# Frustrated peptide chains at the fibril tip control the kinetics of growth of amyloid-β fibrils

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Amyloid fibrillization is an exceedingly complex process in which incoming peptide chains bind to the fibril while concertedly folding. The coupling between folding and binding is not fully understood. We explore the molecular pathways of association of Aβ40 monomers to fibril tips by combining time-resolved in situ scanning probe microscopy with molecular modeling. The comparison between experimental and simulation results shows that a complex supported by nonnative contacts is present in the equilibrium structure of the fibril tip and impedes fibril growth in a supersaturated solution. The unraveling of this frustrated state determines the rate of fibril growth. The kinetics of growth of freshly cut fibrils, in which the bulk fibril structure persists at the tip, complemented by molecular simulations, indicate that this frustrated complex comprises three or four monomers in nonnative conformations and likely is contained on the top of a single stack of peptide chains in the fibril structure. This pathway of fibril growth strongly deviates from the common view that the conformational transformation of each captured peptide chain is templated by the previously arrived peptide. The insights into the ensemble structure of the frustrated complex may guide the search for suppressors of Aβ fibrillization. The uncovered dynamics of coupled structuring and assembly during fibril growth are more complex than during the folding of most globular proteins, as they involve the collective motions of several peptide chains that are not guided by a funneled energy landscape.

amyloid fibrillization | fibril growth | frustrated states | Alzheimer's

The formation of amyloid fibrils of various proteins is a cardinal characteristic of several neurodegenerative diseases that afflict the growing aging populations worldwide (1, 2). Alzheimer's patients display neurofibrillary tangles made up of tau protein and accumulate plaques of the protein fragment amyloid-β (Aβ) outside neurons in the brain (3–6). The precise mechanism of pathogenesis remains unclear. According to one view, neurotoxic Aβ oligomers are the culprits (7–9), but much attention has focused on the structures and formation mechanisms of the Aβ fibrils and plaques, which are the most-characteristic disease markers (10–15). Notably, abundant plaque formation by the Aβ peptide mutated at positions Glu-22 (associated with the Dutch and Arctic types), Asp-23 (Iowa type), Leu-34 (Piedmont type), and others has been found to cause severe cerebral amyloid angiopathy (16). Cleaving the amyloid precursor protein generates a population of different-length polypeptide chains (17, 18). Two Aβ peptide isoforms, composed of 40 or 42 amino acid residues, Aβ40 and Aβ42, are the dominant components of fibrils in vivo (11, 19, 20). Here, we focus on the shorter peptide, Aβ40, which is present at 5- to 10-fold–higher concentrations than Aβ42 (21) and is overrepresented in mature fibrils and plaques (22, 23).

The assembly of Aβ peptides into fibrils is exceedingly complex (24). After the peptides diffuse toward each other, they establish contacts between their amino acid residues and realign these contacts to find conformations that minimize the free energy of the emerging structure (25). The first stage of this process, referred to

as nucleation, engenders a population of small oligomers that may themselves be pathogenic (25–28). Nucleation is succeeded by growth, in which peptides remaining in solution associate with the existing fibrils (29–31). After further growth, the longer fibrils may fracture, doubling the number of growing tips and releasing peptide oligomers, which in turn boost nucleation (28, 32). The fibrils also can branch by secondary nucleation. Both processes autocatalytically accelerate fibrillization (33). This complex series of events can lead to explosive growth of both oligomers and fibrils and encourage the spread of the aggregates and disease in patients' brains.

The entangled fibrillization processes are typically studied in bulk assays (28, 33–35), which often employ agents such as Thioflavin T (35) that fluoresce at a specific wavelength when bound to an amyloid structure (36). The fluorescence signal is assumed to scale with the total fibril mass, which increases due to fibril nucleation, growth, fragmentation, and branching occurring in parallel (37). The presence of a fluorescent tag that binds to the fibrils, however, may modify the kinetics. In bulk assays, the contributions of each of the constituent processes to the evolution of the signals cannot be directly evaluated but must be assessed from fits to models in which each step is expected to obey simple kinetic laws (28, 33, 35). Usually, fibril growth has been approximated as a simple bimolecular reaction between the fibril tips and solute peptides (28, 31). Recent studies have employed atomic force microscopy (AFM) (37–44) and fluorescence microscopy (45–49) to monitor the growth of individual amyloid fibrils of diverse proteins and peptides. In contrast with the steady bimolecular reaction at the fibril tips, usually assumed

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in the models of bulk fibrillization, the growth trajectories sometimes incorporate periods of complete stagnation and the two ends of a fibril often grow at distinct rates. Asymmetric, unsteady, stagnant, and nonbimolecular fibril growth modes have not been considered in the current models. In consequence, the molecular mechanisms of fibril growth have remained elusive, severely restraining the search for ways to suppress amyloid fibrillization.

In the studies reported here, we synergistically join experiments with simulations. We examine the mechanisms of Aβ fibril growth by monitoring individual fibrils (50). Of the two reagents in the bimolecular reaction between fibril tips and the dissolved peptides that leads to fibril growth, the fibril tips are at a concentration lower by orders of magnitude than the peptide concentration. Hence, insight into the structures and dynamics of the fibril tips may potentially provide a route to block fibrillization by agents operational at substantially lower concentrations than those needed to suppress other stages of fibrillization. Our most-powerful tools are the correlations between the measured growth rates, peptide concentration in the solution, and the effect of denaturant, which directly illuminate the mechanism of peptide incorporation and can be compared quantitatively to computer simulations. For insights on the energetics of the intermediate and transition states for fibril incorporation, we study the effect of added urea, whose impact on the formation of interchain and intrachain contacts is relatively well understood from studies of protein folding. The direct determination of individual fibril growth rate that we employ affords the opportunity to elucidate the mechanistic complexity arising from the concurrent binding and folding of the incoming peptide chains.

