Solid-support Electron Paramagnetic Resonance (EPR) Studies of Aβ40 Monomers Reveal a Structured State with Three Ordered Segments

Alzheimer disease is associated with the pathological accumulation of amyloid-β peptide (Aβ) in the brain. Soluble Aβ oligomers formed during early aggregation process are believed to be neurotoxins and causative agents in Alzheimer disease. Aβ monomer is the building block for amyloid assemblies. A comprehensive understanding of the structural features of Aβ monomer is crucial for delineating the mechanism of Aβ oligomerization. Here we investigated the structures of Aβ40 monomers using a solid-support approach, in which Aβ40 monomers are tethered on the solid support via an N-terminal His tag to prevent further aggregation. EPR spectra of tethered Aβ40 with spin labels at 18 different positions show that Aβ40 monomers adopt a completely disordered structure under denaturing conditions. Under native conditions, however, EPR spectra suggest that Aβ40 monomers adopt both a disordered and a structured state. The structured state of Aβ40 monomer has three more ordered segments at 14–18, 29–30, and 38–40. Interactions between these segments may stabilize the structured state, which likely plays an important role in Aβ aggregation.

Alzheimer disease (AD), a fatal neurodegenerative disorder, is the most common cause of senile dementia and represents a global healthcare problem as the world population is aging. AD is characterized with the pathological accumulation of amyloid-β peptide (Aβ) in the brain. Aβ peptides are the normal proteolytic products of amyloid-β precursor protein (1). Depending on the exact proteolytic cleavage site of amyloid-β precursor protein, different lengths of Aβ monomers have been extensively investigated (2). Aβ42 is the most common cause of senile dementia and represents a precursor protein (1). Depending on the exact proteolytic cleavage site of amyloid-β peptide (Aβ), proteolytic products containing different fractions of secondary structural elements remain speculative. Many conformations of Aβ monomers containing different fractions of secondary structural elements...
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have been suggested, but little consensus was found in these studies. One potential factor contributing to the discrepancies in previous work is sample homogeneity. It has been shown that Aβ forms small oligomers almost instantly upon dissolving in aqueous solution (20). Therefore, it is very difficult to obtain a homogeneous solution of Aβ monomers. To address the homogeneity issue, here we employ a solid-support approach to study the structure of Aβ40 monomers. In the solid-support approach, Aβ monomers are tethered on nickel-charged agarose beads via a 6-residue His tag introduced at the N terminus of Aβ sequence. The non-covalent interaction between nickel and His tag is strong enough to withstand denaturing conditions, allowing tethering to take place under conditions unfavorable for Aβ aggregation, such as 8 M urea in a buffer of pH 11 in this work. Both high concentration of urea (21) and high pH (22) are known to inhibit Aβ aggregation. Because Aβ monomers are tethered on the solid support, interactions among monomers for further aggregation are prevented. Thus, a homogeneous sample of Aβ monomers can be obtained.

Previous studies have shown that tethering generally does not perturb protein structure. For example, Amirgoulova et al. (23) developed a cross-linked, six-arm, star-shaped polyethylene glycol surface, which was shown to allow reversible unfolding and refolding, and also has negligible interaction with the immobilized proteins. Zhuang et al. (24) developed a modified riboyme which was immobilized to a surface via a streptavidin-biotin interaction. It shows that immobilized protein resulted in identical rate and equilibrium constants of catalytic reaction as obtained from unmodified protein, indicating that immobilized riboyme was functionally indistinguishable from the unmodified free riboyme in solution. Previously, EPR studies of T4 lysozyme (T4L) labeled at various sites show that the EPR spectral lineshape remains largely unchanged upon tethering on solid support (25, 26), suggesting that tethering on solid support does not disrupt protein structure.

