Distinct Early Folding and Aggregation Properties of Alzheimer Amyloid-β Peptides Aβ40 and Aβ42

STABLE TRIMER OR TETRAMER FORMATION BY Aβ42

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The amyloid β peptide (Aβ), composed of 40 or 42 amino acids, is a critical component in the etiology of the neurodegenerative Alzheimer disease. Aβ is prone to aggregate and forms amyloid fibrils progressively both in vitro and in vivo. To understand the process of amyloidogenesis, it is pivotal to examine the initial stages of the folding process. We examined the equilibrium folding properties, assembly states, and stabilities of the early folding stages of Aβ40 and Aβ42 prior to fibril formation. We found that Aβ40 and Aβ42 have different conformations and assembly states upon refolding from their unfolded ensembles. Aβ40 is predominantly an unstable and collapsed monomeric species, whereas Aβ42 populates a stable structured trimeric or tetrameric species at concentrations above ~12.5 μM. Thermodynamic analysis showed that the free energies of Aβ40 monomer and Aβ42 trimer/tetramer are ~1.1 and ~15/~22 kcal/mol, respectively. The early aggregation stages of Aβ40 and Aβ42 contain different solvent-exposed hydrophobic surfaces that are located at the sequences flanking its protease-resistant segment. The amyloidogenic folded structure of Aβ is important for the formation of spherical β oligomeric species. However, β oligomers are not an obligatory intermediate in the process of fibril formation because oligomerization is inhibited at concentrations of urea that have no effect on fibril formation. The distinct initial folding properties of Aβ40 and Aβ42 may play an important role in the higher aggregation potential and pathological significance of Aβ42.

Alzheimer disease (AD) is a progressive neurodegenerative disease occurring in the elderly resulting in the accumulation of misfolded proteins and cognitive dysfunction (1). The major pathological hallmarks of AD are the deposition of senile plaques and accumulation of neurofibrillary tangles in specific regions of the brain. The plaque deposits consist predominantly of insoluble amyloid fibrils formed by amyloid-β peptide (Aβ). The mutations associated with inherited forms of AD provide strong evidence that the aggregation of Aβ42 is a causative factor in etiology of AD because the mutations increase the relative amount of Aβ42 (2–4). A growing body of evidence indicates that prefibrillar oligomeric forms of Aβ may represent the primary pathological species and not the mature amyloid fibrils that accumulate in plaque deposits (4, 5).

The Aβ peptide is generated from limited proteolysis from a type 1 transmembrane protein, amyloid precursor protein (APP) (6). The two most common isoforms of Aβ are Aβ40 and Aβ42, which vary by the length of the C terminus. Although secreted Aβ40 is much more abundant, Aβ42 is the major component in senile plaques (7, 8). Biochemical studies show that Aβ42 aggregates and forms fibrils more rapidly than Aβ40 (9, 10). Recent studies have demonstrated that overexpression of high levels of Aβ40 alone do not result in overt amyloid pathology transgenic mice; however, expression of low levels of Aβ42 results in a broad range of amyloid pathology (11). Therefore, Aβ40 and Aβ42 possess different biochemical properties and Aβ42 is believed to be the major etiologic agent in pathogenesis of Alzheimer disease due to its enhanced aggregation or oligomerization properties.

The aggregation or oligomerization of Aβ has been the subject of numerous studies employing a variety of experimental approaches. Early studies indicated that Aβ42 forms non-covalent, SDS-resistant species with apparent molecular weights of dimer, trimer and/or tetramer, whereas under the same conditions Aβ39 and Aβ40 migrate as a monomeric species (9, 12, 13). However, gel filtration analysis under physiological conditions indicates that the smallest Aβ40 and Aβ42 species co-elute at a position corresponding to an apparent molecular weight expected for a dimer (12, 13). Fluorescence resonance energy transfer experiments suggest that low molecular weight Aβ40 exists as a dimer (14), whereas NMR diffusion measurements suggest that it is primarily monomeric (15). Photochemical oxidative cross-linking studies suggest that monomer, dimer, trimer, and tetramer species of Aβ40 exist in a rapid equilibrium (16). In contrast, the same method applied to Aβ42 suggests that pentameric or hexameric aggregates are preferentially formed that represent a fundamental building block for higher order assembly (17). Although some of these results appear inconsistent, different experimental conditions and analytical methods could conceivably account for the inconsistencies.

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2 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β peptide; Bis-ANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt; APP, amyloid precursor protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Although extensive research has been done on the structural and kinetic properties of Ab fibrillogenesis, the thermodynamic and initial folding properties have not been studied in detail. Unlike some amyloidogenic proteins that aggregate rapidly, seed-free Ab at low concentration exhibits “nucleation-dependent” kinetics with a significant lag phase on the order of hours to days to form fibrils (10). Taking advantage of this property, we examined the equilibrium folding properties of early species of Ab40 and Ab42 and determined their experimental energy values. We found the early species of Ab40 and Ab42 are different not only in tertiary and secondary structures but also in thermodynamic stability.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Ab peptides and the short peptides indicated in text were synthesized by using fluorenlymethoxy carbonyl solid phase chemistry using a continuous flow semiautomatic instrument described previously (9). Peptides were purified by reverse phase high performance liquid chromatography, and the purity was analyzed by matrix-assisted laser desorption ionization mass spectrometry. Puriﬁed peptides were dissolved in 50% acetonitrile/water, aliquotted, and relyophilized.