## Results and Discussion

**Kinetics of Fibril Growth.** To examine the growth of Aβ40 fibrils, we use time-resolved in situ AFM (Fig. 1 and *SI Appendix*, Fig. S1) (50). We deposit fibril seeds on mica and monitor the growth of both fibril ends toward fixed reference points (Fig. 1*A*) in solutions of Aβ40 monomer (*SI Appendix*, Fig. S2) with known concentrations. We evaluate the fibril growth rate as the slope of the time correlation of the fibril tip displacement (Fig. 1*E*) (50). Our previous work employing this method (50) revealed that the fibril growth rates and solubility measured using in situ AFM are close to those determined from time-dependent bulk growth of fibrils in solution (31). This comparison certifies that interactions with the substrate that may strain the fibrils or assist the supply of monomers to the fibril tip do not modify the growth rates, in contradistinction to results with Aβ42, the short amyloid peptide Aβ (12–28), and amylin (43, 51, 52). We also established that the opposite ends of individual fibrils grow at similar rates, and the growth was relatively steady (50). Time-resolved in situ AFM monitoring of fibril growth revealed that the fibrils readily dissolved in quiescent peptide-free solutions (Fig. 1 *B* and *F*).

The addition of urea led to significant increase of the fibril solubility: the fibrils dissolved at concentrations at which they otherwise grew in urea-free solutions (Fig. 1*C*). Urea-induced thermodynamic fibril destabilization, manifested as higher solubility, is consistent with urea's known activity as a universal protein denaturant (53) owing to its favorable interaction with the amide groups of the peptide backbones (54). This interaction impairs not only the formation of contacts between segments of a single chain that support folded protein structures but also the formation of contacts between distinct chains within amyloid fibrils (55). Increasing the peptide concentration in the presence of urea, however, leads to growth rates significantly faster than the values recorded at the same peptide concentration in the absence of urea (Fig. 1*D*). The acceleration of fibril growth in the presence of urea would appear contrary to the destabilization of the contacts that support the fibril structure (56, 57).

The correlation between the fibril growth rate *R* and the Aβ40 concentration *C* is linear (Fig. 2*A*). The observed linearity
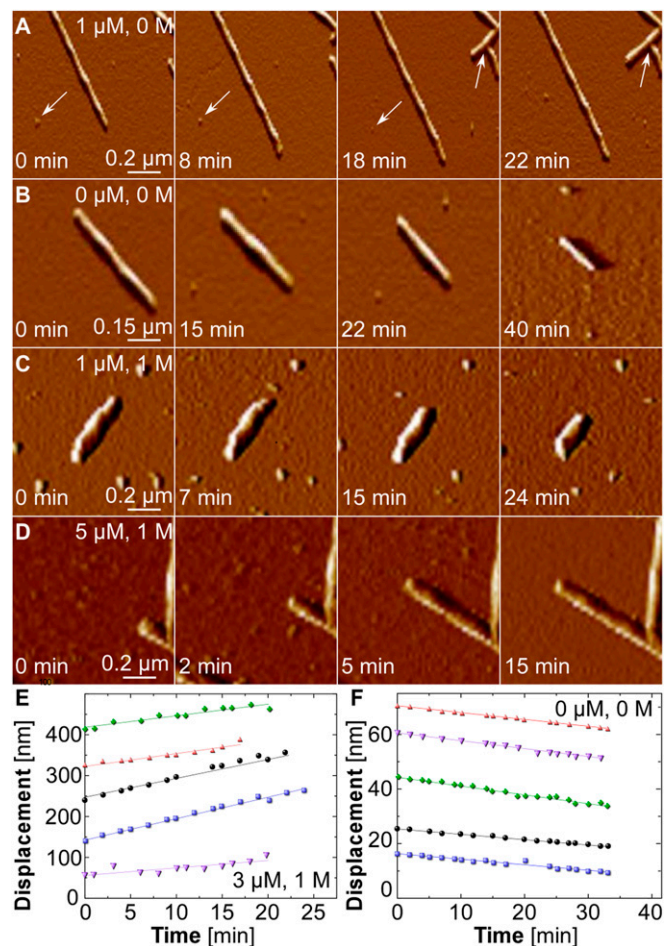


**Fig. 1.** Growth and dissolution of Aβ40 fibrils. (*A–D*) Time-resolved in situ AFM monitoring of the evolution of growth and dissolution of Aβ40 fibrils at different concentrations of Aβ40 in μM and urea in M. The arrows in *A* point to immobile objects used as reference to measure the displacement of fibril tips. (*E* and *F*) Evolutions of the displacements of 10 fibril ends. The straight lines represent best fits that were used to determine the fibril growth rates. (*E*) Fibril growth at Aβ40 solution concentration 3 μM in the presence of 1 M urea. (*F*) Fibril dissolution in peptide-free and urea-free buffer.

implies that the fibrils grow by association of the dominant solution Aβ40 species, whether it be monomer, dimer, or a heavier oligomer (58). Whereas oligomers of varying compositions are present in Aβ solutions, they reside in equilibrium with the monomers, which capture the majority of the peptide mass in the solution (59, 60). Growth by association of oligomers in equilibrium with a majority of monomers would manifest as a superlinear (e.g., quadratic, for growth by dimer association) $R(C)$ correlation (58). We tentatively conclude that Ab40 fibrils grow by association of monomers.