Upon tethering on solid support, we use EPR spectroscopy to study the structure of Aβ monomers. EPR has been proven to be a powerful technique in studying protein structure, dynamics, and protein-protein interactions (27–29). In Aβ studies, EPR has been used to investigate the structure of different aggregates (30–33). Because EPR signal originates from the magnetic resonance of spin labels introduced at specific residues in protein sequence, use of solid support does not affect EPR analysis. Rather, tethering on solid support has the advantage of reducing the overall tumbling of the whole protein, allowing EPR analysis to be focused on intrinsic protein dynamics. Spin labels are introduced to protein sequence through site-directed spin labeling. The basic strategy of site-directed spin labeling is to first substitute a specific residue with a cysteine by site-directed mutagenesis. Then the SH group of the cysteine residue is reacted with a spin labeling reagent to generate a spin-labeled side chain. The spin-labeled side chain used in this work is named R1 (supplemental Fig. S1). Three major pieces of information can be obtained from EPR studies of spin-labeled proteins: spin label mobility, solvent accessibility, and inter-residue distance. In this work, we use spin label mobility to analyze the structure of Aβ monomers.

EXPERIMENTAL PROCEDURES

Preparation of T4 Lysozyme, Spin Labeling, and Tethering on Agarose Beads—Cysteine was introduced into T4L sequence at residue 72 using the QuikChange site-directed mutagenesis kit (Agilent). For protein expression, the mutant construct was transformed into BL21(DE3) cells (Novagen). The cells were cultured to an absorbance of A600nm = 0.6 in LB broth at 37 °C and then induced with 1 mM IPTG at 28 °C for 4–6 h. The cells were collected by centrifugation. For purification of T4L protein, the cells were resuspended in PS buffer (50 mM phosphate, 50 mM NaCl, pH 7.4) and then sonicated on ice. The cell debris was pelleted by centrifugation. The supernatant was filtered using 0.45-μm filter (Whatman) and loaded onto a 5-ml HiTrap column (GE Healthcare) equilibrated with PS buffer. Protein was eluted with a linear imidazole gradient (50 to 500 mM) in 10 column volumes. Protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 24.1 mM⁻¹ cm⁻¹.

For spin labeling, T4L was first incubated in 10 mM DTT for 20 min to break any disulfide bonds and then desalted using a 5-ml HiTrap desalting column (GE Healthcare) equilibrated with a buffer containing 20 mM MOPS, 50 mM NaCl, pH 6.8. The spin labeling reagent MTSSL (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate; Enzo Life Sciences) was used at 5-times molar excess and incubated at 4 °C for 24 h. Free spin labels were removed by a 5-ml HiTrap desalting column (GE Healthcare) equilibrated with PS buffer. MALDI-TOF mass spectrometry was performed to ensure that the mass is correct and the extent of labeling is >95%.

For denaturation of untethered T4L, T4L D72R1 was diluted to 0–5 M guanidine hydrochloride (GdnHCl) with a stock solution containing 15 mM phosphate, 7 M GdnHCl, and pH 6.8 for EPR spectroscopy.

For denaturation of tethered T4L, T4L D72R1 was added to 100 μl of agarose beads charged with nickel (Agarose Bead Technologies) and the mixture was incubated at room temperature on a nutating mixer for 1 h to allow attachment of peptides onto the beads. The beads were then washed six times with 500 μl of buffer containing 15 mM phosphate, pH 6.8, with 0–5 M GdnHCl for EPR spectroscopy.

The unfolding data were analyzed with nonlinear least-squares fitting as described in Santoro and Bolen (34) to obtain Cmv, the concentration of GdnHCl at the mid-point of unfolding transition.