**Sample Preparation**—Folded Ab peptides were prepared as follows except where indicated. A denatured stock solution was prepared by dissolving lyophilized peptides at a concentration of 10 mg/ml in buffer containing 10 mM sodium phosphate, pH 7.4, and 10 M urea freshly before use. The urea-containing buffer was prepared as described (18) by weight measurement and refractive index. The denatured peptide stock in 10 M urea-containing buffer was centrifuged at 15,000 × g for 10 min at room temperature. The desired amount of denatured Ab in 10 M urea was added into buffer A (10 mM sodium phosphate, pH 7.4) for refolding. The sample was immediately vortexed and centrifuged at 15,000 × g for 10 min at room temperature and ﬁltered through a 0.22-μm ﬁlter. The supernatant was collected, and the concentration of Ab was determined by UV absorption at 280 nm (ε = 1280 cm⁻¹ M⁻¹) based on the Edhochn equation (19). The refolding yield calculated by absorbance before and after centrifugation is generally 80–100%.

**Native and SDS Polyacrylamide Gel Electrophoresis (PAGE)**—Tris-Tricine Ready Gels, 16.5%, (Bio-Rad) without SDS were used for monitoring the Ab absorption at 280 nm (10). Native PAGE was electrophoresed at 100 V for 5–6 h at 4 °C. Filtered through a 0.22-

**Urea Denaturation**—Both fluorescence and CD were employed to monitor the structural changes of Ab upon urea denaturation. Three concentrations of Ab, 50, 25, and 12.5 M, were examined. A titration method was performed by titrating the unfolded protein stock in 8 M urea-containing buffer A to the folded protein stock in <0.2 M urea with a constant protein concentration. The folded protein stock was prepared by refolding the unfolded peptide in buffer A through rapid dilution of urea. Reversed titration was performed with titration of the folded protein stock to the unfolded stock. The final urea concentration ranged from 0.08 to 8 M urea depending on the experimental design. The experimental time is less than 1 h for one set of denaturation experiments. For urea denaturation by Bis-ANS ﬂuorescence, the emission wavelength from 450 to 600 nm was monitored. The emissions at 508 nm for Ab40 and at 500 nm for Ab42 were collected, normalized, and plotted against urea concentration. For urea denaturation by far-UV CD, the signals at 220 nm were collected every 0.5 s for 60 s. The data were averaged, normalized, and plotted against urea concentration. Both Bis-ANS and CD data were normalized using the native signals in the lowest urea concentration as unity. The experiments were performed at 25 °C.

**Data Fitting and Analysis**—The denaturation data were plotted and ﬁtted by KaleidoGraph 3.0 (Synergy Software) or Igor Pro 5 (Wave Matrix Inc.). The data of the denaturation of Ab40 were ﬁtted to a two-state mechanism (N ↔ U) described by Santoro and Bolen (21). Briefly, total signal is contributed from fractions of the folded species N, fN, and the unfolded species U, fU, with their signal properties, P. Slopes for pre- and post-transition are also incorporated into Equation 1.

\[
P_{\text{total}} = f_N P_N + f_U P_U = f_N (P_{\text{so}} + m_\text{N(urea)}) + f_U (P_{\text{so}} + m_\text{U(urea)}) \quad (\text{Eq. 1})
\]

The sum of the folded and unfolded fraction is 1, \(f_N + f_U = 1\).

For a two-state mechanism with a folded monomer and an unfolded monomer (N ↔ U) the equilibrium constant between N and U, \(K_{\text{UN}}\), as shown in Equations 2 and 3, is

\[
K_{\text{UN}} = \frac{[U]}{[N]} = f_U (1 - f_N) \quad (\text{Eq. 2})
\]

and

\[
K_{\text{UN}} = \exp(-\Delta G_{\text{UN}}/RT) \quad (\text{Eq. 3})
\]

where \(R\) is the gas constant, \(T\) is the absolute temperature, and \(\Delta G_{\text{UN}}\) is the free energy between N and U states in the presence

**Fluorescence Emission and Circular Dichroism Spectroscopy**—Fluorescence emission spectra were obtained with a SPEX Fluorolog spectrofluorometer (Jobin-Yvon). The fluorescence of Bis-ANS (4,4’-dianilino-1,1’-binaphthyl-5,5’-disulfonic acid dipotassium salt; Sigma) was obtained by exciting the samples at 400 nm in buffer A containing 5 μM Bis-ANS and different urea concentrations as indicated. The fluorescence emission from 450 to 600 nm was monitored. CD spectroscopy was done using a Jasco Model J-800 (Jasco Inc.) spectropolarimeter for the far-UV CD spectra. The samples were in buffer A containing different urea concentrations as indicated. The samples were placed in a cylindrical quartz cell (Hellma) with the path length of 1 cm. The spectral data were collected from 250 nm and below.
of denaturant. Because \( \Delta G_{UN} \) is dependent on denaturant concentration linearly, Equation 4 describes the relationship between the free energy and denaturant.

\[
\Delta G_{UN} = \Delta G_{UN}^{H=0} - m[\text{urea}]
\]

(Eq. 4)

The fittings were done with Equation 1 after incorporating the Gibbs free energy of unfolding in the absence of denaturant, \( \Delta G_{UN}^{H=0} \) by Equations 2–4.

