lock}} \approx \tau_0(N)M^3$ [28•], where M is the number of peptides, and the prefactor $\tau_0(N)$ depends on the length of the peptide. Second, the description of growth dynamics in terms of a two-stage DL mechanism is simplistic. When examined carefully, the assembly of the oligomers consists of multiple stages characterized by a range of time scales. In addition, there is considerable structural heterogeneity in the growth of oligomers (and indeed fibrils [63••]) that cannot be captured by the DL mechanism. The nuances discovered in computer simulations can only be captured using single molecule experiments [64••] and theoretical models that capture the structural fluctuations in the monomers and the oligomers (or fibrils) as they grow.

The role of water in Aβ fibril formation

Simulations of $(A\beta_{16-22})_3$ formation [13] showed the ordered state can form in multiple ways. The rapid formation of disordered oligomers is typically driven by the interaction between hydrophobic residues in the CHC. The observation that the interior of the small orientationally disordered structures is dry implies that expulsion of water molecules occurs on timescales that are far shorter than the timescale on which ordering of the peptides occurs. Mutation of F19 renders the oligomers unstable [13], which further supports the conclusion that the lack of water in the interior of $A\beta_{16-22}$ is largely due to side chain contacts (see Figure 3a and c in [13]) between the residues in the CHC ($L^{17}VFFA^{21}$). The antiparallel orientation requires the formation of the salt-bridge between K16 from one peptide and E22 from another,

Figure 3



Structure of the hydrated monomer in $A\beta_{1-40}$ fibril. The blue shade represents trapped water molecules that are localized in a hydrophobic pocket. Reprinted from [68••].

which underscores the importance of both the hydrophobic and electrostatic interactions in stabilizing the ordered state. In contrast, there are very few stable hydrogen bonds that persist between the peptides [15••].

The expectation that water might play a subtle role as n increases was clearly demonstrated in the assembly of protofilaments of $A\beta_{16-22}$ [65•]. In some of the trajectories water is expelled early before assembly. In other trajectories, the two processes are observed to be coincident. The predominant interactions that mediate protofilament formation are hydrophobic with interactions involving Phe playing a major role, as was previously shown in the context of oligomer formation [13,66,67].

It is remarkable that atomically detailed structures of fibrils grown from a large number of small peptides [11] show that the interior of two sheets is 'bone dry.' This finding has led to the suggestion that a key structural motif of fibrils could be pair of peptides held together by a 'steric zipper' in which the side chains are fully interdigitated. As the size of the peptide increases the complexity of the assembly dynamics must increase, including the way water molecules mediate oligomer and fibril formation. In addition, given the presence of multiple fibril morphologies and the observed heterogeneities in the oligomer formation, we expect many variations in the way water mediates amyloid formation. Subtle roles played by water are starting to be elucidated. Using 2D IR spectroscopy it was recently shown that water molecules (roughly 1.2 per monomer) are trapped in $A\beta_{1-40}$ (Figure 3) fibrils [68••]. The formation of water channels near the salt-bridge (D23–K28) has been observed in simulations of a solid-state NMR-derived structural model [69,70]. However, the experimental finding that there are water molecules in the hydrophobic pocket (L17, V18, L34, and V36) that interact with the amide backbone of L17 and L34 is a surprise [68••]. There are two possible explanations for this finding. If mobile water molecules are not part of the fully mature fibrils, it is likely that the fibril structures with trapped waters are metastable. Only by performing careful kinetic experiments can one assess if the water-trapped fibrils undergo further rearrangements. Alternatively, it is possible that these structures represent another fibril morphology. In light of these results [68••], it is likely that in Luhrs model incorporating interpeptide D23–K28 salt-bridge is soaked with mobile water molecules. The disparate experimental reports show that many of the questions pertaining to the roles discrete water molecules play in the formation of oligomers and fibrils formed from full length Aβ-peptides stand unanswered. In this regard, oligomer formation in reverse micelles with varying hydration levels [71••] should provide a quantitative basis for describing how water mediates amyloid assembly.

Conclusions

Studies of amyloid-forming small peptides have provided valuable lessons, which may be useful in shedding light on the intriguing questions surrounding the considerably more complex process of fibril formation in proteins. Revealing the additional complexities that invariably arise when considering longer proteins will require a combination of new computational tools and experiments that can provide detailed structural and kinetic data. It is also necessary to bridge the gap between the biology and biophysics of fibril formation in order to decipher the structural basis of functional amyloids as well as those implicated in diseases. Finally, we suspect that the idiosyncratic role water plays in leading to distinct strains, which is surely one of the most perplexing aspects of fibril formation, will challenge researchers from all disciplines.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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