The $R(C)$ correlation crosses the interpolated line of zero growth at $C_e = 0.44 \pm 0.07$ μM (50). For $C$ below $C_e$, the negative values of $R$ correspond to fibril dissolution (Figs. 1*B* and 2*A*). A solution with concentration $C_e$ is in equilibrium with the fibrils (i.e., $C_e$ is the Aβ40 solubility with respect to the fibrils). The equilibrium $F_n + M \rightleftarrows F_{n+1}$, where $F_n$ and $F_{n+1}$ denote fibrils that differ in length by one monomer $M$, is characterized by a constant $K = [M]_e^{-1}$, since the addition of a monomer does not modify the fibril concentration and $[F_n] = [F_{n+1}]$. Considering the dominance of monomers in the solution, we approximate the equilibrium monomer concentration $[M]_e$ with the total peptide concentration $C_e$ at equilibrium with the fibrils and arrive at $K = C_e^{-1}$.
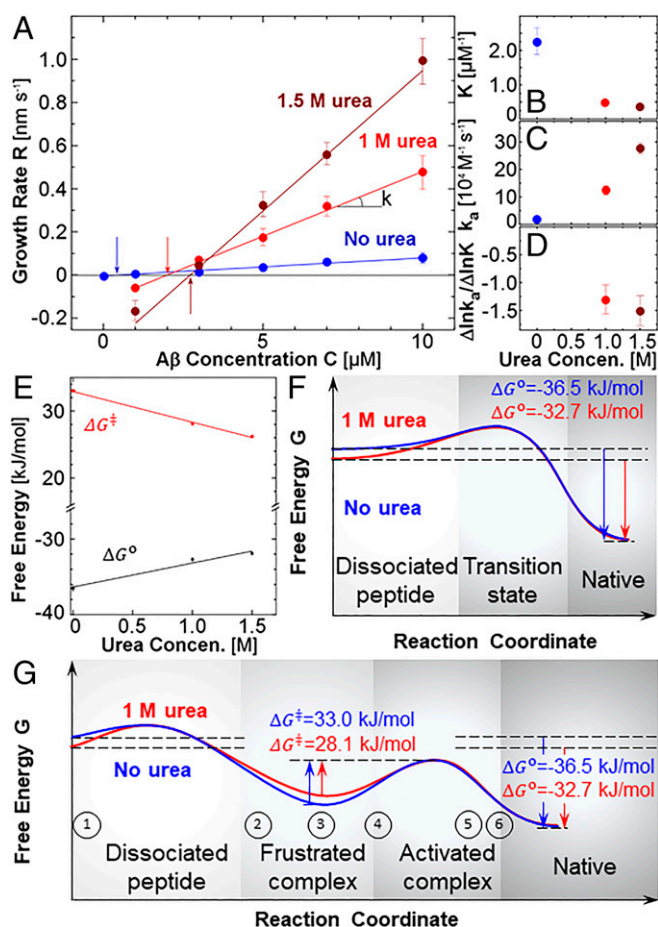
**Fig. 2.** The kinetics of Aβ40 fibril growth. (*A*) The dependences of the growth rates *R* of individual fibrils on the concentration *C* of Aβ40 in the absence and the presence of urea at two concentrations. The error bars correspond to the SD from the mean for 20 to 50 measurements illustrated in Fig. 1 *E* and *F*. The arrows mark the respective solubilities $C_e$. The rate constants $k_a$ are determined from the slopes of the linear correlations. Data in the absence of urea are from ref. 50. (*B* and *C*) The equilibrium constant $K = C_e^{-1}$ in *B* and the rate constant $k_a$, in *C* for fibrillization in the absence and presence of urea at two concentrations determined from the *R(C)* correlations in *A*. (*D*) The ratio $\Delta\ln k_a / \Delta\ln K$, evaluated from the data in *B* and *C*. The error bars in *B–D* indicate SDs from the mean values evaluated from the *R(C)* correlations in *A*. (*E*) The correlations of the free energies for fibrillization $\Delta G^o$ and of the transition state for incorporation $\Delta G^{\ddagger}$ with the urea concentration. The error bars indicate the SDs of $\Delta G^o$ and $\Delta G^{\ddagger}$ and are smaller than the symbol size for most data points. (*F*) Schematic of the free energy landscape along a direct pathway of incorporation of a peptide chain into a fibril tip that does not involve any intermediate states. (*G*) Schematic of the free energy landscape along a pathway of incorporation of a peptide chain into a fibril tip that passes through a frustrated intermediate state. The encircled numbers denote approximate locations along the reaction coordinate of the conformations depicted in Fig. 3*A*. In *F* and *G*, blue curves and blue $\Delta G^{\ddagger}$ and $\Delta G^o$ values characterize association to fibrils in the absence of urea, red curves and red $\Delta G^{\ddagger}$ and $\Delta G^o$ values, in the presence of 1 M urea.