The solvent-exposed surface area (\( \Delta \text{ASA} \)) and the estimated number of residues can be obtained from Equations 5 and 6 (22).

\[
m_{\text{cal/mole}} = 374 + 0.11(\Delta \text{ASA}) \quad \text{(Eq. 5)}
\]

\[
\Delta \text{ASA} = -907 + 93(\#\text{residue})
\]

(Eq. 6)

For two-state mechanism with a trimeric species to unfolded monomeric species, \( \text{Tri} \leftrightarrow 3\text{U} \), or tetrameric species to unfolded species, \( \text{Tetra} \leftrightarrow 4\text{U} \), the equilibrium constants are expressed as Equation 7 and Equation 8, respectively,

\[
K_{\text{UTri}} = [U]^3/[[\text{Tri}]] = 3P_1 f_u/(1 - f_u)
\]

(Eq. 7)

\[
K_{\text{UTetra}} = [U]^4/[[\text{Tetra}]] = 4P_1 f_u/(1 - f_u)
\]

(Eq. 8)

where \( P_1 \) is the total subunit concentration. By solving the third-order equation of \( f_u \) for the trimer model (23, 24), we obtain the solution as follows in Equations 9 and 10.

\[
f_u = [0.5c + (13^{1/2}/18)c(4c + 27)^{1/2}]^{1/3}
\]

\[-c/\{[0.5c + (13^{1/2}/18)c(4c + 27)^{1/2}]^{1/3}\}
\]

\[
c = K_{\text{UTri}}/(3P_1)
\]

(Eq. 9)

(Eq. 10)

For the tetrameric model, we obtain the physically meaningful root as follows by solving the fourth-order equation of \( f_u \) as shown in Equations 11–13 (25).

\[
f_u = \{-(s^{1/2}) + [-s + 2/(as^{1/2})]^{1/2}\}/2
\]

\[s = -4/[2/[27a + (729a^2 + 6912a^2)^{1/2}]]^{1/3}
\]

\[+ 1/[3a(2/[27a + (729a^2 + 6912a^2)^{1/2}])^{1/3}]
\]

\[a = 4P_1/\exp(K_{\text{Tetra}})
\]

(Eq. 11)

(Eq. 12)

(Eq. 13)

The fitting of the denaturation data of Aβ42 for the trimer model was done by Equation 1 incorporating Equations 4, 7, 9, and 10, whereas for the tetramer model the fitting was done by Equations 1, 4, 8, 11–13. The parameters including \( \Delta G_{\text{UTri}}^H \), \( \Delta G_{\text{UTetra}}^H \), m-value, \( f_u, f_{\text{Tri}}, f_{\text{Tetra}} \), and the slope of pre- and post-transitions were obtained. Moreover, the fitting of this model was performed using the global fitting package in Igor Pro 5 to increase the accuracy where \( \Delta G_{\text{UTri}}^H \) and m-value were set as global parameters, meaning only one value is generated while fitting to various data sets. The other parameters can be varied.

To describe the Aβ42 folding with the three-state model either \( \text{Tri} \Leftrightarrow 3\text{M} \Leftrightarrow 3\text{U} \) or \( \text{Tetra} \Leftrightarrow 4\text{M} \Leftrightarrow 4\text{U} \), we plotted various free energies as a function of urea and employed linear extrapolation to obtain the free energy in the absence of denaturant (26). Briefly, the first transition describes multimer dissociation. The monomer fraction, \( f_{\text{M}} \), can be obtained by the experimental data using Equation 14.

\[
f_{\text{M}} = (\lambda_{\text{obs}} - \lambda_t)/(\lambda_{\text{M}} - \lambda_t)
\]

(Eq. 14)

\( \lambda_{\text{obs}}, \lambda_M, \) and \( \lambda_T \) are the observed trimeric/tetrameric and monomer signals. The signal at 2 M urea was used as the monomeric signal. By using Equations 14, 7, and 8, we obtained various free energies of multimer dissociation, \( \Delta G_{\text{dissociation}} \), in different concentrations of urea. The second transition represents the monomeric species unfolding (M ↔ U); the unfolded fraction, \( f_u \), can be obtained by the experimental data using Equation 15.

\[
f_u = (\lambda_{\text{obs}} - \lambda_M)/(\lambda_u - \lambda_M)
\]

(Eq. 15)

By using Equations 15 and 2, we were able to plot the free energies of unfolding, \( \Delta G_{\text{UMP}} \), as a function of urea. The data were fit linearly by Equation 4 to obtain the free energy in the absence of urea and the m-value.

