Alzheimer’s Aβ40 Studied by NMR at Low pH Reveals That Sodium 4,4-Dimethyl-4-silapentane-1-sulfonate (DSS) Binds and Promotes β-Ball Oligomerization*§

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The Alzheimer’s Aβ40 peptide forms soluble oligomers that are extremely potent neurotoxins and strongly impede synapses function. In this study the formation and structure of the large, soluble, neurotoxic Aβ40 oligomer called “β-ball” were characterized by two-dimensional NMR, circular dichroism, fluoresence spectroscopy, hydrogen exchange, and equilibrium sedimentation. In acidic aqueous solution, half the Aβ40 molecules are in the β-ball state; the remainder are monomeric. The equilibrium between the two states is slow as judged by NMR linewidths and is stable for months. The kinetics of β-ball formation from monomer are biphasic with τ1 = 7 min and τ2 = 80 min with no transient helix formation. Monomeric Aβ40 is essentially devoid of stable secondary structure, although the central, Leu7–Ala25, and C-terminal, Gly29–Val40, hydrophobic regions show propensity toward adopting extended structure, and residues 22–25 tended to form a turn. We found that sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) binds to the central hydrophobic region of monomeric Aβ40. DSS binds β-balls more strongly and caused them to double in size. Plausible micelle-like models for the β-ball structure with and without bound DSS are presented.

Amyloid plaques, one of two classic histological hallmarks of Alzheimer’s disease (1), contain as their major protein component 39–42 residue Aβ peptides (Aβ). These peptides are produced in vivo when the large, membrane-bound amyloid precursor protein is cleaved by β- and γ-secretase complexes (2, 3). Harmless, monomeric Aβ can associate in vitro to form a series of long lived soluble oligomers before adopting the distinct fibril conformation present in amyloid plaques (4). The last years have witnessed a paradigm shift away from amyloid fibrils and toward soluble oligomers of Aβ as the conformation responsible for the loss of synapse function occurring in the earliest stages of Alzheimer’s disease (5). These soluble oligomers induce acute electrophysiological changes in neurons (6) and are neurotoxic at much lower concentrations than amyloid fibrils (7). Moreover, these soluble oligomers form rapidly in vivo within mildly acidic intracellular compartments and can block long term potentiation, a widely used model for the formation of new memories, at extremely low concentrations (8). Interestingly, non-disease-related proteins can also form highly toxic soluble oligomers, which later form relatively innocuous amyloid fibrils (9). Strikingly, it has been reported that the toxicity of the soluble oligomers of Aβ and other proteins can be blocked by an antibody that binds to them but not to their monomeric or insoluble amyloid fibrils (10).

To date, at least two soluble neurotoxic Aβ oligomers have been identified and characterized: 1) a large spherical form called “β-ball” present at pH 2.5 containing about 200 monomers and a high content of β secondary structure (10, 11) and 2) smaller β-rich spherical or protofibril oligomers formed at neutral pH containing an SDS-resistant core (7, 8, 10, 12–14). An additional oligomeric state forming at pH 1 that shares certain similarities with the β-balls has been extensively characterized (15).

Structural studies of Aβ oligomers should reveal insights into the biological origins of Alzheimer’s disease and may yield new approaches for treatment. The study of Aβ structure at low pH offers the advantage that the β-balls do not evolve to form larger aggregates, such as fibrils (11), as occurs at neutral or endosomal pH (4). Moreover, at low pH, the direct measurement of hydrogen exchange rates is possible. One plausible mechanism for Aβ neurotoxicity is the accumulation of the peptide and its oligomers inside the lysosome (pH 4.7), which leads to the rupture of the lysosomal membrane and cell death (16). Therefore, a comparison of the structural tendencies of Aβ at pH 2.5 and neutral pH could give insight into its conformation at the pH that is physiologically significant inside the lysosome. The direct study of the Aβ structures formed at pH 4.7 is very difficult because of the extreme insolubility of the...
peptide near its isoelectric point (pH 5.5). Finally the ability of an antibody to recognize and block the neurotoxicity of β-sheet oligomers and the formation of soluble Aβ40 oligomers at neutral pH is good evidence that they share a structural motif that is key for toxicity (10). For all these reasons, we decided to study the structure of Aβ at low pH.

Our first objective was to characterize the equilibrium between the monomeric and β-sheet forms of Aβ40 using equilibrium sedimentation (ES), CD, and NMR. In addition, the kinetics of β-sheet formation were studied by fluorescence resonance energy transfer and CD. These experiments provided the data necessary to address the proposal of Zagorski and Barrow (17) that a helical intermediate gives rise to the formation of toxic β-sheet rich oligomers of Aβ40. The data obtained in the present study together with previous results serve as the basis for a plausible model of the β-sheet structure.

β-Ball formation could be aids by nascent structure in the Aβ monomer. NMR is an excellent technique for determining the high resolution structure of peptides, but early NMR studies of the Aβ were hindered by the strong tendency of this peptide to aggregate, particularly near pH 5, where it forms a β-sheet-rich oligomer that precipitates. This obstacle was first surmounted by the pioneering studies of Zagorski and co-workers, who used cosolvents (18) and detergents (19) to solubilize monomeric Aβ. NMR studies of monomeric Aβ in organic solvents (20), in cosolvents (18, 21), or associated with detergent micelles (19, 22–24) revealed extensive helix formation. In contrast, no helical structure was detected in water without cosolvents; when more soluble fragments of Aβ peptides and large sample volumes in a 10-mm NMR probe were used to overcome low solubility (25). Recent studies reveal that the full-length Aβ monomer is mostly random coil in aqueous solution near neutral pH, although some segments tend to adopt defined structures (26–28). Our second goal was to characterize structurally the Aβ40 monomer by NMR at low pH in aqueous solution without cosolvents. In addition, the presence of stable secondary structure was tested by direct measurements of hydrogen exchange (HX) that are possible only at low pH. Low peptide concentrations (300 μM), detectable with a high sensitivity cryosopore, were used to avoid precipitation. These measurements revealed further details of the interconversion between monomer and β-sheet states and established conditions to test for small molecule binding.

