Following the aggregation of amyloid-forming peptides by computer simulations

Adrien Melquiond
Laboratoire de Biochimie Théorique, UPR 9080 CNRS, Institut de Biologie Physico-Chimique et Université Paris 7, 13 rue Pierre et Marie Curie, 75005 Paris, France

Geneviève Boucher and Normand Mousseau
Département de Physique and Regroupement Québécois sur les Matériaux de Pointe, Université de Montréal, C.P. 6128, succursale centre-ville, Montréal (Québec), Canada

Philippe Derreumaux
Laboratoire de Biochimie Théorique, UPR 9080 CNRS, Institut de Biologie Physico-Chimique et Université Paris 7, 13 rue Pierre et Marie Curie, 75005 Paris, France

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There is experimental evidence suggesting that the toxicity of neurodegenerative diseases such as Alzheimer’s disease may result from the soluble intermediate oligomers. It is therefore important to characterize extensively the early steps of oligomer formation at atomic level. As these structures are metastable and short lived, experimental data are difficult to obtain and they must be complemented with numerical simulations. In this work, we use the activation-relaxation technique coupled with a coarse-grained energy model to study in detail the mechanisms of aggregation of four lys–phe–phe–glu (KFFE) peptides. This is the shortest peptide known to form amyloid fibrils in vitro. Our simulations indicate that four KFFE peptides adopt a variety of oligomeric states (tetramers, trimers, and dimers) with various orientations of the chains in rapid equilibrium. This conformational distribution is consistent with all-atom molecular-dynamics simulations in explicit solvent and is sequence dependent; as seen experimentally, the lys–pro–gly–glu (KPGE) peptides adopt disordered structures in solution. Our unbiased simulations also indicate that the assembly process is much more complex than previously thought and point to intermediate structures which likely are kinetic traps for longer chains. © 2005 American Institute of Physics. [DOI: 10.1063/1.1886725]

I. INTRODUCTION

The deposition of amyloid fibrils sharing a common cross β-sheet structure with the β-strands perpendicular to the fiber axis and β-sheets propagating along the direction of the fiber is a hallmark of several fatal diseases, including Alzheimer’s disease and the spongiform encephalopathies.1,2 It is still not known which features cause proteins and peptides differing in amino acid composition and length to form amyloid fibrils in vivo,3,4 although the effects of mutations on the rates of aggregation of unfolded polypeptide chains can be correlated with changes in simple physico-chemical properties.5

Evidence implicating the β-amyloid protein (Aβ) in Alzheimer’s disease has accumulated. The main form of Aβ counts 40 amino acids but Aβ with 42 amino acids is also produced. Irrespective of the real role of amyloidosis in Alzheimer’s disease, the distribution of amyloid plaques in the brain correlating poorly with the affected-diseases regions, detailed knowledge of the early steps of amyloid-fibril formation, and structural characterization of the soluble Aβ oligomers are still missing at the atomic level.6 Yet, soluble oligomers of amyloid-forming peptides have been shown to have toxic effects in cell cultures and have been proposed to be an important contributor to neurodegeneration in amyloid diseases.7,8 The goal of this study is to understand the basic mechanisms leading to fibril formation by molecular simulations and to complement experiments. We know from photo-induced crosslinking of unmodified proteins (PICUP) studies coupled to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) that crucial intermediates consist of dimers and tetramers in rapid equilibrium for Aβ1–40.9 However, this transient distribution varies with the type of Aβ and the solution conditions. For instance, the nucleus for Aβ1–42 consists of pentamers/hexamers. Other intermediates between dimers and fibrils, the so-called protofibrils, have also been observed.10

Simulating the formation of ordered aggregates on computers is not an easy task because the experimental aggregation time exceeds by several orders of magnitude the time that can be explored by all-atom aggregation molecular-dynamics (MD) simulations in explicit solvent. Considering this obstacle, several computational methods have been used on a variety of amyloid-forming peptides. These include simulations with simplified protein chains: Monte Carlo (MC) simulations of lattice protein models11,12 or discontinuous MD simulations13–15 using from one to four particles per amino acid, biased all-atom MD simulations with interactions between the peptides16 or chemical shift constraints,17 and traditional all-atom MD simulations to study the stability of various oligomer sizes packed in different ways18–20 or the
early steps of aggregation.\textsuperscript{21-23} Complementing these studies, the aggregation pathways of \(\text{A}\beta_{16-22}\) dimers\textsuperscript{24,25} and trimers\textsuperscript{26} have been investigated using the activation-relaxation technique (ART) coupled to the OPEP coarse-grained force field. ART differs from MC and MD simulations in two respects. ART events are defined directly in the space of configurations which allows to generate moves of any complexity in contrast to MC moves. Moreover, ART efficiency is not related to the height of the energy barrier at the transition point, allowing the system to move through the conformation space rapidly without having to wait for the rare thermal fluctuations to occur as in MD simulations.