Fibril and Prefibrillar Oligomer Assay—Amyloid fibrillization was monitored by thioflavin T binding (27) using a fluorescence plate reader (XS; Molecular Devices Inc.). Folded Aβ peptides at 50 μM in buffer A containing 0.02% sodium azide in different urea concentrations were prepared. The samples were stirred with Teflon-coated microstar bars at 300 rpm at room temperature. An aliquot of 10 μl was taken at the indicated time and mixed with 90 μl of thioflavin T solution (30 μM thioflavin T in buffer A) to detect thioflavin T fluorescence. The samples were excited at 442 nm, and the emissions at 485 nm were recorded. The emission was plotted against time as indicated under “Results.” The background fluorescence of the buffer is subtracted. Dot blotting was also employed to monitor the appearance of oligomeric species in the fibrillation process. Aliquots of 2 μl from the samples were evenly pipetted and dotted on nitrocellulose membranes at the indicated time. Then, the membranes were stained by direct blue staining or blotted with Aβ antibody A11 to detect prefibrillar oligomers (28).

RESULTS

The experimental paradigm we used to analyze the early conformations and aggregation states of Aβ was to refold Aβ from 10 M urea denatured stock solutions. This strategy has been commonly used to study the folding properties of proteins (18). We refer to the refolded Aβ as described under “Experimental Procedures” as “folded” Aβ (<0.2 M urea) and the Aβ in 6 M urea as denatured Aβ.

First, we performed analytical size-exclusion chromatography to examine the assembly and hydrodynamic properties of folded Aβ40 and Aβ42 at 50 μM. We found that both Aβ40 and Aβ42 co-elute as single peaks with an apparent molecular mass of ~11 kDa as previously reported (12, 17) (supplemental Fig. S1). This indicates that the hydrodynamic radius of Aβ40 is indistinguishable from that of Aβ42. We also confirmed that the aggregation kinetics of 50 μM folded Aβ40 and Aβ42 displayed a significant lag time before
species that are not in rapid equilibrium because they separate into distinct bands on the time scale of the electrophoretic separation. The faster migrating bands of Aβ40 and Aβ42 closely co-migrate, although the Aβ42 band migrates slightly slower than that of Aβ40. When the same samples were electrophoresed under denaturing conditions in the presence of SDS on Tris-Tricine gels, we found both samples ran predominantly as monomers (Fig. 1B) but Aβ42 contains a higher molecular mass band of 14.5 kDa that is recognized by Aβ-specific antibody. This is the approximate size expected of a trimer (13.5 kDa). We have previously reported that this higher molecular mass Aβ42 band runs as a size consistent with a tetrameric species on Tris-glycine gels (9, 12). The results indicate that Aβ40 and Aβ42 have different assembly states after dilution from urea stock solutions. The fact that Aβ42 runs as a single symmetrical peak on gel filtration, but runs as two distinct species on native gel electrophoresis, suggests that the two species have approximately the same hydrodynamic radius and do not separate by gel filtration.

To examine the possible differences in the conformations of Aβ40 and Aβ42, we employed fluorescence and circular dichroism spectroscopy. We used Bis-ANS, which is known to bind to hydrophobic surfaces of partially folded proteins, to monitor the conformational differences. Bis-ANS has been previously used to characterize soluble Aβ conformations that are distinct from fibrils (29). We found the maximum emission of Bis-ANS fluorescence of 50 μM folded Aβ40 was shifted to ~508 nm compared with that of the denatured Aβ40, which was ~520 nm (Fig. 1C). The differences of Bis-ANS binding between folded and denatured Aβ40 show that the folded Aβ40 contains hydrophobic clusters on the protein surfaces, indicating it is able to form tertiary structures rather than completely random coils. In addition, the fluorescence intensity of Bis-ANS significantly increased in the presence of folded Aβ40.

In contrast, the fluorescence intensity of Bis-ANS in the presence of folded Aβ42 is ~10-fold higher than that of folded Aβ40 (Fig. 1C). The emission maximum of Aβ42-bound Bis-ANS is blue-shifted to ~495 nm, and the intensity of folded Aβ42 is much higher than denatured Aβ42. The differences in the Bis-ANS emission suggest that either there are different Bis-ANS binding sites between the folded Aβ40 and Aβ42 due to structural differences or it is simply the result of different partition of bound and free Bis-ANS. Previous studies of Bis-ANS fluorescence indicate that Bis-ANS does not bind significantly differently to Aβ40 and Aβ42 at pH 2.4, suggesting that Aβ structures and aggregation states are substantially similar at low pH (29).