Aβ40 is positively charged at low pH and has stretches of hydrophobic residues that are likely to be important for the formation of the neurotoxic oligomers. DSS ((CH₃)₂Si-(CH₃)₂SO₄Na⁺) has recently been reported to bind to basic peptides rich in hydrophobic residues (29). The third objective of this study was to characterize by NMR, equilibrium sedimentation, and fluorescent labeling experiments the ability of DSS to bind to monomeric and β-sheet forms of Aβ40. Moreover the toxicity of DSS and its effect on Aβ40 toxicity toward pheochromocytoma (PC-12) cells were determined. On the basis of these data, a plausible, hypothetical structural model for DSS-bound β-sheet is proposed and is presented here.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Aβ40 was synthesized using the Merrifield solid-phase technique with a modified cleavage protocol that minimizes aggregation (11). Guanidinium hydrochloride followed by gel filtration at high pH was used to ensure that stock solutions of peptide were completely monomeric (33) and free of oligomers that could act to seed aggregation. Aβ40 was stored at 4 °C in aqueous solution at pH 10 until use. DSS was obtained from Stohler Isotope Chemicals. All other reagents used were of the highest purity grade commercially available.

Equilibrium Sedimentation—The association state of the Aβ40 samples was determined by ES using a Beckman XLI analytical ultracentrifuge. The data were obtained and analyzed as described previously (11).

Circular Dichroism Spectroscopy—Far UV-CD spectra were recorded in a 0.1-cm cuvette at 25.0 °C using a JASCO J-810 spectrometer equipped with a Peltier temperature control unit. The scan speed was 10 nm/min, and four accumulations were acquired and averaged to give the final spectrum. Blank spectra were acquired on buffer solutions using identical parameters and subtracted.

The kinetics of β-sheet formation were followed at 218 or 222 nm and 25.0 °C in a 1-mm cuvette with a slit width of 1.3 nm and an instrument averaging time of 8 s. The reaction was started by manually mixing 35 μl of Aβ40 (pH 10) with 215 μl of NaH₂PO₄ buffer (50 mM). The kinetic dead time was about 50 s. Far UV-CD and UV absorbance spectra were recorded after equilibrium was reached (3–4 h), and the final pH was measured. One- and two-exponential decay functions were fit to the kinetic data using non-linear least squares algorithm. The uncertainties given are the standard deviation (1σ) of three trials; the fitting errors from each experiment were larger (about 35%).

Fluorescence Resonance Energy Transfer Experiments—Aβ40 peptides were conjugated with a fluorescence donor, Trp, or alternatively to a fluorescence acceptor, ethlyxidaminophenylalane-1-sulfonic acid (EDANS), at the N terminus using a single Gly residue as a flexible spacer as described previously (4). Upon oligomerization of mixed-concentration Aβ40, the formation of Trp-EDANS complexes can be monitored by fluorescence emission. The increased fluorescence emission ratio of EDANS:Trp groups leads to fluorescence resonance energy transfer, producing an increase in the fluorescence emission ratio of EDANS:Trp.

NMR Spectroscopy—To prepare samples for NMR spectroscopy, Aβ40 peptide stock solutions were rapidly mixed with an excess of 5 mM NaH₂PO₄ buffer, pH 2.5. Rapid transfer from alkaline to acidic pH minimizes the time that Aβ40 spends near pH 5 where its solubility is minimal. Small pH adjustments were made by adding HCl, DCl, NaOH, or NaOD. All NMR spectra were recorded at 25.0 °C in 9% H₂O, 10% D₂O, 75% D₂O and used the most upfield resonance of DSS (60 μM) as the chemical shift reference. The final peptide concentration was determined using the absorbance of the lone Tyr residue of Aβ40 at 275 nm (ε₂₇₅ = 1,390 cm⁻¹ M⁻¹, Ref. 30). Two-dimensional ¹H correlation spectroscopy (31), TOCSY (mixing time, 60 ms; Ref. 32), NOESY (mixing time, 150 ms; Ref. 33), and natural abundance ¹⁵C heteronuclear single quantum correlation spectra (34) were acquired on a Bruker 600 MHz NMR spectrometer equipped with a triple resonance (¹H, ¹³C, and ¹⁵N) cryoprobe and Z-gradients. The spectra were assigned using standard methodology (35).

To detect secondary structure, patterns and intensities of NOE backbone signals were examined, and the chemical shift index (36) for ¹H, ¹⁵C, and ¹³C nuclei were determined by applying the procedure and using the reference ¹H cross-relaxation values of Wishart et al. (37) except for Asp, Glu, and His in which case the reference ¹H values of Schwarzinger et al. (38) obtained at pH 2 were used. The Δ profile for NΗ and Cα were based on the reference ¹H values of Wang and Jardetzky (39).