Preparation of Aβ40 Peptides and Spin Labeling—The DNA constructs of GroES-ubiquitin-Aβ40 construct and the deubiquitylating enzyme Usp2cc were kindly provided by Dr. Il-Seon Park at Chosun University (South Korea) and Dr. Rohan T Baker at Australian National University (Australia). DNA sequence containing codons for amino acids HHHHHHHGGG was introduced into the N terminus of Aβ40 sequence using the QuikChange site-directed mutagenesis kit (Agilent). Two Gly residues were intentionally inserted between the His tag and Aβ sequence to allow Aβ to move freely after tethering on beads. Single cysteine mutations at various sites were then introduced into the Aβ40 sequence using QuikChange kit. Mutations were confirmed with DNA sequencing.
For protein expression, the plasmid constructs of Aβ40 fusion protein and Usp2cc were transformed into C41(DE3) cells (Lucigen). The cells were cultured to an absorbance of $A_{600 \text{nm}} = 0.6$ in LB broth at 37 °C and then induced with 1 mM IPTG at 28 °C for 4–6 h. Aβ fusion protein was purified using a nickel column as previously described (35). For purification of Usp2cc, the cells were resuspended in TG buffer (50 mM Tris, 5 mM β-mecaptoethanol, 30% glycerol, pH 7.5) and then sonicated on ice. The cell debris was pelleted by centrifugation. The supernatant was filtered through 0.45-μm filter (Whatman) and loaded on a 1-ml HiTrap SP HP column (GE Healthcare) equilibrated with TG buffer. The protein was eluted using a step gradient of 50 and 100 mM NaCl. Protein concentration was determined by UV absorption at 280 nm using extinction coefficients of 3.84 and 40.84 mm$^{-1}$ cm$^{-1}$ for Aβ fusion protein and Usp2cc, respectively.

Full-length Aβ protein was cleaved from the fusion protein with Usp2cc by mixing 2 ml of Aβ fusion protein with 1.95 ml of distilled water, 50 μl of purified Usp2cc at ~200 μM concentration, and 8 μl of 1 M TCEP stock (in 100 mM CAPS, pH 11). Therefore, the Usp2cc digestion was always performed in a buffer containing 25 mM phosphate, 3 mM urea, 2 mM TCEP, pH 10. The digestion reaction was allowed to proceed at 37 °C for 30 min. The reaction mixture was then immediately filtered with 0.2-μm filter (Whatman) and loaded on a PRP-3 reverse phase column (Hamilton) equilibrated with 30 mM ammonium acetate, pH 10. Aβ40 protein was eluted using acetonitrile in a linear gradient over 10 column volumes. Purified Aβ was lyophilized and stored at ~80 °C. The mass of purified Aβ40 was checked with MALDI-TOF mass spectrometry, and no other impurities were detected on mass spectrum.

For spin labeling, powders of single-cysteine mutated Aβ40 were dissolved in 10 mM sodium phosphate, 8 mM urea, pH 7.4 and the spin labeling reagent MTSSL was used at 10-times molar excess. The spin-labeled Aβ40 was further purified by reverse phase HPLC as described above. MALDI-TOF mass spectrometry was performed to ensure that the mass is correct, and the extent of labeling is >95%. Spin-labeled Aβ proteins were lyophilized and stored at ~80 °C.

**Tethering Aβ40 Monomers on Agarose Beads**—Lyophilized spin-labeled Aβ40 mutant powder was first dissolved in 400 μl of 20 mM CAPS buffer, 8 mM urea, pH 11 to a concentration of 25 μM. It was shown previously Aβ40 was rendered monomeric and denatured in 8 mM urea, pH 10 (21). The solution was added to 100 μl of agarose beads slurry charged with nickel (Agarose Bead Technologies), and the mixture was incubated at room temperature on a nutating mixer for 1 h to allow attachment of peptides onto the beads. The beads were then washed six times with either 500 μl of PG buffer (15 mM sodium phosphate, 7 mM GdnHCl, pH 7.4) or PBS buffer (50 mM sodium phosphate, 140 mM NaCl, pH 7.4) to remove unattached peptides.

**EPR Spectroscopy**—EPR measurements were performed at X-band frequency on a Bruker EMX spectrometer equipped with the ER 4102ST cavity. A modulation frequency of 100 kHz was used. Measurements were performed at 20 milliwatt microwave power at room temperature. Modulation amplitude was optimized to 1 G. For each sample, ~20 μl of solution sample or beads slurry was loaded into glass capillaries (Vitro-Com) sealed at one end. EPR spectra in each figure panel were normalized to the same number of spins.