For insight into the mechanisms that guide faster growth in the presence of urea despite the fibril destabilization that this denaturant enforces, we measured the *R(C)* correlations at two concentrations of urea, 1 and 1.5 M, and compared them to *R(C)* data in urea-free solutions (Fig. 2*A*). At the three tested compositions, fibrillization was reversible. The growth and dissolution dynamics revealed by AFM images (Fig. 1 *C* and *D*) and the *R(C)* correlations (Fig. 2*A*) demonstrate that urea acts as an apparent catalyst for growth and dissolution. It leads to both

faster fibril growth and faster dissolution (Fig. 2*A*), while increasing the Aβ40 solubility with respect to the fibrils.

The solubility boosts with added urea define gains of standard free energy of fibrillization, $\Delta G^o = -RT\ln K = RT\ln C_e$, from $-36.5 \pm 0.4$ kJ · mol$^{-1}$ in the absence of urea to $-32.7 \pm 0.3$ kJ · mol$^{-1}$ at 1 M urea and $-31.9 \pm 0.2$ kJ · mol$^{-1}$ at 1.5 M urea [i.e., about 3 kJ · mol$^{-1}$(mole urea)$^{-1}$ (Fig. 2*E*)]. The increasing $\Delta G^o$ announces the expected urea-enforced destabilization of the fibrils relative to the solute monomers. We use fibril seeds that were generated without urea, and previous work has established that the structure of a fibril persists after the growth conditions deviate from those during fibril nucleation (11, 29, 61). The uniformity of the fibril structure during growth in the presence and absence of urea ascribes the destabilization of the fibrils relative to the solution to urea-imposed lower free energy of the solute peptide chains (Fig. 2 *F* and *G*).

We model the linear *R(C)* correlation as $R = ak_a(C - C_e)$, where $a = 0.47$ nm is the contribution of an incorporated monomer to the protofilament length (29), and $k_a$ is the bimolecular rate constant for the reaction between monomers and fibril tips. We define protofilament as a single stack of peptide chains (*SI Appendix*, Fig. S3) (62, 63); we discuss alternative definitions (29, 61, 64) in *SI Appendix*. The expression for *R* is akin to the result of a model, which assumes a two-step reaction of monomer association to the fibril tip, followed by incorporation into the fibril, under conditions where the first step is rate limiting (31). The constant $k_a$ assumes values between $1.8 \times 10^4$ and $2.8 \times 10^5$ M$^{-1}$ · s$^{-1}$, depending on the urea concentration (Fig. 2*C*). These values are significantly slower than the expected diffusion limit for association of about $10^{10}$ M$^{-1}$ · s$^{-1}$ (65). The rate constant can be written as $k_a = k_0\exp(-\Delta G^{\ddagger}/k_BT)$, where $k_B$ is the Boltzmann constant and *T* is temperature. We assume $k_0 = 10^{10}$ M$^{-1}$ · s$^{-1}$ as the diffusion limit (65). It has been argued that $k_0$ for Aβ40 fibril growth should be closer to $10^9$ M$^{-1}$ · s$^{-1}$ (31); the exact value of $k_0$, however, does not modify the arguments presented in *A Frustrated Complex at the Fibril Tip*. We assume that the rotational and orientational entropy contributions to $k_0$ are not substantially affected by urea. We note that urea increases the solution viscosity by 4% at 1 M and 6% at 1.5 M (66). Accounting for this increase would depress the values of $G^{\ddagger}$ in the presence of urea by about 0.1 kJ · mol$^{-1}$, which is within the experimental uncertainty of this variable (Fig. 2*E*). With this, the free energy barrier $\Delta G^{\ddagger}$ decreases from $33.0 \pm 0.1$ kJ · mol$^{-1}$ in the absence of urea to $28.1 \pm 0.2$ kJ · mol$^{-1}$ at 1.0 M urea and to $26.2 \pm 0.2$ kJ · mol$^{-1}$ at 1.5 M urea; that is, the barrier reduces by about 4 kJ · mol$^{-1}$(mole urea)$^{-1}$ (Fig. 2 *E* and *G*). Importantly, the correlation between $\Delta G^{\ddagger}$ and the urea concentration is linear (Fig. 2*E*). In analyses of protein folding kinetics, such linearity is taken as evidence that urea does not greatly modify the conformation of the transition state. In folding, observed kinetics nonlinearities have been shown to correlate to flatter free energy profiles with "malleable" transition states (67). The linearity here, while based on only three concentrations, suggests that lack of urea-enforced structure change in the transition state is a good first approximation.

**A Frustrated Complex at the Fibril Tip.** The opposing activation free energy $\Delta G^{\ddagger}$ and fibrillization free energy $\Delta G^o$ responses to urea eliminate certain mechanisms of amyloid fibril growth that have previously been suggested based on simulations. One such proposal assumes that monomeric peptides undergo a conformational transformation into an aggregation-prone state, whose lifetime is longer than the time required for collision with a fibril tip (24). In this scenario, the depression of the free energy of the peptides in the solution enforced by urea would boost the free energy barrier for incorporation (Fig. 2*F*) and impose slower fibril growth, contrary to actual observations. From a broader perspective, the

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opposing $\Delta G^{\ddagger}$ and $\Delta G^o$ trends defy any mechanism that constrains the urea impact to the peptide chains in the solution.