For HX experiments, Aβ40 peptide samples were first concentrated to about 1 mg/ml in pH 2.5, 5 mM NaH₂PO₄ buffer by ultrafiltration using a YM-3 Amicon filter with a 3-kDa molecular weight cut-off. Then 150 μl of this solution was added to 450 μl of D₂O buffered D₂O, and HX was monitored by recording a series of one-dimensional ¹H NMR spectra (dead time = 270 s, 80 transients/spectrum, acquisition time/spectrum = 120 s at 25.0 °C). Spectra were recorded until equilibrium was reached (70 min). The HX rates were determined by fitting an exponential decay function to the area or height of each peak in the backbone amide region of the onedimensional ¹H spectra versus time. The observed HX rates were corrected for back-exchange due to 25% ¹H in the solution. The HX experiment was repeated once. Theoretical "κ_MD" rates for HX from short, unstructured peptides were calculated at the same conditions using the parameters of Bai et al. (40).

Sulfhydryl Rhodamine B Cytotoxicity Assay—PC-12 cells were plated at 1,000 cells/well in a 96-well plate with Dulbecco's modified Eagle's media (Invitrogen) containing 10% fetal bovine serum and incubated overnight. Cells were then differentiated for 4 days in serum-free media containing 10 nM nerve growth factor and X2 supplement. Separately Aβ40 (25 μM) samples with or without 1 mM DSS were stored for 24 h at pH 5 at room temperature. After differentiation, cells were incubated with the Aβ40 samples for 24 h. Toxicity was assessed using the sulfhydryl rhodamine B assay. Briefly, cells were fixed with 10% trichloroacetic acid for 0.5 h, washed with H₂O, and air-dried. Protein was stained with 0.4% sulfhydryl rhodamine B (Molecular Probes, Inc.) in 1% acetic acid for 0.5 h, washed with 1% acetic acid, and air-dried. The dye was extracted in 10 mM Tris base, and the cell survival, as absorbance at 560 nm, was assayed on a Tecan microtiter plate reader.
RESULTS

Aβ40 Exists as Monomers and β-Balls at Low pH—The oligomerization state of 300 μM Aβ40 at pH 2.5, 25.0 °C was determined by ES. About half the Aβ40 molecules were monomeric, whereas the rest were in large, soluble oligomers whose molecular mass, 764 kDa, corresponds to 176 Aβ40 monomers; similar results have been obtained previously (11). A representative CD spectrum of Aβ40 in these same conditions shows a broad minimum between 210 and 220 nm characteristic of β-structure (Fig. 1A). The magnitude of the minimum, θ_{217} = −2,000 ± 50 degrees cm² dmol⁻¹, was significant as the signal of a 100% β-structure is about −12,000 degrees cm² dmol⁻¹ (41). Thus, at pH 2.5, 25.0 °C, Aβ40 forms large oligomers rich in β-structure. These oligomers have been characterized previously and named β-balls (11).

To determine the kinetics of β-ball formation, the pH of a monomeric solution of Aβ40 was dropped from 10 to 2.5 (final pH), and the CD signal was followed. Biphasic kinetics with lifetimes (τ) of τ₁ = 6.9 ± 1.2 min and τ₂ = 88 ± 4 min were observed, and no “overshoot” indicative of transient helix formation was detected (Fig. 1B). Changes in CD are sensitive to secondary structure formation. As an alternative approach, the kinetics of quaternary structure formation was studied by fluorescence resonance energy transfer. Two solutions with 300 μM Aβ40, one with untagged Aβ40 peptides and the other with Aβ40 peptides labeled with Trp or EDANS, were mixed after preincubation at pH 2.5 at 25.0 °C. The fluorescence emission decrease with time as unlabeled Aβ40 enters and labeled Aβ40 exits the initially fully labeled β-balls showed biphasic kinetics with τ₁ = 7 ± 1 min and τ₂ = 70 ± 40 min (Supplemental Fig. 1). A control CD spectrum revealed that Trp or EDANS labeling did not significantly affect β-ball formation (data not shown).

Structural Characterization of Aβ40 by NMR at Low pH—The region of the two-dimensional 1H TOCSY spectrum containing the N^1H to αH and side chain correlations of 300 μM Aβ40 recorded at 25 °C at pH 2.5 is shown in Fig. 2A. The linewidth of the αH of His^{14} in D_2O was about 9 Hz. This signal corresponds to the monomer; those corresponding to the β-balls were broadened beyond the detection limit due to their slow correlation time. The monomer linewidth is consistent with slow (≤αs) interconversion of the monomer and β-ball states. The line shapes and intensities of Aβ40 NMR resonances remained essentially constant over a period of several months, and the linewidths and chemical shift (δ) values did not change when Aβ40 was diluted to 40 μM. While the peaks were generally well resolved, the range of N^1H δ-values was relatively small (8.74–8.04 ppm), and Gly αH and αH_{i+1} signals were essentially degenerate; these are symptoms of random coil backbone structure. No fine splitting of the 1H peaks was observed, and therefore no vicinal coupling constants were measured. The backbone Φ assignments were complete with the lone exception of the N^1H of Asp{1}. The δ-values of the side chain protons showed little dispersion with variations generally being ≤0.03 ppm for homologous nuclei of the same type of amino acid. The larger δ-value differences (0.10–0.27 ppm) observed for Val{12} and Val{18} side chain protons relative to those of other Val residues are likely due to ring current effects from His{13} and Phe{19}, respectively.