Lys–phe–phe–glu (KFFE) is the shortest peptide known to form fibrils as determined by electron microscopy, circular dichroism analysis, and congo red staining, and it is believed to adopt an antiparallel \(\beta\)-sheet conformation on the basis of energy minimizations.\textsuperscript{27} Another tetrapeptide of sequence DFNK was also shown to aggregate though to a lesser extent.\textsuperscript{28} The KFFE motif was also used to generate a 12-mer peptide of the sequence KFEAAAKKKFFE which forms antiparallel \(\beta\)-sheets in a cross-\(\beta\) arrangement.\textsuperscript{29} Although the KFFE motif is not found in the \(\text{A}\beta_{1-40}\) and \(\text{A}\beta_{1-42}\) of Alzheimer’s disease, its simplicity makes it an attractive model for understanding amyloid aggregation and conformational states. Furthermore, it has been recently shown that the variation from parallel to antiparallel arrangement in \(\text{A}\beta\) peptides (the \(\text{A}\beta_{16-22}\) is antiparallel\textsuperscript{30} and the full-length \(\text{A}\beta\) is parallel\textsuperscript{31}) is not only controlled by peptide length, but also by peptide amphiphilicity: the octanoyl-\(\text{A}\beta_{16-22}\) forms parallel \(\beta\)-sheets.\textsuperscript{32}

ART-OPEP simulations on hexamers of KFFE were presented recently.\textsuperscript{33} We found that the KFFE peptide is unstructured in solution and six KFFE peptides populate three distinct arrangements in solution: two double-layer \(\beta\)-sheet organizations, in agreement with the generic structure of amyloid fibrils as observed by x-ray diffraction, and one curved single-layer hexamer.\textsuperscript{33} Here, we continue our study on the KFFE peptide blocked with acetyl and amide groups and study the detailed mechanism of the aggregation of four KFFE peptides and the effect of sequence variations on the soluble lowest-energy structures. To this end, we generated 30 ART-aggregation trajectories at 300 K for the KFFE tetramer starting from randomly chosen states. In a second series of simulations, we repeated the ART simulations on the variant lys–pro–gly–glu (KPGE); here as expected from experiment, no \(\beta\)-sheet structure was seen. Finally, we carried out two explicit solvent all-atom MD simulations at 298 and 330 K for a total of 50 ns to determine the stability of four KFFE peptides packed in a fully antiparallel \(\beta\)-sheet.

Together, the MD and ART simulations demonstrate that the peptides exist in rapid equilibrium between a variety of oligomeric states, with various orientations of the chains and hydrogen-bond (H-bond) patterns, and that the aggregation process is much more complicated than the addition of a chain to a predefined assembly.

II. MATERIAL AND METHODS

A. All-atom MD simulations in explicit solvent

Simulations were carried out at pH 7 using the GROMACS program and the all-atom force field GROMOS96.\textsuperscript{3} The fully four-stranded antiparallel \(\beta\)-sheet was solvated in a rectangular box \(45 \times 45 \times 32\) Å containing 1995 SPC (simple point charge) water molecules. No ion was added since the peptide is neutral. MD simulations were performed with periodic boundary conditions at constant temperatures (298 and 330 K) and constant pressure (1 atm), with coupling constants of 0.1 and 0.5 ps, respectively. Bond lengths were constrained with the SHAKE algorithm and the time step for dynamics was 2 fs. The particle mesh Ewald method was used with a cutoff distance of 12 Å for the electrostatic interactions and the nonbonded interactions were updated every five time steps.

B. ART-OPEP simulations in implicit solvent

ART explores the energy landscape by searching local minima connected by first-order saddle points.\textsuperscript{35-38} A similar approach has been followed by Munro and Wales\textsuperscript{39} and the difference between both approaches have been described.\textsuperscript{24} One ART event consists of four steps. The configuration is first displaced outside the minimum in a random direction until a negative eigenvalue appears in the Hessian (matrix of the second derivative of the energy) and then pushed along the corresponding eigenvector until the total force is close to zero (reaching a transition state characterized by a first-order saddle point). Subsequently, the configuration is pushed over the saddle point, then is relaxed to a new minimum using standard minimization technique. This event is eventually accepted or rejected according to the Metropolis criterion\textsuperscript{40} at the desired temperature (\(P_{\text{accept}} = e^{-\Delta E/\Delta T}\)).