**Spectral Simulations**—Spectral simulations were performed using a LabVIEW (National Instruments) interface (36) of the program NLSL developed by Freed and co-workers (37, 38). A microscopic order macroscopic disorder model was used as previously described (38). A least-squares fit of the user-defined spectral parameters was performed using the Levenberg-Marquardt algorithm. For all fits, the values for the magnetic tensors $A$ and $g$ were fixed as $A_{xx} = 5.7$, $A_{yy} = 6.0$, $A_{zz} = 37.5$, and $g_{xx} = 2.0078$, $g_{yy} = 2.0058$, $g_{zz} = 2.0023$, which were determined previously for R1 (39). Two parameters were allowed to vary: isotropic rotational diffusion constant ($R$), and $A_{0}$ (average of $A_{xx}$, $A_{yy}$, and $A_{zz}$). Here $A_{0}$ is used as a variable parameter to account for the effect of solvent polarity on $A_{0}$, but varying $A_{0}$ does not affect $R$. Rotational correlation time ($τ$) was calculated from $τ = 1/(6R)$. One and two component fits were performed as indicated in the text. Values of $τ$ obtained from simulation are plotted in the figures.

**RESULTS**

**Solid-support EPR**—The general strategy of solid-support EPR is to tether a protein on agarose beads. Agarose is a cross-linked form of polysaccharide polymer, and is the most widely used solid support matrix for affinity chromatography (40). Agarose has many properties of an ideal solid support including low nonspecific binding, hydrophilic characterstics, and chemical stability under conditions of coupling as well as under various conditions of pH, ionic strength, temperature, and presence of denaturants. We choose to use the non-covalent interaction between nickel and His tag for the following reasons. (i) His tag allows tethering at either N- or C-terminal end. Tethering at the end of a protein generally does not affect protein structure. (ii) His tag is small, containing six His residues in this work, and it normally does not interact with other parts of the protein (41). (iii) Binding between nickel and His tag is reversible, allowing the protein to be released from agarose beads if needed. Fig. 1A shows a schematic drawing of a protein tethered on agarose beads.
Previous studies have shown that tethering on solid supports in general does not disrupt protein structure (23–26, 42). Here we also investigated how protein structure changes upon tethering on agarose beads. Fig. 1B shows that T4L D72R1 tethered on agarose beads has similar EPR spectrum as untethered T4L D72R1 in 40% glycerol. Because the overall tumbling of the whole protein is diminished on agarose beads, 40% glycerol was added to untethered T4L to reduce the effect of the protein tumbling. Under these conditions, the EPR spectrum reflects the protein backbone fluctuations and internal bond rotation of R1. Similar EPR spectra for the tethered and untethered T4L suggest that the structure is not affected by tethering.

To further investigate the effect of tethering on protein structure, we studied the protein stability of both tethered and untethered T4L D72R1 using EPR. The unfolding of T4L obeys a two-state model without intermediates. If solid support affected protein structure, we reasoned that it would affect the native and denatured states differently because the denatured state has a much larger conformational space. Fig. 2A shows the EPR spectra of tethered T4L D72R1 in the presence of various concentrations of GdnHCl. The EPR spectra of untethered T4L D72R1 are shown in supplemental Fig. S2. It is clear from the EPR spectra that the unfolding of T4L is a two-state process because the EPR spectrum at higher concentration of GdnHCl contains two spectral components. Fitting of the unfolding data to a two-state model is shown in Fig. 2, B and C. The GdnHCl concentration at mid-point of the unfolding transition is 2.58 M for the tethered protein, and 2.56 M for the untethered protein, suggesting that the solid support has little effects on the stability of T4L. Because the native structure is unaffected by solid support (Fig. 1B), the stability results suggest that tethering does not significantly affect the conformational ensemble of the unfolded state.

EPR studies of tethered T4L suggest that a folded structure is not affected by the solid support. This is consistent with the general findings from numerous studies on immobilization of various proteins using different solid supports (23–26, 42). However, because Aβ40 is an intrinsically disordered protein, it is important to know how a disordered polypeptide chain interacts with solid support. In particular, do agarose beads have nonspecific interactions with proteins and thus stabilize some non-native structures? Our solid-support EPR studies on the intrinsically disordered yeast prion protein Ure2 suggest that there are no detectable nonspecific interactions between Ure2 prion domain and the agarose beads. EPR spectra of Ure2 with spin labels at every 5th residue position from residue 10 to residue 75 show extremely similar residue mobility profile in 7M GdnHCl and in PBS buffer, suggesting that residue-specific dynamics remain unchanged when switching from a denaturing buffer (7 M GdnHCl) to a native buffer (PBS). These results suggest that Ure2 prion domain adopts a completely disordered structure in the native buffer. Therefore, we conclude that agarose beads generally do not interact with a disordered polypeptide chain.