Comparison with the assigned Φ spectra and intrinsic 13C δ-values permitted the assignment of all the 13Cβ and many 13C side chain and 13Ca backbone nuclei in the 13C heteronuclear single quantum correlation spectra. The assignment of other 13Ca nuclei was impeded by their overlap or proximity to the water line. The ΦH side chain assignments are complete except for rapidly exchanging protons. The δ-values of the ε-methyl group of Met{35}, 1H (2.09 ppm) and 13C (17.1 ppm), are consistent with its sulfur being unoxidized. The δ-values of protons in Asp and Glu residues indicate that they are in the neutral, carboxylic acid form, whereas those in His, Lys, and Arg show they are charged. These charged states are consistent with the thorough study of Aβ pK_a values reported previously (42). All of the resonances of Aβ40 assigned here have been deposited in the BioMagResBank data base (www.bmrb.wisc.edu) under accession number 6257.

In general, strong N^1H to CaH_{i+1} NOEs were observed in the NOESY spectra, whereas N^1H to N^2H_{i+1} NOEs were generally weaker or absent (Fig. 2B). These relative NOE intensities are characteristic of extended structures such as β-strands (35). However, medium-strong NH to NH_{i+1} NOEs, which are indicators of helix or turn regions, were observed in residues 12–13, 22–25, and 33–35 (Fig. 2B). NOEs were also observed.
between the aromatic side chains of Phe19 and Phe20 with the alkyl side chains of Val18, Ala21, and Val24 (Fig. 2C), indicating that these apolar side chains lie close together and form a hydrophobic cluster, which is at least partially populated. Several different backbone conformations for the turn detected by NOEs between residues 22–25 could position the side chain of Val24 near those of residues 18–21. Given the low number of medium and long range NOEs observed no structure calculations were undertaken.

Rules based on the differences between reference and experimental δ-values, known as the chemical shift index, permit the identification of stable secondary structural elements (36, 43). This approach was applied to the δ-values of A40 obtained here, and no groups of three or four consecutive residues were found to exceed the threshold values; this indicates there is no stable secondary structure in A40 in H2O at pH 2.5 at 25 °C (Fig. 3). In polypeptides, like monomeric A40, that lack stable backbone structure, conformational shifts (Δδ observed – δ reference) can indicate tendencies to adopt partly populated secondary structural elements. According to the ΔδHα values, some residues in the N-terminal half of A40 appeared to favor the helical conformations, whereas a somewhat stronger trend toward β-conformations was observed from Val24 to the C terminus (Fig. 3A). A general propensity to adopt helix was detected by the ΔΔ13Ca values, whereas the ΔΔ13Cβ values showed that the central hydrophobic region of A40, Leu17–Ala21, tended to adopt β-structure (Fig. 3, B and C). The ΔΔN1H values also showed a general tendency of the polypeptide chain to adopt β-structure (Fig. 3D). The 13Ca conformational shifts discern helix from random coil better than β from random coil, whereas the opposite is true for the other three nuclei (39). This could account for the different tendencies detected by the ΔΔ13Ca values. The magnitudes of these shifts were small; a change of 0.5 ppm in δ13Ca is one-sixth that observed for protein helices (44), and the ΔΔ13Cβ of residues 17–21 corresponded to a β-population ~25% (45). Overall, while these results suggest a tendency for the central and C-terminal non-polar residues to adopt a small population of β-conformations, they clearly indicate the lack of stable secondary structure in these conditions.

Hydrogen Exchange—The HX data could be well fit by a single exponential equation, indicating monophasic kinetics, and essentially identical rates were obtained if peak heights were fit instead of peak areas (data not shown). The final peptide concentration was 0.24 mM. The HX rates are given in Table I. Each peak monitored contained contributions from one to several amide protons. The HX rates from the two experiments agreed within a factor of two. For comparison, the theoretical kcoil rates for exchange of unprotected amide protons are listed in Table I. The experimental rates were very similar to the kcoil rates, indicating that there are no protected amide protons in monomeric A40. If β-balls contained protected amide protons and if β-balls dissociated to form monomeric A40 on a time scale of minutes to tens of minutes, an extra phase in the HX kinetics would have been observed. Such a phase, however, was not seen.

DSS Binds to Monomeric Aβ40—The trimethylsilyl moiety of DSS, \((\text{CH}_3)_3\text{Si-CH}_2\text{-CH}_2\text{-SO}_3\text{Na}^+)\), produces a sharp
resonance in the upfield region of NMR spectra that is frequently used as the δ reference. However, when 60 μM DSS was present in the Aβ40 NMR sample, a broadened signal with a linewidth of 7 Hz, which is similar to those of monomeric Aβ40 resonances, was observed at this upfield position, and its δ-value was provisionally taken as 0.00 ppm (Fig. 4A). This signal was observed in the COSY or TOCSY spectra, but produced no cross-peaks away from the diagonal even when the TOCSY mixing time was increased from 60 to 90 ms (data not shown). These results are compatible with the assignment of the broadened peak to the trimethyl moiety of DSS, which would not give rise to COSY or TOCSY cross-peaks with the methylene protons because the corresponding scalar couplings are negligible. Moreover, the 1H assignments of Aβ40 obtained in the present study are essentially complete, and no Aβ40 1H resonated below 0.75 ppm. Meanwhile, in the NOESY spectrum, the 0.00 ppm peak produced broadened cross-peaks at 0.67, 1.81, and 2.96 ppm (Fig. 4B); these are δ-values of DSS's methylene groups. To confirm that the broadened signal at 0.00 ppm was due to DSS and did not arise from the peptide, the amount of DSS in the sample was reduced by ultrafiltration, and the NMR spectrum recorded afterward revealed that the broad 0.00 ppm peak was much decreased relative to Aβ40 resonances (Fig. 4C). On the basis of these results, we conclude that DSS binds to Aβ40.