The essential identity of the bulk fibril structure in the presence and absence of urea eliminates urea-driven bulk fibril structure modifications as the mechanism that regulates the high sensitivity of $\Delta G^{\ddagger}$ to urea. The exclusion of peptides in the solution and fibril structure as targets for urea attack implies an incoming chain passes through an intermediate state, in which it attaches, but only partially, to the fibril tip. The substantial magnitude of $\Delta G^{\ddagger}$ and its sensitivity to urea together imply that the intermediate complex is bound by strong hydrophobic contacts, distinct from the native contacts that support the fibril structure and dictate $\Delta G^o$. The unraveling of the initial nonnative contacts between the incoming and terminal fibril monomers, in search of the native conformation typical of the bulk fibril, becomes the rate-limiting step for the attachment of the monomer to the fibril tip and contributes to $\Delta G^{\ddagger}$. The extension of the linear $R(C)$ correlation to dissolution in undersaturated solutions (Fig. 2A) indicates that disorder at the tip is an equilibrium feature of the fibril structure. Urea, by interacting with the backbones of monomers at the fibril tip (68, 69), weakens the nonnative contacts and thereby destabilizes the intermediate state, which lowers $\Delta G^{\ddagger}$ (Fig. 2G). Importantly, the linear correlation of $\Delta G^{\ddagger}$ with the urea concentration (Fig. 2E) indicates that the urea-induced weakening of the nonnative contacts likely stops short of modifying the structure of the intermediate and transition states. In protein folding, such energy-rich nonnative contacts have been called frustrated (70–72). Whereas simulations have foreseen frustrated states as amyloid peptides fold to incorporate into fibrils (25, 73, 74), the opposing $\Delta G^{\ddagger}$ and $\Delta G^o$ responses to urea, based on the divergent effects of urea on fibril solubility and growth rate, provide direct experimental evidence for the role of frustration in fibrillization.

The pathway of association of peptides with the fibril tips suggested by the rates of fibril growth (Fig. 2) shares certain features with a mechanism put forth by simulations. In this mechanism, the incorporation of a monomer into a fibril divides in two steps. First, the association of an unstructured monomer to the fibril tip (often called docking), followed by conformational rearrangement toward the peptide structure in the fibril bulk (locking) (24, 65, 75, 76). In the simplest lock-and-dock scenario, the conformational transformation of each captured peptide chain is templated by the previously arrived peptide. The magnitude of $\Delta G^{\ddagger}$ and the opposing $\Delta G^{\ddagger}$ and $\Delta G^o$ trends that we observe advocate a more complex picture whereby the docked state evolves to a frustrated complex, in which a monomer makes many nonnative contacts in the fibril tip.

Additional characteristics of the frustrated complex emerge when we quantitatively compare the urea-induced weakening of the frustrated contacts to what is seen for protein unfolding (77). Statistics over 45 proteins have revealed that urea-induced lowering of the free energy of unfolding scales with the protein surface area exposed to solvent upon unfolding (77). This proportionality indicates that the thermodynamic effect of urea of about $3 \text{ kJ} \cdot \text{mol}^{-1}$ (mole of urea)$^{-1}$ is associated with the loss of about 3,200 Å$^2$ of solvent-accessible surface area (SASA) upon monomer incorporation into the fibril; we designate this loss as $\Delta SASA^0 = -3{,}200$ Å$^2$. The urea-induced activation free energy drop of about $4 \text{ kJ} \cdot \text{mol}^{-1}$ (mole of urea)$^{-1}$ corresponds to the exposure of $\Delta SASA^{\ddagger} = 5{,}300$ Å$^2$. The evaluations of $\Delta SASA^0$ and $\Delta SASA^{\ddagger}$ afford the opportunity to compare the results of the kinetics experiments to those of simulations and thus attain additional insights in the incorporation pathway.

**Interaction of the Monomer with the Fibril Tip during Binding and Folding.** To achieve greater molecular-level understanding of how an incoming peptide chain acquires the structure typical of the fibril bulk as it incorporates into a fibril, we carried out simulations

using the associative memory, water-mediated structure, and energy model for molecular dynamics (AWSEM-MD) (78). The coarse-grained nature of the AWSEM simulation Hamiltonian leads to much more rapid sampling than models with explicit solvent molecules. Owing to the lack of solvent, 1 ps of simulation time compares to longer than a nanosecond of laboratory time. We used the Protein Database entry 2LMN, the polymorph examined with AFM (50), to construct the fibril structure. We evaluated the free energy profile for an incoming monomer to associate with a fibril composed of five chains arranged in a single stack (Fig. 3A). We characterized the conformational ensembles in terms of the distance between the center of mass of the peptide chain and the fibril end and by the similarity of the structure of the incoming monomer to that of a chain in the fibril bulk (Fig. 3B). We explored about 30 select configurations, divided into six groups, along the reaction pathway (Fig. 3 A and B). We then computed the SASA of fully atomic models generated from the coarse-grained structures (Fig. 3C).