EPR Studies of Tethered Aβ40 Monomers under Strong Denaturing Conditions—We first investigated the structure of tethered Aβ40 monomers in the presence of 7 M GdnHCl. Measurement of the peak-to-peak amplitude (A_{pp}) of the center line is shown. GdnHCl results in the increase of center line amplitude A_{pp}, indicating elevated flexibility at the labeling site as the protein is unfolded. B, denaturation curve of tethered T4L D72R1. C, denaturation curve of untethered T4L D72R1. C_m, GdnHCl concentration at midpoint of unfolding transition. Similar C_m values between tethered and untethered T4L suggest similar stability. a.u., arbitrary unit.

EPR Studies of Tethered Aβ40 Monomers under Strong Denaturing Conditions—We first investigated the structure of tethered Aβ40 monomers in the presence of 7 M GdnHCl. It has been suggested that proteins under strong denaturing conditions adopt random coil structures (43). Spin labels are introduced at 18 positions in Aβ sequence as shown in Fig. 3A. Fig. 3B (black lines) shows the EPR spectra of three representative mutants. The full set of EPR spectra of all mutants are shown in supplemental Fig. S3. The spectral lineshape suggests high
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We fitted the experimental spectra to obtain the isotropic rotational correlation time ($\tau$) of the spin label. The simulated spectra of the three mutants were shown as red lines in Fig. 3B and full set of spectra in supplemental Fig. S3. All spectra are fitted well with only one spectral component, suggesting Aβ40 monomers adopt only one conformation. The values of $\tau$ are plotted in Fig. 3D. Smaller values of $\tau$ correspond to higher mobility of the spin label. Therefore, the plot of $\tau$ shows the same trend as the plot of $A_{pp}$ with a relatively flat N-terminal segment (residues 1–10) and a gradual decrease of $\tau$ from residue 12 to C terminus.

**EPR Studies of Tethered Aβ40 Monomers under Native Conditions**—To study the structure of Aβ40 monomers under near-physiological conditions, we performed EPR studies of the same spin-labeled mutants in PBS buffer. Our rationale is that, if Aβ adopts a structured state in the native buffer, we would observe changes in the EPR spectra between the PBS and GdnHCl buffers. The effect of tethering on a given residue position may have subtle differences between the two conditions, which are difficult to evaluate. To simplify the explanation, we assume that the effect of tethering on the local and global dynamics in PBS and GdnHCl remains the same.

EPR spectra of three representative mutants are shown in Fig. 4A and the full set of spectra from all mutants are shown in supplemental Fig. S4. We first noticed that the center line amplitude in PBS is generally smaller than that in 7 M GdnHCl. This is consistent with the formation of collapsed structures in PBS, because spin label in structured state has lower mobility than disordered state and thus lower signal amplitude. To evaluate site-specific structural information, we used the difference in center line amplitude between 7 M GdnHCl and PBS (Fig. 4C). Our rationale is that ordered structure in PBS leads to a decrease in center line amplitude compared with the spectrum in 7 M GdnHCl, so the extent of amplitude decrease can be used as a measure for ordered structure at a specific site. Fig. 4C shows that there are three regions with largest decrease in center line amplitude when changing from 7 M GdnHCl to PBS buffer: residues 14–18, 29–30, and 38–40, suggesting that ordered structures are formed at these segments.