Addition of a capillary containing DSS to an Aβ40 sample allowed the δ reference to be established in the absence of binding. This DSS resonated with a narrow linewidth of 2.1 Hz at 0.00 ppm in the same position as the broadened signal observed for Aβ40-bound DSS (Fig. 4D). This shows that Aβ40 binding does not significantly alter the δ-values of DSS and suggests that the trimethyl moiety of DSS is not rigidly fixed.
against Aβ40. Moderately strong NOE cross-peaks were observed between the DSS (60 μM) signals at 0.00 and 0.67 ppm and Aβ40 (300 μM) signals corresponding to the ring protons of Phe19 and Phe20, and additional NOEs were detected between DSS and methyl groups that could belong to Leu17 or Val18 (Fig. 4, B and E). These data indicate that DSS binds specifically to the central hydrophobic region. In the presence of 200 μM Aβ40, 60 or 240 μM (trimethylsilyl)-propionic acid, which is neutral at pH 2.5, also showed a broadened lines, whereas 1 mM dioxane, which lacks a trimethylsilyl group, shows sharp lines (data not shown).

The interaction between DSS and Aβ40 could induce δ-values changes in both molecules. As a test, the titration of the 200 μM Aβ40 with 0–1,000 μM DSS was followed by one-dimensional 1H NMR spectroscopy. As expected, no signal at 0.00 ppm was observed for Aβ40 without DSS. No significant DSS-induced changes in the δ-values or linewidths of the peptide were seen. A broad DSS signal was observed at the lowest DSS concentration tested (20 μM), and its linewidth decreased only slightly with increasing DSS (Fig. 4F), which suggests that at 1,000 μM DSS more than one DSS molecule is bound per Aβ40 monomer. At the end of this titration, a known amount of Trp was added as a concentration standard. By comparing the NMR peak integrals, the concentrations of Aβ40 and DSS were found to be 70 and 470 μM when their actual concentrations were 120 and 730 μM, respectively. Taking the NMR-invisible fraction of Aβ40 and DSS as being incorporated into β-balls indicates that about 40% of Aβ40 molecules were in β-balls. The ratio of NMR-invisible DSS:Aβ40 was 260 μm:50 μm, which suggests that about five DSS molecules are bound per Aβ40 monomer in β-balls.

**Table 1**

| Peak | Residues in peak | k^β | k^γ | k^δ |
|------|------------------|-----|-----|-----|
| 1    | Ala^5            | >1  | >1  | 2.4 |
| 2    | His^14           | ND  | 0.01| 0.075|
| 3    | Asp^7, Gly^27    | 0.06±0.01 | 0.05±0.01 | 0.074, 0.28, 0.49 |
| 4    | His^10, His^11, | 0.11±0.02 | 0.06±0.01 | 0.13, 0.18, 0.22 |
| 5    | Lys^16, Asp^23, | 0.10±0.02 | 0.07±0.01 | 0.079, 0.13, 0.21 |
| 6    | Ser^8, Gly^9     | 0.09±0.01 | 0.059±0.06 | 0.067, 0.11, 0.20 |
| 7    | Glu^15, Leu^16, | 0.083±0.006 | 0.076±0.04 | 0.016, 0.081, 0.155 |
| 8    | Phe^19, Arg^20, | 0.066±0.003 | 0.047±0.002 | 0.056, 0.076, 0.089 |
| 9    | Ile^31           | 0.071±0.005 | 0.047±0.003 | 0.057 |
| 10   | Val^41           | 0.091±0.006 | 0.059±0.004 | 0.067 |
| 11   | Val^36, Ala^37, | 0.065±0.003 | 0.062±0.002 | 0.057, 0.105, 0.18 |
| 12   | Tyr^14, Val^12, | 0.082±0.006 | 0.042±0.004 | 0.060, 0.077, 0.10 |

a The experimental exchange rate, adjusted for back-exchange due to 25% 1H. The uncertainties obtained from the fitting analysis are given.
b The k^γ, k^δ rates calculated for pH 2.5 and 25 °C using the parameters of Bai et al. (40).
c Not determined.
d For composite peaks, three calculated rate values are shown: the left value is the k^δ rate for the slowest exchanging amide, the central value (in bold) is the mean k^γ rate, and the right value is k^β for the fastest exchanging amide.

**DSS Modulation of Aβ40 Cytotoxicity**—As a first approach to evaluate the possible in vivo effects of DSS, the sulphydryl rhodamine B assay was used to test the toxicity of Aβ40, DSS, and DSS + Aβ40 toward PC-12 cells. DSS was found to be non-toxic at concentrations up to 1 mM and not to affect the toxicity of Aβ40 toward PC-12 cells (data not shown).

**DISCUSSION**

Monomeric Aβ40 Forms β-Balls without Apparent Helical Intermediates—ES and CD clearly detected the presence of large, soluble, neurotoxic β-ball oligomers at pH 2.5, 25 °C. The molar ratio of Aβ40 molecules in the monomeric:β-ball states was about 1:1 in these conditions. The invariability of the NMR spectrum over several months indicates that the equilibrium between the monomeric and β-ball forms is stable once constituted, and no additional oligomers or aggregates are present in significant amounts. Previous work at 4 °C at pH 3 also showed that β-balls have significant β-secondary structure and are stable as they give essentially identical ES results after storage at 0 °C for 1 month (11).