We also employed far-UV CD to compare the secondary structural properties of the folded and denatured Aβ40 and Aβ42. Folded Aβ40 is mostly random coil (Fig. 1D) as previously reported (30, 31). However, it is not completely unfolded because there are significant intensity differences between the folded and denatured Aβ40. In comparison with Aβ40, folded Aβ42 contains some β-structure that displays a minimum at 216 nm. Denatured Aβ42 contains significantly less structure than folded Aβ42. Because of the presence of high urea concen-

FIGURE 1. Native gel and SDS-PAGE electrophoresis, Bis-ANS fluorescence spectra, and far-UV CD spectra of Aβ40 and Aβ42. A, native gel of folded Aβ40 and Aβ42 by direct blue staining. Unlike Aβ40, Aβ42 migrates as two distinct bands (arrows). B, SDS-PAGE of folded Aβ40 and Aβ42 by direct blue staining and Western blot. The nitrocellulose membrane was stained by direct blue staining. Unlike Aβ40, Aβ42 migrates as two distinct bands on the time scale of the electrophoretic separation. The faster migrating bands of Aβ40 and Aβ42 closely co-migrate, although the Aβ42 band migrates slightly slower than that of Aβ40. When the same samples were electrophoresed under denaturing conditions in the presence of SDS on Tris-Tricine gels, we found both samples ran predominantly as monomers (Fig. 1B) but Aβ42 contains a higher molecular mass band of 14.5 kDa that is recognized by Aβ-specific antibody. This is the approximate size expected of a trimer (13.5 kDa). We have previously reported that this higher molecular mass Aβ42 band runs as a size consistent with a tetrameric species on Tris-glycine gels (9, 12). The results indicate that Aβ40 and Aβ42 have different assembly states after dilution from urea stock solutions. The fact that Aβ42 runs as a single symmetrical peak on gel filtration, but runs as two distinct species on native gel electrophoresis, suggests that the two species have approximately the same hydrodynamic radius and do not separate by gel filtration.

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Early Stages of Aβ Folding

Âβ40 and Âβ42 have reached a quasi-equilibrium within the time for each titration (<30 s) where no further folding, unfolding, or aggregation events are rapidly proceeding. The reversibility of Aβ also allows us to examine the thermodynamics of the peptide assembly.

For Âβ40 (Fig. 2A), the Bis-ANS emission at 500 nm and CD signal at 220 nm were plotted against urea concentration. Similar denaturation curves were obtained with both Bis-ANS binding and far-UV CD. This indicates the changes of the exposed hydrophobic surface (tertiary structures) and secondary structures are concomitant. The consistency between Bis-ANS binding and CD data also supports the conclusion that Bis-ANS binding monitors the overall conformational changes rather than selective species under these conditions. We examined different concentrations of Âβ40 to determine whether the structural changes are due to quaternary associations. If multimers are present at equilibrium, the denaturation curves will change accordingly due to dissociation of the multimeric species, whereas if the protein exists as a monomer the denaturation curves will not change as a function of peptide concentration.

Âβ40 at 50, 25, and 12.5 μM were examined for both Bis-ANS binding and far-UV CD in different urea concentrations. The data from three concentrations obtained by both methods overlay with no apparent concentration dependence (Fig. 2A). A single transition between ~0.75 and ~4 M urea was obtained. The pretransition (<1 M urea) and the post-transition (>4 M) are also affected by urea concentration but in linear fashions. The cooperative change is due to the structural changes of Aβ at intermediate urea concentrations. The data were accurately fit by a two-state model (N ⇌ U), where a folded, or native, monomer and an unfolded or denatured monomer are present at equilibrium. The free energy (ΔG^{N→U}_{folding}) of Âβ40 obtained from the fit is ~1.1 kcal/mol. The m-value, a cooperativity parameter, is ~0.7 kcal/mol/m. An unfolded fraction of 0.18 was obtained in the absence of urea. The midpoint of the transition, [urea]_{0.5}, calculated from the fit is at 1.6 M, which is close to the experimental data. This indicates that fitting the data to a two-state model is precise. The residual of the fit is shown in Fig. 2A.

To further analyze the assembly and equilibrium folding properties of Aβ, we examined Âβ40 and Âβ42 as a function of urea and peptide concentration. First, we performed denaturation studies by titration as described under "Experimental Procedures." Bis-ANS binding to Aβ in different urea concentrations was monitored. We also performed the denaturation by reverse titration to examine the reversibility. If the data generated by titration and reverse titration are equivalent, the folding of the protein is considered to be reversible where no aggregation or irreversible reaction is involved in the folding process (18). The results demonstrate that Aβ folding is reversible because the Bis-ANS emission spectra from titration and reversed titration are identical (supplemental Fig. S2). The results also indicate that both

![Image](60x354 to 396x733)