ART is coupled to the OPEP energy function (optimized potential for efficient peptide-structure prediction in solution) which uses a reduced off-lattice protein representation. All amino acids are represented by their N, H, C, C, and O atoms and each side chain is modeled by one sphere with an appropriate van der Waals radius, geometry with respect to the main chain and hydrophobic/hydrophilic character.\textsuperscript{41-43} The OPEP energy function is expressed as a function of four types of interactions: harmonic potentials for maintaining the geometry of peptides (bond lengths and bond angles for all particle, and improper dihedral angles of the peptide bond and side chains with respect to the backbone), excluded-volume potential, backbone two-body and four-body hydrogen bonding interactions,\textsuperscript{44,45} and pairwise potential between side chains, considering all 20 amino acid types, represented by a 12-6 potential if the interactions are hydrophobic in character and by a 6 potential if otherwise. OPEP has been used successfully to predict the equilibrium structures of several polypeptides with all-\(\alpha\), all-\(\beta\), or mixed-\(\alpha/\beta\) characters in solution.\textsuperscript{31-43}

Each generated ART-OPEP trajectory is physically based as it is a fully connected sequence of local minima separated by a shared transition point. ART in its current form, however, does not describe the thermal entropic contributions and the OPEP force field cannot reproduce the full complex-
ity of interactions between all-atom side chains (it includes only one sphere per side chain) and between the solute and the solvent (it uses an implicit solvent model). This means that ART-OPEP cannot describe accurately the extended conformations that would occur at a higher temperature, for example. This limitation allows us, on the other hand, to set freely the Metropolis temperature we use. Moreover, it allows us to have at all times fully relaxed conformations, providing a much clearer understanding of the underlying dynamics. Previous results show that neglecting these entropic contributions still allows us to produce folding and aggregation patterns consistent with MD for systems much larger than those studied here.

In effect, in spite of these approximations, there is strong evidence that the trajectories generated are also representative of dynamical paths. Whether or not all the trajectories are equivalent to the most likely ones remain to be determined, but we find that the ART-generated pathways are very similar to those identified by long molecular dynamics in explicit or implicit solvent for monomeric $\alpha$-helices and $\beta$-hairpins. Moreover, our ART-OPEP simulations on dimers and trimers of $\mathrm{A}\beta_{16-22}$ helped clarify the experimental dependency of $\beta$-sheet registry on pH conditions. This propensity to adopt several H-bond patterns was also found by long all-atom MD simulations on the peptide fragment 105-115 of transthyretin in explicit solvent. Using the dimeric structure of $\mathrm{A}\beta_{16-22}$, we also described the mechanisms propagating the conformational changes between in-register and off-register arrangements of H bonds; some were confirmed by recent long all-atom MD simulations on $\mathrm{A}\beta_{16-22}$ and related peptides at high $T$. Finally, using six KFFE chains, we also predicted that fibril growth proceeds.

**FIG. 1.** All-atom MD simulations in explicit solvent starting from the fully antiparallel $\beta$-sheet: evolution of the $C_\alpha$ RMSDs (in angstrom) at 298 K (A) and 330 K (B) and evolution of the number of native (i.e., within the starting conformation) and non-native (dashed) H-bonds at 298 K (C) and 330 K (D).

**FIG. 2.** Representative snapshots from the all-atom 20-ns MD simulations in explicit solvent at 298 K starting from the four-stranded antiparallel $\beta$-sheet. AP and P stand for antiparallel and parallel chains. The $N$-terminal end of each chain is located by a sphere. Figs. 2–4 were produced using the VMD package (Ref. 47).
by both β-sheet elongation and lateral association,33 this was confirmed by discontinuous MD simulations on model polyalanine peptides.15

The MD simulations covering a total of 50 ns and the 35 ART simulations took 4 months on a cluster of five 1.3-GHz processors.