The NMR linewidths indicate that the interconversion of monomeric and β-ball forms is slow on the time scale of seconds or more. Both fluorescence resonance energy transfer and CD detected biphasic kinetics with similar lifetimes (τ1 = 7 min and τ2 = 80 min), which strongly suggests that oligomerization, as detected by fluorescence resonance energy transfer, and acquisition of β structure, as monitored by CD, occur simultaneously. These results also suggest that an oligomeric intermediate with a moderate amount of β structure forms. The population of this intermediate at equilibrium must be low as it was not detected by NMR or ES. The lack of an additional kinetic phase in the HX kinetics indicates that either the amide protons in β-balls are unprotected or that the dissociation of β-balls into Aβ40 monomers has a time constant slower than tens of minutes. The minimal scheme accounting for the kinetic results presented here is below.

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\begin{array}{c|c|c}
\text{monomer} & \text{intermediate} & \text{β-ball} \\
\tau_1 = 7 \text{ min} & \tau_2 = 80 \text{ min} & \\
\end{array}
\]

The transient intermediate seems to be “on-pathway” (46) since its oligomerization state and content of β-structure are between those of the monomer and β-ball states. Although the data do not provide support for the “off-pathway” model of Ref. 17, for the formation of β-sheet-rich oligomers via a highly


\( \alpha \)-helical intermediate, a helical intermediate still might be present if it formed and disappeared within the kinetic dead time. The repeated finding of helical structure in A\( \beta \) dissolved in fluorinated alcohols (17, 18, 21) or associated with detergent micelles, which mimic some membrane features, (19, 22–24), may well be due to the ability of these substances to promote helix formation in peptides with a certain innate helix propensity (47, 48). Moreover the inability of SDS to disrupt the potently neurotoxic oligomeric conformations of A\( \beta \) that disrupt synapse function (7, 10) suggests that the helical conformation formed by monomeric A\( \beta \) associated with SDS micelles is not akin to pathogenically relevant structures. In contrast, when A\( \beta \) peptides interact in vitro with authentic membrane lipids such as gangliosides (49) or 1-palmitoyl-2-oleoyl-sn-

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\text{FIG. 4. DSS binds to monomeric A\( \beta \)40. A, upfield region of the one-dimensional } ^1\text{H NMR spectrum showing a broadened peak (t) at 0.00 ppm where DSS normally resonates as a sharp line. The sharp line at 0.15 ppm is a small molecule impurity. B, one-dimensional row extracted from the two-dimensional } ^1\text{H NOESY spectrum of A\( \beta \)40 (300 } \mu\text{M total peptide concentration) and DSS (60 } \mu\text{M). The lowercase letters a, b, c, and t refer to methylene groups in DSS: } \text{O}_3\text{S-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-Si(CH}_3)_3. \text{ C, upfield region of the spectrum of A\( \beta \)40 after ultrafiltration to deplete DSS. D, A\( \beta \)40 sample after DSS depletion by ultrafiltration with DSS added within a capillary. E, NOEs arising from the interaction of the trimethylsilyl moiety of DSS and the aromatic ring protons of Phe19 and Phe20. F, the dependence of DSS linewidth on its titration with A\( \beta \)40. The linewidth of free DSS, filled circles and solid line, and the expected dependence for tight 1:1 A\( \beta \)40-DSS binding, open squares and broken line, are indicated.}
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\text{FIG. 5. DSS binds to } \beta \text{-balls. A, enhancement in the acceptor:donor fluorescence ratio when DSS was incubated overnight with 40 } \mu\text{M A\( \beta \)40 labeled with Trp or EDANS at pH 2.5 at 25 °C. A line is shown to guide the eye. B, equilibrium sedimentation results of A\( \beta \)40 solution (pH 2.5 at 25 °C) before (top panel) or after (bottom panel) addition of 1 mM DSS.}
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glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPC/POPG) vesicles (50), a rapid induction of fibrils or β-structure, respectively, is observed. Gangliosides and cholesterol are present at high concentrations in lipid raft domains of the cell membrane (51), and studies have recently correlated cholesterol levels with in vivo Aβ production and oligomerization (52) and with the risk for developing Alzheimer’s disease (53).

**Structure Preferences in Monomeric Aβ40 Are Similar at Neutral and Low pH**—One aim of this work was to characterize the nascent structure in monomeric Aβ40 that could play important role(s) in the formation of soluble neurotoxic oligomers. The NMR results showed that the backbone structure of monomeric Aβ40 is generally random coil with the central and, perhaps to a lesser extent, C-terminal hydrophobic regions showing some propensity to adopt β-structure. Medium-strong backbone NH to NH+1 NOEs revealed turnlike backbone conformations for residues 22–25 and also 33–35. The close similarity of the observed HX rates for Aβ40 and the theoretical rates for unprotected NH means that there is no stable secondary structure in monomeric Aβ40. The lack of long range NOEs is consistent with an absence of stable tertiary structures. The side chains of Val18, Phe19, Phe20, Ala21, and Val24 form a local hydrophobic cluster as detected by NOEs. The lack of significant δ-values deviations for these residues, except Val18, suggests that the packing of their side chains is loose.

There are notable similarities between the structural propensities detected here for Aβ40 in aqueous solution at pH 2.5 and those obtained by others near neutral pH. Working with the fragment Aβ-(10–35), Zhang et al. (26) observed an extensive hydrophobic patch formed by side chains from the central hydrophobic region (Leu17–Ala21) together with the side chains of Tyr10, Val12, and Ile32. They described the backbone conformation as a “compact random coil” with residues 22–33 forming a series of turns. The more extended backbone conformation reported here could result from increased charge-charge repulsion in Aβ40 at pH 2.5 (Z = +7) versus Aβ-(10–35) at pH 5.7 (Z = +1). There is good evidence that denatured protein chains expand as their net charge increases (54). Working with Aβ40 or Aβ42 at pH 6.4, Riek et al. (27) reported a defined backbone conformation for residues 16–24, a helical turn for residues 20–24, and hydrophobic contacts among the side chains of Leu17, Phe19, Phe20, Ala21, and Val24 with the rest of the peptide being random coil. A comparison of the δ-value differences of the δH resonances of Aβ40 at pH 2.5 and those reported at neutral pH (26, 27) revealed a close resemblance that suggests the backbone conformations are similar at pH 2.5 and neutral pH. Hou et al. (28) also concluded that Aβ40 and Aβ42 are mostly unfolded at pH 7.2 in water but have two partial populated β-strands spanning residues 17–20 and 31–35 and turns encompassing residues 7–11 and 20–26. The fact that similar structural tendencies were found here at pH 2.5 as near neutral pH corroborates the hypothesis that these nascent structures in monomeric Aβ40 represent the first step toward the formation of soluble, neurotoxic oligomers.