The computed change, $\Delta SASA_{eq} = SASA_6 - SASA_1$ (Fig. 3C), defines the sensitivity of the equilibrium $\Delta G^o$ to the addition of denaturant, and the model result $\Delta SASA_{eq} = 3{,}400$ Å$^2$ agrees
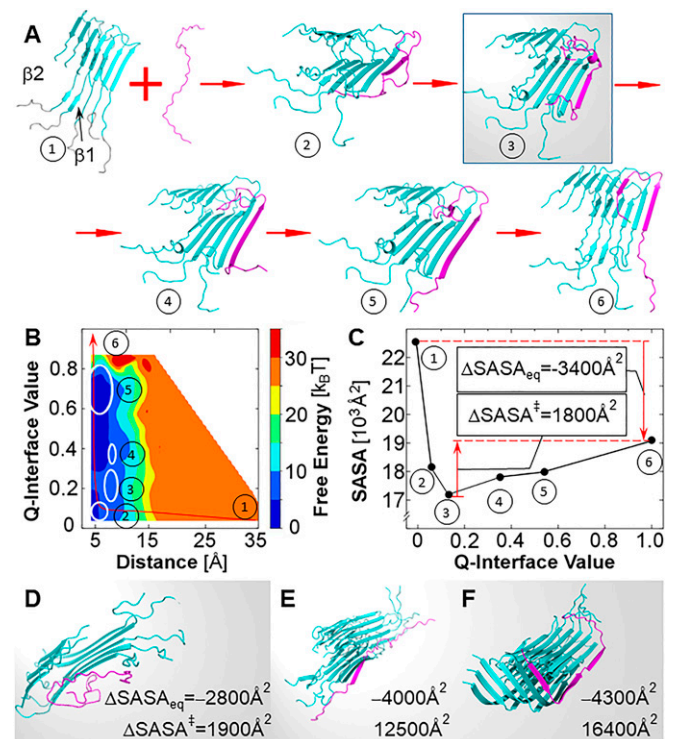


**Fig. 3.** Microscopic view of monomer association to a fibril tip. (A) Select successive conformations of a monomer peptide (magenta) associating to an even end (where the β1 strand of a 2LMN protofilament is open) of a fibril comprised one protofilament (cyan), computed using AWSEM-MD simulations. 1, a dissociated peptide; 2 to 5, intermediate conformations; 6, peptide fully integrated in the fibril. (B) The free energy as a function of the distance from monomer end and Q-interface, which measures the similarity of the monomer structure to that in the fibril bulk. The white ovals next to 2 to 5 highlight pools of conformations, from which respective SASAs were sampled and averaged. The red arrow schematically depicts a reaction pathway. (C) Total SASA of the fibril and monomer in positions 1 to 6. Computed $\Delta SASA_{eq}$, corresponding to equilibrium, and $\Delta SASA^{\ddagger}$, representative of the activation barrier, are shown. (D–F) Conformations of a monomer peptide (magenta) associating with three different fibril tips (cyan) at which SASA is minimal, as conformation 3 in A. Listed negative areas represent $\Delta SASA_{eq}$ and positive areas, $\Delta SASA^{\ddagger}$. (D) The odd end of a 2LMN fibril comprised of one protofilament. (E) A fibril comprised of two protofilaments of equal length. (F) A fibril comprised of two protofilaments, of which one is longer by one monomer.

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well with the estimate based on the measured urea dependence of the fibril solubility. The simulations reveal that the SASA passes through a minimum at position 3 (Fig. 3 *A* and *C*). In this group of structures, an incoming monomer partially binds to itself forming nonnative inter- and intrachain contacts rather than forming native contacts with the fibril tip (Fig. 3 *A* and *B*). These frustrated contacts must unravel as the peptide reconfigures to fully integrate into the fibril in the cluster 6 structures, labeled in Fig. 3*B*. Remarkably, the finding of an intermediate frustrated complex at the fibril tip agrees with the conclusions implied by the responses of experimentally measured $\Delta G^{\ddagger}$ and $\Delta G^{o}$ to added urea.

The positive $\Delta$SASA between positions 3 and 6 represents only a rough estimate of the effects of urea on the activation free energy $\Delta G^{\ddagger}$. All-atom simulations of the conformations of the peptides at the fibril tip yield $\Delta$SASA$^{\ddagger}$ = 1,800 Å$^2$. The results for monomer association to the opposite, odd, fibril end (Fig. 3*D* and *SI Appendix*, Fig. S5) are similar. Importantly, both values of $\Delta$SASA$^{\ddagger}$ are only about one-third of the value inferred from the response to urea of the growth kinetics of individual fibrils, measured by AFM. The discrepancy between the simulated and measured $\Delta$SASA$^{\ddagger}$s suggests that the structure of the frustrated complex at the fibril tip is probably more elaborate than what has emerged from these initial simulations.

We envision two possible models of a more elaborate frustrated intermediate. First, the frustrated complex may involve more than one peptide chain within a single protofilament; the ratio between the experimental and simulated $\Delta$SASA$^{\ddagger}$ suggests that three or four monomers would need to be involved. Simulations of a peptide chain association with a disordered fibril tip would involve characterizing a multidimensional free energy landscape and require significant additional efforts. Alternatively, the intermediate frustrated state may involve binding of the incoming monomers to both protofilaments in a filament. The experimentally measured thicknesses of the fibrils monitored by AFM indicate that the majority of the filaments are built of two parallel protofilaments, represented by the 2LMN structure (50).