III. RESULTS AND DISCUSSION

A. Dissociation/aggregation events of KFFE in explicit solvent MD simulations

We first examine the stability of the tetrameric conformation proposed by Tjernberg et al.27 The root-mean-square deviations (RMSDs) of the backbone Cα atoms with respect to the minimized four-stranded antiparallel β-sheet are reported for the 20-ns at 298 K and 30-ns at 330 K MD runs in Fig. 1. Figures 2 and 3 report representative snapshots of the trajectories at 298 and 330 K, respectively.

In Fig. 1, we see that the four chains unpack and reassociate twice within 20 ns at 298 K (see maxima at 7.4 and 15.4 ns with RMSDs of 4.8 and 5.2 Å, and minima at 11.6 ns with RMSD of 0.8 Å and at 19.8 ns with RMSD of 1.8 Å). At 330 K, the experimental temperature often used to incubate amyloids, the four chains unpack within 8.8 ns, reaching a maximum of 8.2-Å RMSD, and refold to their initial states at 14.6 ns (RMSD of 1.5 Å). Subsequently, the peptides fluctuate between 1.5- and 4.5-Å RMSDs for 5 ns and finally explore unfolded states (RMSD of 7 Å at 26 ns).

To analyze the trajectories, we also grouped the conformations into clusters using a Cα RMSD cutoff of 2.5 Å. Cluster analysis at 298 K shows that the peptides are in equilibrium between a tetrameric architecture with antiparallel β-strands (population of 76% within 20 ns; see snapshots at 11.6 and 19.8 ns in Fig. 2) and two perpendicular dimers with antiparallel β-strands (population of 16%; see snapshots at 7.2 and 14.3 ns in Fig. 2). The rotation of one dimer with respect to another is thus thermally accessible at room temperature. As expected, the cluster analysis shows more arrangements in equilibrium at 330 K: dimers–monomers (snapshots at 5.8 and 22.3 ns in Fig. 3), trimer–monomer (snapshots at 8.1 and 24.5 ns) with distinct orientations and conformations of the monomer with respect to the trimer, and tetramers with antiparallel (snapshot at 15.3 ns) or mixed antiparallel–parallel β-strands (snapshots at 10.6 and 28 ns). The population of tetramer, trimer–monomer, and dimers–monomers is 71%, 21%, and 8%, respectively. Further analysis of the MD-generated structures at 298 and 330 K as a
function of the number of H bonds in Figs. 1(C)–1(D) shows that each dominant oligomeric arrangement consists of states with non-native H-bond patterns.

As seen, the change in conformation takes place rapidly at both temperatures. Even though the rate of conformational change is rapid, it does not mean that the simulations have reached equilibrium within 20–30 ns. Furthermore, because the simulations were not repeated using different initial velocities, the MD-generated population in explicit solvent has only qualitative significance. This explains why a two-dimer conformation has not been detected at 330 K.

B. Soluble oligomeric structures of KFFE and KPGE as determined by ART

As we have seen Sec. III A, the topological organization proposed by Tjernberg et al. is a local minimum and is not the only visited conformation. In order to identify these structures, we turn to ART-OPEP. We have carried out 30 runs (R1–R30) of 8000 events on KFFE at 300 K using various random seeds and starting from two randomly chosen organizations and conformations of the four chains [Figs. 4(A) and 4(B)], separated by at least 6 or 10 Å from each other. We find four possible low-energy arrangements with the following properties: tetramers with fully antiparallel β-sheets [nine simulations or 30% of the runs and energy of −45 kcal/mol; Fig. 4(D)], tetramers with mixed parallel–antiparallel β-sheets and mixed trimers with one monomer [30% of the runs and energy of −43 kcal/mol; Fig. 4(C)], and two perpendicular dimers with anti-parallel conformations of the chains [10% of the runs and energy of −42.6 kcal/mol; Fig. 4(E)]. Metastable states consisting of disordered dimers–monomers and disordered trimer–monomer are also detected in 30% of the runs, with energies varying between −33 and −39 kcal/mol.

Our lowest-energy conformation is thus fully consistent with the prediction of Tjernberg et al. based on energy minimizations of four β-strands packed in antiparallel or parallel registry. In addition, we also predict that tetramers, trimer–monomer, and dimers–dimers are nearly isoenergetic and these oligomeric states consist of several minima with distinct H-bond patterns. The marginal stability of the ordered structures explains why even a short 20-ns MD trajectory at room temperature can sample a large distribution of conformations. These findings are in line with previous aggregation and dissociation MD simulations on related peptides using various solvent models.