**DSS Binds to the Central Hydrophobic Cluster of Monomeric Aβ40**—The broadened linewidth of the DSS signals and NOEs between DSS and the aromatic rings of Phe19 and Phe20 in Aβ40 indicate that DSS binds to monomeric Aβ40. DSS was reported to bind positively charged, hydrophobic peptides (29); and β-cyclodextrin (55) also binds the central hydrophobic region of Aβ. The ability of (trimethylsilyl)-propionic acid, which lacks a negative charge at pH 2.5, to bind the hydrophobic segment of Aβ (residues 12–28) (56) suggests that the trimethylsilyl moiety is key for DSS binding to Aβ40. Additional NOEs were observed in the present work between the aliphatic methyl groups in Aβ40 and the DSS trimethyl moiety. If these methyl groups belonged to Leu17 or Val18 the sulfonate moiety of DSS would be positioned to form a favorable electrostatic interaction with the charged amine group of Lys16. The weak concentration dependence of the DSS linewidth as well as excess NMR-invisible DSS relative to Aβ40 suggest that, at high DSS concentrations, there are multiple binding sites for DSS in Aβ40. Plausible additional binding sites could be groups of hydrophobic residues like Leu34, Met35, and Val36; Val39 and Val40; or combinations of nonpolar plus positively charged groups such as Phe4, Arg6, and His8; Val12, His13, and His14; or Lys26–Ile32.

**DSS and Aβ40 Toxicity Toward PC-12 Cells**—There is intense interest in finding molecules that block the formation of neurotoxic, oligomeric species of Aβ peptides (55, 57), and here DSS was found to bind to both monomeric and oligomeric Aβ40. In an initial series of experiments, DSS was found neither to be toxic nor to affect Aβ40 toxicity toward PC-12 cells. Future experiments in more sophisticated model systems, such as nerve tissue or transgenic mice, could further define the potential of DSS to affect Aβ neurotoxicity.
Hypothetical Plausible Models for Aβ40 β-Ball Structure—In developing models for the β-ball structure in the presence and absence of DSS, the following structural data were considered. 1) At pH 2.5, all the carboxylate groups, including the C-terminal carboxylate group, are almost completely in the neutral carboxylic acid form. 2) The Arg, Lys, and His residues, as well as the N-terminal amine, are essentially completely charged. These charged groups are concentrated in the N-terminal third of the molecule with the only charged group beyond Lys16 being Lys28 (Fig. 6A). 3) The β-ball conformation is well populated at pH 2.5–3.0 but is not found above pH 4 where different, insoluble oligomers rich in β-secondary structure form and precipitate (11). 4) The range of the number of monomers in the β-balls is limited; sizes of 150–260 monomers per β-ball were observed in our Toronto laboratory for samples with slightly different buffer conditions and monomer concentrations. 5) β-Balls have a significant β-secondary structure as judged by CD. 6) The Aβ40 sequence has two regions that are rich in nonpolar residues: Leu21–Ala21 and the last 11–12 residues at the C terminus. 7) These two regions have a slight tendency to adopt extended backbone structures at pH 2.5 and 25°C as revealed by the conformational shifts and backbone NOE intensities in monomeric Aβ. 8) A turn, residues 22–25, stabilized by a small hydrophobic cluster involving Val24, Ala21, Phe20, Phe19, and Val18, detected by NOEs in monomeric Aβ, may form between these two hydrophobic regions. It should be borne in mind that the NMR data reported here correspond to the Aβ monomer. 9) β-Balls, as resolved by atomic force microscopy, appear spherical and have a diameter that ranges from 8 to 18 nm with a mean of 15 nm (11). 10) The trimethylsilyl and first methylene moieties of DSS bound specifically to the aromatic rings of Phe19 and Phe20 and to additional alkyl side chain(s), which most likely belong to the central or C-terminal hydrophobic regions. 11) DSS was found to promote the oligomerization of Aβ40 and cause the number of Aβ40 monomers in the β-balls to double.

β-Balls with No DSS—Based on these data, we propose that β-balls have a spherical micelle structure with the C-terminal 12 residues buried inside a hydrophobic micelle core and the rest of the peptide, including the charged Lys28, exposed to solvent (Fig. 6B). The peptide groups within the β-ball core are hydrogen-bonded forming β-sheet structures. In an extended conformation, these last 12 residues of Aβ40 would be about 4.1 nm long; therefore the radius of the proposed β-ball model corresponds well with its minimal diameter detected by atomic force microscopy (8 nm). With the last 12 residues of Aβ40 modeled as a cylinder about 4.1 nm long with a radius of 0.25 nm, it can be estimated that about 350 Aβ40 C-terminal tails could fit into the micelle core.