**FIGURE 2.** Urea denaturation of Aβ by Bis-ANS fluorescence and far-UV CD. A, denaturation of Âβ40 by Bis-ANS fluorescence at 50 (●), 25 (■), and 12.5 (○) μM and far-UV CD signals at 50 (○), 25 (■), and 12.5 (○) μM. The solid line is the fit for two-state model (N ⇌ U). The residuals of the fit are plotted above the denaturation curves. B and C, denaturation of Âβ42 and the fits of the two-state models, Trimer ⇌ 3U (panel B) or Tetramer ⇌ 4U (panel C). The data were labeled as in panel A. The fits of Bis-ANS signals (solid curves) and CD signals (dashed curves) were generated from global fitting. D, extrapolation of the free energies of trimer/tetramer dissociation ΔG^{dissociation}_{40} and monomer unfolding ΔG^{folding}_{40} of Âβ42. The calculated free energies for the three-state models (Tri ⇌ 3M ⇌ 3U or Tetra ⇌ 4M ⇌ 4U) were plotted against urea concentration. The ΔG^{dissociation}_{40} of the tetramer model from 50 (●) and 25 (■) μM and of the trimer model from 50 (○) and 25 (□) μM are shown. The second free energy ΔG^{folding}_{40} obtained from 50 (▲) and 25 (●) μM are also shown. Lines are linear fits of the data.
Many small globular proteins (<100 kDa) adopt two-state folding mechanisms at equilibrium. The statistical study of proteins adopting a two-state mechanism provides calculations of the exposed surface area upon protein unfolding and the estimated number of residues in the protein using the $m$-value (22). The exposed surface area of the folded monomeric $\alpha$B40 is predicted to be 2963 Å$^2$, and the estimated number of residues of $\alpha$B40 is 41.6, which is close to the actual number.

The denaturation of $\alpha$B42 at 50, 25, or 12.5 $\mu$M was also examined (Fig. 2B). As observed for $\alpha$B40, the Bis-ANS spectrophotometric titration curves were fully reversible (supplemental Fig. 2B), indicating that the sample reaches equilibrium. The Bis-ANS and far-UV CD curves give similar results, indicating that the tertiary and secondary structure changes coordinately. The denaturation of $\alpha$B42 shows a concentration dependence with a midpoint of transition that varies from ~1.4, ~1.1 to ~0.6 M urea for 50, 25, and 12.5 $\mu$M, respectively.

Comparing the denaturation data of $\alpha$B42 at 50 $\mu$M with that of $\alpha$B40 at the same concentration, $\alpha$B42 shows a flatter pretransition, indicating the structure in the presence of 0 to 0.75 M urea is not significantly affected by urea. The data show one cooperative transition suggesting $\alpha$B42 adopts an apparent two-state mechanism. The midpoint of the transition, ~1.4 M urea, is slightly lower than that of $\alpha$B40, 1.6 M urea.

The concentration dependence and the apparent single transition of $\alpha$B42 denaturation suggest a model of multimer and unfolded monomer equilibrium. Because we observed a higher molecular weight band on SDS gel electrophoresis that has an apparent molecular weight of a trimer, (Fig. 1B), we fit the data globally to a model of a trimer to three unfolded monomers (Tri $\Rightarrow$ 3U). Both the Bis-ANS fluorescence and far-UV CD data of $\alpha$B42 at 50 and 25 $\mu$M fit well to the trimer-unfolded monomer model (Fig. 2B). The residual of the fit is shown in Fig. 2B. We obtained a $\Delta G^{H_2O}_{\text{dissociation}}$ of ~14.9 kcal/mol and an $m$-value of ~2.8 kcal/mol. The data for $\alpha$B42 at 12.5 $\mu$M cannot be fit to the model because it lacks a pretransition state. This indicates that at 12.5 $\mu$M $\alpha$B42 multimer is not significantly populated or it is not stable in the presence of low concentrations of urea. Because the higher molecular weight $\alpha$B42 band has also been reported as a tetramer (9, 12), we fit the data to a tetramer to four unfolded monomer model (Tetra $\Rightarrow$ 4U) (25) and found they fit equally well with little difference on the $\chi$ square value, 0.145 and 0.069 for trimer and tetramer model, respectively. With the tetramer model, we obtained $\Delta G^{H_2O}_{\text{dissociation}}$ of ~21.6 kcal/mol and an $m$-value of ~3.4 kcal/mol (Fig. 2C). However, both fits reveal a large amount of unfolded species populated in the absence of urea by linear extrapolation of the post-transition.

An alternative three-state model is also possible with the presence of a folded monomeric intermediate. It is possible that a transition at higher urea concentration is present despite a large population of unfolded species. The three-state model either Trimer $\Rightarrow$ 3M $\Rightarrow$ 3U or Tetramer $\Rightarrow$ 4M $\Rightarrow$ 4U includes a transition of multimer dissociation from 0 to ~2 M urea and an unfolding of a monomeric intermediate from ~2 to 4 M urea. Due to the complexity of the fitting, we used the plotting method and extrapolation described under "Experimental Procedures" to obtain the energy values (Fig. 2D). We obtained free energy values for trimer dissociation $\Delta G^{H_2O}_{\text{dissociation}}$ of 16 kcal/mol and for tetramer dissociation $\Delta G^{H_2O}_{\text{dissociation}}$ of 23.2 kcal/mol, where close values were obtained from two $\alpha$B concentrations (50 and 25 $\mu$M). The monomer unfolding $\Delta G^{H_2O}_{\text{dissociation}}$ of 5.8 kcal/mol was obtained from the second transition. Therefore, we suggest that trimer/tetramer dissociation is the major energetic cost rather than the unfolding of the monomeric species. Overall, the distinct equilibrium folding properties of $\alpha$B40 and $\alpha$B42 indicate that the early species differ by assembly state, structure, and stability.