To determine the effect of sequence variations on the lowest-energy structures, ART-OPEP simulations were repeated on the variant KPGE which replaces the two phenylalanines by a glycine and a proline. Here, as expected from the β-sheet breaker property of the proline amino acid used for designing antiamyloid agents reducing amyloid plaques and cerebral damages in transgenic mouse models [e.g., Ac-LPFFD-NH2 (Ref. 45)], only disordered structures are detected. The lowest-energy structure is shown in Fig. 4(F). This result demonstrates that ART-OPEP is not biased toward the formation of oligomeric topologies with high β-strand and H-bond composition.

C. Aggregation mechanisms of KFFE as determined by ART

To follow the formation of the lowest-energy structures, we analyzed the total energy, the number of native and non-native H bonds between the chains as determined using the DSSP conditions, the orientation of the chains calculated using the scalar product between the end-to-end unit vectors of each chain, the end-to-end Cα distance of the four chains, and the percentage of native side-chain–side-chain contacts. Native contacts are those present in the lowest-energy structure for each run. Two side chains, k and l, of van der Waals radii Rk and Rl are defined in native contact if they deviate by less than Rk + Rl + 1 Å from their positions in the native state. Two peptides are considered as ordered and associated when they have at least three H bonds formed and their Cα1–Cα4 distances are >8 Å.

Figure 5 shows a detailed analysis of the run R2 leading to the four-stranded antiparallel β-sheet structure. We see that, within 500 events, the system explores two dimers [chains 1–3 and 2–4; Fig. 5(B)] of very low energy [−40 kcal/mol; Fig. 5(A)] with the chains extended [Fig. 5(E)] and hydrogen bonded in a non-native registry [Fig. 5(C)]. Then, at event 1400, the system assembles into a non-native tetramer with chains 1 and 4 in interaction [Fig. 5(B)]. Once this ordered non-native tetramer is found, the peptides
fluctuate about this local minimum and find the native tetramer by reptation moves of the chains: see the abrupt increase of native H bonds at event 3899 (move of chain 2 with respect to chain 4) and at event 4388 (move of chain 3 with respect to chain 1) in Fig. 5(C), which can be accompanied by a cooperative increase in the number of native side-chain–side-chain contacts in Fig. 5(F). The final assembly is only very weakly driven by the total energy as it falls by less than 2 kcal/mol from events 3000 to 4388. In this simulation, we see that the chains explore essentially antiparallel configurations [Fig. 5(D)]. Convergence to the tetrameric β-sheet structure shows, however, significant differences from one run to another starting from the same point. For instance in runs R12 and R25, we find that the region of ~40 kcal/mol is explored after 5000 events, the chains can pass from antiparallel to parallel orientations and vice versa and the oligomeric state preceding the native state is not a double dimer, but rather a trimer–monomer.

Figure 6 shows a detailed analysis of the aggregation trajectory R6 leading to two perpendicular dimers. We see in Fig. 6(B) that aggregation proceeds through intermediates of trimer–monomer-type (trimer 1–2–4 and monomer 3) within 1800 events, followed by the formation of a dimer (chains 2–4) with two monomers (chains 1 and 3) within 1800–3200 events. Subsequently, the system shifts temporarily to a new trimer–monomer (the trimer is formed from chains 3, 4, and 2) within 3200–4000 events before forming two dimers (chains 1–3 and 2–4) at ~ event 5000 which stabilize in a perpendicular orientation [Fig. 6(D)]. Further analysis shows that aggregation in run R6 involves intermediates with chain 1 not fully extended [see Fig. 6(E) within 2000 and 5000 events], transitions between parallel and antiparallel of the chains 2–4 [Fig. 6(D)], and between non-native and native registry of H bonds [Fig. 6(C)]. As in the aggregation trajectory R2, the final assembly from events 6000 to 7850 is associated with a very small decrease in the total energy of the system.

The ensemble of ART-generated aggregation mechanisms is shown in Fig. 7. These pathways are found with various probabilities in our simulations: pathway (a) is observed in 5/30 runs, pathway (b) in 9/30 runs, pathway (c) in 4/30 runs, and pathway (d) in 3/30 runs. We see that formation of ordered β-sheet complexes can be described by the sequential addition of one chain [monomers → dimer + 2 monomers → trimer + 1 monomer → tetramer, pathway (b)], or by the addition of two chains [monomers → 2 dimers → tetramer, pathway (c)]. This finding is fully consistent with discontinuous MD simulations of simplified protein models under the Gō approximation (energetic biases favoring the native state). However, our aggregation process is higher in complexity than that provided by Gō-based simulations. Firstly, the process is reversible, as seen for the 3/30 runs following pathway (d). Secondly, transitions from trimer–monomer to dimer–dimer and vice versa are found in 7/13 runs following pathways (b) and (c). Finally, as seen in Figs. 5(C) and 6(C), aggregation involves the formation of transient non-native dimeric, trimetric, and tetrameric states. This limitation of Gō simulations in exploring all folding mechanisms has already been discussed for a 16-residue monomeric β-hairpin.