For quantitative analysis, we also performed spectral simulations on the spectra in PBS. Spectral simulations show that satisfactory fits can be obtained only with two spectral components (Fig. 4A), while the one-component fits are much worse as judged by residuals (Fig. 4B). By fitting the spectra to two spectral components, we can separate a slow component from a fast component as shown in Fig. 4A. The values of $\tau$ for both the slow and fast components are plotted in Fig. 4D. The simulated fast component (Fig. 4D, magenta trace) has similar correlation time as the spectrum in 7 M GdnHCl after correcting for the viscosity effect of GdnHCl (44) (Fig. 4D, gray trace), suggesting that the fast component is the same disordered state as in 7 M GdnHCl. The slow component, therefore, represents a structured state of Aβ40 monomers. The plot of $\tau$ of the slow component shows three peaks around residues 12–14, 29, and 38–40. These local maxima in $\tau$ suggest that these residues are more structured and thus have lower mobility than their neighboring residues. More importantly, these three peaks approximately correspond to the three peaks in the plot of center line mobility of the spin label, consistent with a disordered polypeptide chain.

**FIGURE 3.** Aβ40 is unstructured under strong denaturing condition. A, amino acid sequence of Aβ40 with positions for spin labeling indicated by arrows. B, EPR spectra of three tethered Aβ40 mutants in 7 M GdnHCl. The experimental data were fit with a single spectral component, corresponding to one state of the protein. Measurement of the peak-to-peak amplitude ($A_{pp}$) of the center line is shown. C, plot of center line amplitude ($A_{pp}$) versus residue positions. The nearly linear increase of $A_{pp}$ from residue 12 to C-terminal residues is consistent with a disordered polypeptide chain. a.u., arbitrary unit. D, correlation time $\tau$ determined from simulations is plotted versus residue positions. The changes of $\tau$ indicate similar trend of mobility as the plot of $A_{pp}$.
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One potential issue about the two spectral components in EPR spectra is whether they reflect two conformations of the protein or two conformations of the spin label. Specific interactions between the spin label side chain and adjacent side chains in space can result in two-component spectra as shown in studies of T4L (45, 46). Two conformations of the protein can also be resolved from the EPR spectra as previously shown (26). If the two-component spectrum resulted from two spin label conformations, one of the conformations must be stabilized by some interactions between the spin label and neighboring structures to account for the difference in mobility. Here we observed two-component spectra at all spin-labeled sites, and it is unlikely that the spin label at all labeled sites interacts with neighboring groups to a similar extent. Therefore, we conclude that the two-component spectrum corresponds to two conformations of the Aβ40 monomer.

There is a possibility that the structured state arises from interactions between neighboring Aβ monomers on the beads when the attachment sites are saturated. We investigated how changing the coverage of attachment sites on agarose beads would affect the EPR spectrum for the G38R1 mutant. Supplemental Fig. S7A shows that when 20 μM of Aβ40 was used to bind agarose beads, low level of EPR signal was detected in the solution fraction, suggesting that the attachment sites were saturated at 20 μM protein concentration. When Aβ40 concentration was decreased to 10 and 5 μM, no traces of EPR signal were detected in the solution fraction, suggesting that the agarose beads were undersaturated. The EPR spectra under different labeling conditions, however, remain exactly the same (supplemental Fig. S7, B and C). This result suggests that the low-mobility component does not result from near-neighbor intermolecular interactions.

We also checked the possibility of inter-molecular spin-spin interaction. Spin-spin interactions could give rise to a low mobility-like broadened EPR spectrum when the two spin labels are within ~20 Å. Supplemental Fig. S8 shows that a spin-diluted sample with a mixture of spin-labeled Aβ and wild-type Aβ at 1:4 ratio gave rise to identical EPR spectrum as a sample of 100% labeled Aβ. This suggests that the low mobility component does not result from inter-molecular spin-spin interactions.