We explore the possibility where the incoming peptide chain associates to both protofilaments in a filament. We note that the addition of monomers to a double filament must ultimately preserve the structure of the twinned protofilaments, and this involves at least two alternating conformations of the fibril tip: one where the two protofilaments exactly match in length and one where one of the protofilaments is ahead by one monomer. AWSEM simulations of monomer attachment to the tip of a two-protofilament structure reveal that, instead of folding on top of one of the two monomers at the fibril tip, an incoming peptide chain spans both stacks and enforces a stabilized frustrated conformation that may be incapable of further growth (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S6). This state was predicted for both the matched and the mismatched protofilaments (Fig. 3 *E* and *F*). $\Delta$SASA$^{\ddagger}$ from such frustrated states to the final conformation are greater than 12,000 Å$^2$ (Fig. 3 *E* and *F*).

**The Growth of Fibrils with Bulk Structure of Their Tip.** To discriminate experimentally whether the complex frustrated state at the fibril tip recruits more than one disordered peptide chain that crown a single protofilament or structures as a single chain that spans both protofilaments in a filament, we monitored the growth rates of freshly cut fibrils (Fig. 4*A*). In freshly cut fibrils, the peptides at the tip initially should carry the structure of monomers in the fibril bulk, and an intermediate state composed of more than one frustrated chains may not have had time to evolve. Thus, if the slow growth rate observed in the AFM experiments is due to a frustrated complex composed of several chains, freshly cut fibrils will grow faster than fibrils with normal tips. By contrast, if the high $\Delta G^{\ddagger}$ deduced from AFM experiments is due to interactions of a single incoming chain with two

adjacent protofilaments, the frustrated complex that crowns the freshly cut fibril tips will be identical to the one at equilibrated tips, and the freshly cut tips will grow with rates similar to those of normal tips.

The AFM measurements reveal that freshly cut fibrils initially grow about twice as fast as fibrils with equilibrated tips (Fig. 4 *B* and *C*). In five of the cut fibrils, the growth rate transitioned to its "normal" value after 6 to 8 min, during which time the fibrils grew about 10 nm (Fig. 4*B*). The observed faster growth rates indicate that the freshly cut fibrils carry a simpler frustrated complex than equilibrated fibril tips. For a second test of the distinction between the kinetics of growth of freshly cut and mature fibrils, we measured the effects of 1 M urea on the growth of freshly cut fibrils and compare them to the growth rate of mature fibrils (Fig. 4*C*). The results reveal that urea accelerates the growth rate constant of freshly cut fibrils by about threefold, significantly weaker than its ca. sixfold effect on normal tips (Fig. 4*C*).

Collectively, the outcomes of the two tests with freshly cut fibrils indicate that the frustrated complex at equilibrated fibril tips comprises more than one peptide chain but is probably constrained to a single protofilament.

## Conclusions

Time-resolved in situ AFM measurements of the growth kinetics of individual fibrils and molecular simulations suggest a two-step mechanism of growth of Aβ40 fibrils, whereby an incoming monomeric solute peptide first associates to a complex residing at the fibril tip and composed of several other monomers that have nonnative conformations. The unraveling of the frustrated initial contacts during the conformational rearrangement of one of the constituent peptide to the bulk fibril structure constitutes the rate-limiting step for fibril assembly. The proposed reaction pathway should help guide the search for fibrillization inhibitors by finding small molecules that bind to the frustrated complex at the fibril tip and increase the free energy cost of rearranging it. In a broader context, our findings indicate that the coupled dynamics of structuring and assembly during fibril growth are more complex than observed for the folding of most globular proteins, since they involve the collective motions of several peptide
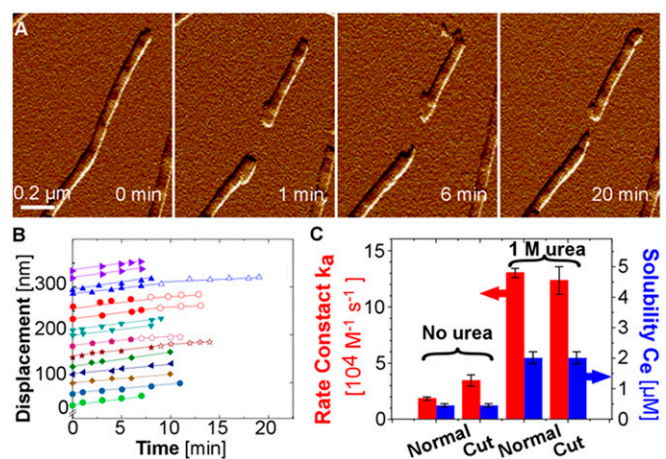


**Fig. 4.** The growth of fibrils with bulk structure of the peptides at the tip. (*A*) AFM micrographs of growth of freshly cut fibrils. (*B*) The displacement of the freshly cut ends. Five of the freshly cut tips transition to slower growth depicted with open symbols. (*C*) The rate constants $k_a$ (red bars and *Left* axis) and the solubilities $C_e$ (blue bars and *Right* axis) of fibrils with normal equilibrated tips and with freshly cut tips in the absence of urea and in the presence of 1 M urea. The error bars represent the SD from the mean evaluated from about 400 fibril tip displacement measurements for the normal fibrils and 100 fibril tip displacement measurements for the freshly cut as in Fig. 2 *A* and *C*.

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chains that comprise the initial frustrated complex and simultaneously strain nonnative contacts in the transition state. Furthermore, the substantial kinetic consequences of this frustrated complex indicate that fibril growth does not enjoy a fully funneled energy landscape.

In the context of Alzheimer's disease, the low concentration of fibril tips in the fibrillization reaction mixture suggests that the tips may be suitable targets for attack by potential suppressors of fibrillization. The distinct structure of the fibril tip proposed here may guide the computational search for small molecule compounds and antibodies that bind to the tip and stunt fibril growth.