In the present work, 30% of the runs fail to locate the ground states within 8000 events, and rather end up in various oligomeric states with non-native alignments of the strands. This behavior, already detected in ART simulations of $A\beta_{16-22}$ dimers and trimers, suggests that the rear-
rangement from non-native to native alignments of the strands is the rate-limiting factor in the aggregation process.

**IV. CONCLUSIONS**

Amyloid fibrils arise from misfolding and assembly of peptides and proteins. Despite recent experimental investigations, the nature of the soluble oligomers and assembly process remains elusive. In this work, we have attempted to follow the aggregation of the KFFE peptide by ART using a coarse-grained energy model (OPEP). This scheme was selected to overcome the long experimental aggregation time and was used for the Aβ_{16-22} fragment.25,26

On the basis of biophysical data and energy minimizations, Tjernberg et al. proposed an assembly of antiparallel β-sheets for the KFFE fibril.27 Although the structure in KFFE fibrils may depend on interactions between many neighboring molecules, both within one β-sheet and between β-sheets, 30% of ART-OPEP simulations on four KFFE peptides lead to a four-stranded antiparallel β-sheet, independently of the initial structure and the random seed. This ground state is sequence dependent, since no ordered structure is found for the variant KPGE, in agreement with the experiment.

ART simulations also predict that the tetrameric β-sheet is in equilibrium with dimers and trimers. Although such a distribution is not fully surprising, the simulations complement experiment by providing a clear picture of the various orientations of the chains and hydrogen-bonding patterns within each oligomer of lowest energy. For example, tetramers and trimers can mix parallel and antiparallel β-strands and two dimers can be perpendicular to each other but cannot form a stable two-layer antiparallel or parallel β-sheet.

Because this distribution of low-energy arrangements can be force field dependent, we also carried out all-atom MD simulations at 298 and 330 K with explicit solvent conditions, and starting in an antiparallel β-sheet, we find a conformational distribution similar to that of ART-OPEP. This consistency of results demonstrates the potential of ART-OPEP for the study of larger systems.

In contrast to all-atom MD simulations in explicit solvent, which explore a limited number of the possible aggregation23 or dissociation20 paths for tetramers within 10–20 ns, ART simulations provide us with a detailed picture leading to the formation of a four-stranded antiparallel β-sheet. Our analysis shows that formation of ordered β-sheet complexes is much more complex than previously established by MD simulations using the Gō approximation.13 In agreement with Gō-based simulations, our aggregation trajectories involve the sequential addition of one chain or the addition of two chains. But they also reveal reversible steps (10% of the aggregation trajectories), transitions from trimer–monomer to dimer–dimer and vice
versa (23% of the aggregation trajectories), and obligatory transitions between non-native and native alignments of β-strands. Because the possible number of non-native β-sheet intermediates scales with \( n^{N-1} \) (\( n \) is the number of H bonds within a dimer and \( N \) is the size of the nucleus) and most of these states are isoenergetic before the nucleus is formed, these non-native β-sheets likely are kinetic traps in the early steps of \( \alpha \beta_{1,40} \) or prion protein aggregation.

Finally, our simulations indicate that KFFE tetramers are not stable at room temperature. This finding is at variance with 10-ns MD simulations of tetramers of the human calcitonin DFNKF pentapeptide in explicit solvent,\(^20\) but is consistent with 2.5-μs MD simulations of tetramers of transthyretin fragments in implicit solvent.\(^17\) It is possible that the MD simulations on DFNKF have not converged yet within the 10-ns time scale, or that the DFNKF peptide has a much higher thermodynamic stability than KFFE and the transthyretin peptide. Since the instability persists for seven KFFE chains in a two-layer parallel or antiparallel β-sheet as determined by MD and ART simulations at 300 K (unpublished results), heptamers likely are below the critical size for nucleation of KFFE amyloid fibrils.

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