EPR Studies of Untethered Aβ40 Monomers—To investigate if the structured state of Aβ40 monomer results from tethering on agarose beads, we performed EPR study on untethered Aβ40 V40R1 in the presence of 1 mM imidazole. We found that, after we added 1 mM imidazole to release Aβ monomers from the agarose beads, no change in EPR spectrum was detected for at least 24 h, suggesting Aβ40 remains as monomers. Fig. 5A shows that, in PBS, the EPR spectrum of Aβ40 V40R1 can be simulated with only one component. However, this may be due to the fact that monomeric Aβ tumbles very fast (~2 ns) (47), and this fast motion makes the mobility difference between the fast and slow components undetectable with EPR. By increasing solution viscosity with high concentration of glycerol at 76%, we show that the EPR spectrum now clearly contains multiple spectral components and can be simulated only with two components (Fig. 5B). The correlation times of the fast and slow components of untethered Aβ are very similar to those of tethered Aβ (compare τ in Fig. 5B to τ in Fig. 4D). Although this solution condition contains 1 mM imidazole and 76% glycerol, detection of two spectral components using untethered Aβ40 monomers sup-

![FIGURE 4. Aβ40 monomers adopt a structured and a disordered states under native condition. A, upper panel, three representative EPR spectra of tethered Aβ40 in PBS, with best fits to a fast and a slow spectral components. Lower panel, the fast and slow spectral components from simulations. B, same three spectra in panel A were fitted with a single component. Note that the fit is significantly worse than the two-component fit, indicating two conformational states existed in Aβ40. C, difference in Ains between in the spectra in 7 M GdnHCl and spectra in PBS is plotted versus residue positions. Three regions, 14–18, 29–30, and 38–40, show a higher Ains difference, suggesting more compact structures in these segments. D, correlation time τ determined from simulation is plotted versus residue positions. Calculated τ of fast components is derived from τ in 7 M GdnHCl normalized to the viscosity of PBS. Three regions, 12–14, 29, and 38–40, show greater τ values for slow components than neighboring residues, suggesting a greater level of ordered structure in these segments.]

amplitude difference in Fig. 4C. Based on spectral simulations, the amount of the structured state is similar across different labeling sites at ~71% (supplemental Fig. S6).
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FIGURE 5. Untethered Aβ40 adopts two conformations. A, EPR spectrum of untethered Aβ40 V40R1 in PBS containing 1 mM imidazole can be fit with one spectral component. Because of fast protein tumbling in solution, the fast and slow components are not resolved by EPR. B, left, experimental spectrum of Aβ40 V40R1 in PBS with 1 mM imidazole and 76% (w/v) glycerol with best fit to two spectral components; right, simulated spectra of the fast and slow components. Glycerol increases solution viscosity and thus slows down protein tumbling, allowing two spectral components to be resolved.

ports the notion that the two structural states identified with solid-support EPR is not an artifact due to the solid support.

DISCUSSION

The findings in this work provide new insights into the structures of Aβ40 monomers. First, EPR data show that Aβ40 monomers adopt two conformations in PBS buffer: a completely disordered state and a structured state (Fig. 4). Based on EPR spectral simulations, the structured state accounts for ~71% of the total monomer population (supplemental Fig. S6). The completely disordered state has residue-specific dynamic properties similar as those in 7 M GdnHCl, a buffer that presumably renders Aβ completely disordered. The structured state is inferred from increased rigidity at all residue positions (Fig. 4D) based on an inverse relationship between protein structure and dynamics. Second, in the structured state, three regions around residues 14–18, 29–30, and 38–40 show higher rigidity than their neighboring residues, suggesting that these segments may form long-range interactions that stabilize this structured state.

Because of two technical advantages of EPR spectroscopy, we were able to detect two structural states of Aβ40 monomers. The first advantage is that EPR studies the nanosecond-timescale dynamics of spin labels that are introduced at specific residue positions. Therefore, EPR relies on the difference in dynamics to detect structural order rather than based on other observables such as chemical shifts or hydrogen bonding. This makes EPR a unique tool and provides otherwise inaccessible structural information. The second advantage is that EPR spectrum can resolve multiple spectral components resulting from multiple structural states. Because the two structural states of Aβ40 are resolved using CW EPR, the interconversion between the two structural states must be slower than the nanosecond timescale. Previous studies on Aβ40 monomers have identified Aβ40 monomer as a largely extended structure with structural propensity at scattered local segments. Some structural techniques are also capable of resolving multiple conformations. For example, if multiple states give rise to different chemical shifts and the interconversion between different states is slow on NMR timescale, NMR is able to detect multiple structural states. Hydrogen exchange combined with mass spectrometry has also been shown to detect multiple structural states if different structural states have difference in their amide proton exchange rates (48). Other ensemble-based spectroscopic methods such as circular dichroism may not be able to resolve different structural states. The fact that only one structured state was detected in previous NMR and hydrogen exchange mass spectrometry studies suggests that the structured state does not have significant difference in their secondary structure propensities and their hydrogen bonding patterns from the disordered state.