Because we found Bis-ANS binds much better to the folded than the denatured $\alpha$B, we explored which regions contain the Bis-ANS binding site. We compared the binding of Bis-ANS to synthetic segments of $\alpha$B including $\alpha$B1–12, 7–18, 13–24, 19–30, and 25–36 (Fig. 3). The short peptides were denatured and refolded as described for the full-length protein. Interestingly, two of the peptides, $\alpha$B13–24 and $\alpha$B25–36, show dramatic Bis-ANS emissions, whereas the others do not interact with Bis-ANS. Bis-ANS does not interact with $\alpha$B19–30, which possesses a protease-resistant segment (32) suggesting that the hydrophobic side chains within the region are buried. Therefore, it is likely that the flanking regions of the protease-resistant segment compose the solvent-exposed hydrophobic surfaces.

Finally, we examined the effect of different urea concentrations on fibril and prefibrillar oligomer formation, using thioflavin T to monitor fibril formation and A11 anti-oligomer antibody (28) to monitor oligomerization. We analyzed the aggregation kinetics of folded $\alpha$B40 and $\alpha$B42 at 50 $\mu$M with continuous stirring at room temperature (Fig. 4). By stirring the folded $\alpha$B40 and $\alpha$B42 individually at 0.2 M urea, a lag phase from 0 to ~6 h followed by an increase in fibril formation was observed (Fig. 4, A and B). The fibrillization of $\alpha$B40 is significantly more sensitive to urea disruption than $\alpha$B42. This result suggests the folded structure of $\alpha$B42 promotes fibrillization. The results are consistent with the reported data on the fibrillization of $\alpha$B40 measuring by light scattering in the presence of urea (33). Similar to $\alpha$B40, $\alpha$B42 fibrillization is slower at 2 and...
Interestingly, we found that Aβ42 fibrillization although the unfolding of Aβ42 is complete. This suggests that the unfolded monomer is also amyloidogenic.

Prefibrillar oligomer formation was determined by immunoblotting with the conformation-dependent, oligomer-specific antibody (A11) (Fig. 4C). This antibody does not recognize oligomers smaller than approximately hexamer (28). Equal amounts of protein were dotted as determined by total protein staining (data not shown). In 0.2 M urea, oligomers begin to appear around 16–52 h of incubation for Aβ40 and Aβ42 and increased with longer incubations, well after the time where fibril formation is maximal as determined by thioflavin fluorescence. The results are not due to limited sensitivity of the dot blot assay because it is at least 10-fold more sensitive for oligomers than the thioflavin T assay is for fibrils. This indicates that the normal kinetic relationship where oligomers are observed prior to fibril formation is reversed in the presence of urea.

At urea concentrations >0.2 M, oligomer formation was not observed for either Aβ40 or Aβ42. The fact that A11-positive oligomers are not observed at early times indicates that the structure of the folded multimer of Aβ42 is immunologically distinct from the oligomeric state. The results also indicate that oligomer formation is significantly more sensitive to disruption by urea than fibril formation. The fact that fibril formation of Aβ42 proceeds efficiently at 2 and 4 M urea, concentrations where no oligomers are detected, also indicates that oligomers are not an obligate intermediate for fibril formation and that oligomers and fibrils represent distinct alternative pathways of aggregation.

**DISCUSSION**

Aβ aggregation is a critical aspect of Alzheimer disease pathogenesis, and increasing evidence points to the role of relatively small aggregates or soluble oligomers as the primary pathogenic species (4). Unlike fibril formation, relatively little is known about the assembly states and energetics of initial folding and early aggregation. We found that the initial folded structures and properties of Aβ40 and Aβ42 are different and these differences are important for the ability to form higher order structures, such as amyloid fibrils. Aβ40 exists as an unstable monomer population containing a large fraction of random coil but is not completely unfolded. It likely adopts a collapsed structure as previously reported (34). Aβ40 displays a simple two-state monomeric to unfolded monomer model similar to most small proteins. Its free energy of folding is marginal with the value of 1.1 kcal/mol and a low m-value, 0.7 kcal/mol/M, indicating the structure is not strongly dependent on the denaturant concentration. The estimated residue number of Aβ40, ~41.6, based on two-state globular proteins is close to the actual number 40, but an over-estimation of the m-value could result from the large slope of the pretransition (35). Therefore, because Aβ40 is unlikely to adopt a globular structure, the actual solvent-exposed surface area exposed upon unfolding can be smaller than the estimation as described under “Results.”

Unlike Aβ40, Aβ42 displays a denaturation profile that depends on the peptide concentration. One apparent transition was observed, and the data fit well with either a two-state trimer...
to unfolded monomer model (Tri \( \Leftrightarrow \) 3U) or tetramer to unfolded monomer model (Tetra \( \Leftrightarrow \) 4U), whereas the data do not fit to a dimer to unfolded monomer (D \( \Leftrightarrow \) 2U) model. Furthermore, the large post-transition could represent an unfolding transition of the monomeric species. Thus, the trimer/tetramer dissociation is the major step compared with the monomer unfolding in the three-state model (Tri \( \Leftrightarrow \) 3M \( \Leftrightarrow \) 3U or Tetra \( \Leftrightarrow \) 4M \( \Leftrightarrow \) 4U). The free energy of \( \beta \)-42 dissociation is much larger than that of \( \beta \)-40. Nevertheless, \( \beta \)-42 at 12.5 \( \mu \)M no longer displays a pretransition and is unable to be fit to the same model, suggesting that very little trimer/tetramer exists at this concentration.