## Materials and Methods

Detailed descriptions of all methods used in this work are provided in *SI Appendix*.

**Aβ40 Expression and Purification.** We have expressed Aβ(M1-40) (MDAEFRHDS GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) in *E. coli* and purified it by size exclusion chromatography (50) following published procedures (79). This method produces Aβ peptide with a methionine attached to the N terminus (*SI Appendix*, Fig. S2), which significantly simplifies purification and contributes to several-fold–greater yields (79). The fibrillization kinetics of the methionine-initiated peptide quantitatively matches that of the methionine-free peptide (79), justifying the wide use of Aβ(M1-40) and Aβ(M1-42) in amyloid-β fibrillization studies (31, 35, 80).

**Fibril Formation.** Fibril seeds grew at 37 °C while stirred at 300 rpm on a table Inkubator 1000 (Heidolph) for 24 h. Seed generation in continuously stirred solutions enforces a twofold symmetric fibril structure, 2LMN (31, 50). The generation of fibril seeds was performed without any exposure to urea even for growth rate determinations in the presence of urea. In those latter runs, urea was added to the solution immediately before fibril growth observations with AFM.

**Sample Preparation and In Situ AFM Imaging.** We used a multimode atomic force microscope (Nanoscope VIII or IV, Bruker) for all AFM experiments. To prepare samples for growth rate measurements, aliquots of 2 μL second-generation fibril seeds was added to the incubation buffer; urea was added based on experiment needs. AFM images were collected in tapping mode.

**The Growth of Freshly Cut Fibrils.** Existing fibrils deposited on the AFM substrate were monitored under AFM for several minutes. To cut a fibril, we chose a relatively long one, which spanned about 50% of the image width. To ensure a good cut, we rotated the scan angle so that the angle between the fibril and fast scan direction angle was at least 45°. To increase the force of interaction between the tip and the fibril, we lowered the amplitude set point to 50 or 60% of the original value and set the scan frequency to 1 Hz to better control the length of the cut out. After the cut was complete, we reset the set point and the scan frequency to their original values to monitor the growth of the freshly cut ends.

**Simulations.** The simulations were carried out using the AWSEM force field (78). AWSEM represents proteins as coarse-grained structures, with every amino acid residue defined by the interactions of three beads: $C_\alpha$, $C_\beta$, and O. The AWSEM Hamiltonian ensures an ideal backbone geometry between the explicit beads and includes terms accounting for secondary and tertiary interactions, implicit solvent effects, and residue burial preferences. To simulate the growth of an Aβ40 fibril, we used the structure of a twofold symmetric polymorph consisting of U-shaped protofilaments with a positive stagger (2LMN) (81). We chose this structure because it likely represents the polymorph that forms under the conditions employed in the AFM experiments (50).

To study the free energy associated with monomer addition, we introduce the order parameter Q-interface in the following form:

$$Q_{interface} = \frac{1}{N_p} \sum_{|i-j|>2} e^{\left(\frac{\left(r_{ij}-r_{ij}^N\right)^2}{2\sigma_{ij}^2}\right)},$$

where $r_{ij}$ is the interatomic distance between the C-α atoms of residues $i$ and $j$, and $r_{ij}^N$ is the distance between the same C-α atoms in the fully aligned fibril n + 1 structure. The term $\sigma_{ij}$ defines the distribution width, which is minimally influenced by the sequence distance between the $i$ and $j$ C-α atoms. The value of Q-interface ranges from 0 (completely dissimilar contacts) to 1 (native contacts), as enforced by the normalization by $N_p$, the number of unique residue pairs that satisfy the $|i − j| > 2$ conditions.

Umbrella sampling simulations were performed with a biasing force between the center-of-mass of the free monomer and the center-of-mass of the chain sitting at the tip of the protofilament (65).

The set of simulations performed for modeling monomer additions at either end of the protofilament—to compare the dynamics of a positive and negative stagger—and across the full range of distance biasing and temperatures, resulted in trajectories totaling 0.46 μs. A timestep of 2 fs was used for all simulations, and each simulation ran for a total of 2,500,000 steps (5 ns), with the first 0.042 ns of each simulation disregarded to allow for equilibration. Potential of mean force plots were generated using the multistate Bennet acceptance ratio (82) to remove the biasing term contribution to the energy calculations from umbrella sampling (Fig. 3B).

To compare the simulations results with results of the fibril growth kinetics experiments, we evaluate degree of burial of sex distinct conformations along the reaction pathway in terms of the total SASA. Even though AWSEM represents each amino acid with three beads, which correspond to the $C_\alpha$, $C_\beta$, and O atoms, it allows for the reconstruction of all heavy atoms of the backbone under the assumption of backbone planarity and ideal geometry. To perform the SASA calculations, the CG conformations were first back-mapped to all-atom representations using Modeler (83), with the lowest-energy predicted structure being chosen from 10 possible conformations. The positions of the rest of the heavy atoms of the side chains, except $C_\beta$, which is explicitly represented in AWSEM, were computed using SCWRL4 (84). The structures generated from this process were then visualized using PyMOL (85). The SASA values were estimated using a native function in PyMOL, in which a rolling a sphere with radius 1.4 Å, representing a water molecule, is used to race over the exposed atoms of the peptides and the fibril.

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