The second major finding of this work is that the structured state of Aβ40 monomers has three regions with increased rigidity: residues 14–18, 29–30, and the C-terminal residues 38–40. The segment 14–18 is near the central hydrophobic cluster (residues 17–21), which has been shown to either adopt a collapsed structure (11) or have increased structural propensities. This segment is also adjacent to the two histidine residues at positions 13 and 14, whose chemical shifts moved upfield with aggregation-promoting condition (7). This suggests that the ordering of residues His-13 and His-14 may participate in the initial structure formation on the pathway to aggregation. Danielsson et al. (12) show that residues 12–19 have high effective correlation time calculated from 15N relaxation NMR data. Yan et al. (13) also show that residues 12, 17, 18, and 21 are more rigid than their neighbors based on the methyl dynamics NMR studies. Molecular dynamics studies show a propensity to form a γ-turn at residues 12–18 (18). Together, these studies support a structural role of residues in the central hydrophobic cluster. Residues His-13 and His-14 may be important for the initial structural ordering of Aβ monomers through electrostatic interactions.

The second segment of higher local structural rigidity is at residues Gly-29–Val-30. Hou et al. (7) detected medium-range NOEs for the Gly-29–Ile-31 region, suggesting local structural order at these residues. Residue 32 was shown to have high effective correlation time based on NMR 15N relaxation data (12). Methyl dynamics also showed relatively high rigidity at residues 30 and 31 (13). The region 29–33 was found to have high potential to form a 310 helix in an MD study of Aβ42 (49). Another MD study of Aβ40 shows interactions between residues Ile-31–Ile-32 and Val-18 (50).

The third segment of higher structural order is at the C-terminal residues Gly-38–Val-40. Local structural order for this region has not been detected by solution NMR in previous studies. EPR spectral simulations together with the experimental strategy of comparing denaturing and native conditions allowed us to identify the local structural order at the C terminus in comparison with its neighboring residues. NMR studies have shown that Aβ42 has increased structural order at C terminus compared with Aβ40 (7–9, 14), suggesting that C terminus may interact with residues at other regions at least in Aβ42. MD simulations suggest that the C terminus of Aβ40 interacts with the central hydrophobic cluster (50), similarly as in Aβ42.
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(51). Our data provide experimental evidence that the C-terminal region has high structural order, supporting its role in tertiary interactions.

Several intra-Aβ mutations have been found to be associated with familial AD. In general, these mutations show stronger toxicity than the wild-type counterparts in toxicity assays, although differences exist among different variants (52). Most intra-Aβ mutations are located in the region of residues 21–23, and these mutations include A21G (Flemish), E22K (Italian), E22G (Arctic), E22Q (Dutch), E22Δ (Osaka), and D23N (Iowa). In our results, residues 21–23 are located in a region with high mobility, suggesting low structural order. We speculate that the role of this region may be less important than other regions in stabilizing the structured state of Aβ40 monomers. In aggregation, this region might play a more important role in stabilizing intermolecular interactions.

Overall, the structured state may be stabilized by interactions between the ordered segments at residues 14–18, 29–30, and 38–40 through mostly hydrophobic interactions. The residues His-13–His-14 may further stabilize the structured state through electrostatic interactions (7). The role of the structured state in the Aβ aggregation is a matter of speculation. The structured state may serve as a folding nucleus to form productive intermediates for oligomers and fibrils. Alternatively, the structured state may be an off-pathway structure for ordered assembly of Aβ oligomers and fibrils. Future studies aimed at distance measurements between these ordered segments may reveal further structural insights about the ordered state and its role in Aβ aggregation.

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