The concentration dependence of \( \beta \)-42 aggregation agrees well with the reported critical concentration for micelle formation in vitro, \( \sim \)20 \( \mu \)M (12). However, \( \beta \)-40 also displays the same critical concentration as \( \beta \)-42 (12), and the stability of \( \beta \)-40 ranging from 12.5 to 50 \( \mu \)M is not affected over this concentration range, indicating that the initial folding and aggregation of \( \beta \)-42 is not related to micelle formation. These results indicate the early folding and aggregation states of \( \beta \)-40 and \( \beta \)-42 are distinct, which may account for their different fibrilization properties and pathological significance.

Differences in the early aggregation states of \( \beta \)-40 and \( \beta \)-42 have also been observed by photochemical cross-linking (17). Whereas the predominant cross-linked products of 30 \( \mu \)M \( \beta \)-40 are dimer, trimer, and tetramer, cross-linking of 30 \( \mu \)M \( \beta \)-42 yields predominantly tetramer, pentamer, hexamer, and heptamer. These data have been interpreted to suggest that \( \beta \)-42 contains higher order aggregates ranging from tetramer to octamer termed “paranuclei” that are in rapid equilibrium and are not observed in \( \beta \)-40. Our data are consistent with this observation and suggest that the trimeric or tetrameric species we observe represent the paranuclei observed by cross-linking. This is also consistent with the observation that paranuclei are not observed by cross-linking at low concentrations of \( \beta \) (1 \( \mu \)M) (36), because the trimeric/tetrameric state is poorly populated at concentrations below 12.5 \( \mu \)M. Our data further indicate that trimers or tetramers are relatively stable because they do not dissociate detectably during native gel electrophoresis and they have a free energy of formation of \( \sim \)15 or \( \sim \)21.6 kcal/mol for trimer and tetramer, respectively.

There are some apparent differences in the size and stability of the paranuclei previously described and the trimer/tetramer reported here. This apparent discrepancy can be simply explained by the possibility that the photochemical cross-linking products are a mixture of products that result from the cross-linking of a trimer/tetramer and the random collisional cross-linking of additional monomeric subunits that are in rapid equilibrium and present in the mixture. Trimer/tetramer formation correlates well with the faster nucleation kinetics of \( \beta \)-42 (10), suggesting that these small oligomers may be important for nucleation. However, their formation is not required for fibril formation because \( \beta \)-40, which does not form such an oligomer at the same peptide concentration, forms fibrils and fibril formation by \( \beta \)-42 occurs at concentrations of urea that disrupt the small oligomers.

We were also able to probe the solvent-exposed hydrophobic area of \( \beta \) by Bis-ANS binding. LeVine (29) has shown that Bis-ANS at pH 3 binds to the early species of aggregation and the kinetics is opposite to the thioflavin T kinetics. The possible reason for the disappearance of Bis-ANS signal is that the hydrophobic surface is buried in the higher aggregative assembly, in which thioflavin T binds to its \( \beta \)-structure. This assumption is consistent with the finding that high concentrations of naphthalene sulfonates including Bis-ANS are able to inhibit \( \beta \) oligomerization (37).

We found that the regions \( \beta \)-13–21, HHQQKLVFFA, and \( \beta \)-30–36, ALIGLMV are the potential Bis-ANS binding sites. The hydrophobic residues in these regions are most likely exposed, whereas the protease-resistant segment containing a structured loop, \( \beta \)-21–30, is rather buried (32, 38, 39). Our results are consistent with the NMR studies showing that a hydrophobic cluster composed of \( \beta \)-17–19 is involved in a hydrophobic patch on the protein surface (34).

\( \beta \) aggregation is critical for the etiology of AD. We found that the degree of “foldness” is important for the aggregation properties of \( \beta \). Both unfolded \( \beta \)-40 and \( \beta \)-42 in 6 M urea cannot fibrillize, although \( \beta \)-42 seems to be more capable of fibrillization under these denaturing conditions. Nevertheless, oligomerization of \( \beta \)-40 and \( \beta \)-42 is strongly affected by denaturant. The appearance of prefibrillar oligomers requires the native, folded structure in the early stages of aggregation because oligomerization is prevented by low concentrations of urea. If the oligomeric pathway is required for toxicity, the folded structure of \( \beta \) is therefore essential for the etiology of AD.

The differences in assembly, stability, and structure between \( \beta \)-40 and \( \beta \)-42 (Fig. 5) are illustrated in the energy landscape. If the fibrillization of amyloid is a downhill energy diagram (40, 41), the stability of folded \( \beta \)-42 is closer to that of the fibrillar state which contains the most stable structure. These results provide a rational basis for explaining the enhanced aggregation, deposition, and pathological significance of \( \beta \)-42.
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