It should be kept in mind that the β-ball model proposed here

### Table II

| Name                          | pH  | Micelle diameter (nm) | Hydrophobic interactions | Electrostatic interactions | Characteristics | Ref. |
|-------------------------------|-----|-----------------------|--------------------------|---------------------------|----------------|------|
| Spherocylindrical micelles β-balls | 1   | 4.8 × 11              | Central and C-terminal or both hydrophobic segments | Repulsive (7+, 0−) | 30–50 monomers/oligomer | 15   |
|                               | 2.5–3.0 | 8                  | C-terminal              | Repulsive (7+, 0−)       | 200 monomers/β-ball. Slow equilibrium with monomer. No evolution toward fibrils. Contain neurotoxic structural motif recognized by antibody (10). | 11; this work |
| Phase 1 and phase 2 spherical particles | 6.0 | Phase 1, 38; Phase 2, 78 | Central or C-terminal or both hydrophobic segments | Generally attractive (7+, 6−) | Likely contain neurotoxic structural motif recognized by antibody (10). Readily evolve toward protofibrils. | 4    |
| Spherical prefibrillar aggregates | 7.0 | 15                  | Central or C-terminal or both hydrophobic segments | Generally attractive (7+, 4−) | Contain neurotoxic structural motif recognized by antibody (10). Readily evolve toward protofibrils. | 14   |
| Aβ-derived diffusible ligands | 7.4 | 5–6                  | Central or C-terminal or both hydrophobic segments | Generally attractive (7+, 4−) | Neurotoxic at concentrations ≈10 nm. Disrupt long term potentiation. Mass, 17 and 27 kDa. | 7    |
| Spherical species            | 7.4 | 4.3                  | Central or C-terminal or both hydrophobic segments | Generally attractive (7+, 4−) | Contain neurotoxic structural motif recognized by antibody (10). Readily evolve toward protofibrils. | 12   |
| Globular assemblies          | 7.4 | 3–6                  | Central or C-terminal or both hydrophobic segments | Generally attractive (7+, 4−) | Contain neurotoxic structural motif recognized by antibody (10). Readily evolve toward protofibrils. Five to six monomers per minimal oligomer. | 13   |
| βamy balls                    | 7.4 | 20,000–200,000       | Generally attractive (7+, 4−) | Generally attractive (7+, 4−) | Very large, insoluble oligomers composed of mature amyloid fibrils; formed at high Aβ concentrations. | 62   |

* The number of positively and negatively charged groups per monomer is given in parentheses.
is hypothetical. Other geometric forms, like elliptical or cylindrical micelles or bilayers, also permit the solvent exposure of a polar head group and the burial of a nonpolar tail. Adapting the approach of Tanford (58) and approximating the last 12 residues of Aβ40 as a cylinder, the surface area available to Lys28, the first exposed residue, would be ~60 Å² in a spherical micelle, 50 Å² in a cylindrical micelle, and just 20 Å² in a bilayer. Thus, the positive charges in the N termini will be the farthest apart and the most solvent-exposed in a spherical micelle. Both cylindrical micelles and bilayers can grow indefinitely on their ends or edges, respectively, whereas the number of monomers in spherical or elliptical micelles is limited geometrically. Therefore, the observation that the size distribution of β-balls is limited is more consistent with spherical or elliptical micelle geometries.

β-Balls have not been detected above pH 4. Our model predicts that β-balls will be unstable above this pH since titration of the C-terminal carboxylate group (pKₐ ~ 3.8) favors its solvent exposure and will break any hydrogen bonds that may form between the neutral carboxylic acid groups and disrupt the hydrophobic β-ball core. A spherocylindrical Aβ micelle forming at pH 1 has been thoroughly studied by Yong et al. (15). Like the β-ball, the pH 1 oligomer could contain buried C-terminal carboxylic acid groups and has a definite size range. Relative to the β-ball, the pH 1 spherocylindrical micelle contains fewer monomers (30–50), and its dimensions suggest that the nonpolar residues in the both the central and C-terminal hydrophobic segments are buried within the micelle core. The higher ionic strength present at pH 1 could more effectively screen the charge-charge repulsions allowing Lys28 from different monomers to be positioned closer together and thus account for its different geometry compared with β-balls. Other spherical Aβ peptide oligomers are present at endosomal (4) or neutral pH (7, 12–14) prior to the formation of amyloid fibrils or spherical oligomers is consistent with the finding that their C-terminal carboxylic acid groups are not buried but are charged and exposed. In contrast to the low pH Aβ oligomers, at neutral pH both positively and negatively charged groups are present and could be arranged to permit favorable electrostatic interactions in the oligomers (59). The positioning of multiple positive charges on the exterior of the β-balls probably keeps them well separated in solution and impedes their evolution to fibrils. In contrast, favorable electrostatic interactions in neutral pH spherical oligomers likely permit them to form progressively higher order aggregates. The existence of a common structural motif in both the β-balls, which lack favorable electrostatic interactions, and the neutral pH oligomers, wherein favorable electrostatic interactions are likely, that is specifically recognized by a neurotoxicity-neutralizing antibody (10) suggests that electrostatic interactions are not key for the stability or formation of this structural motif.

**β-Balls with DSS**—In the presence of DSS, the number of Aβ40 monomers in the β-balls approximately doubled to about 476 monomers. This number of monomers is too large to fit into the hydrophobic core of the micelle but could be accommodated as a second shell (Fig. 6C). In this plausible, hypothetical model, the C-terminal and central hydrophobic regions of each additional molecule are proposed to interact with the central hydrophobic regions of two adjacent Aβ40 molecules whose C termini are buried in the micelle core (Fig. 6C). DSS would promote the oligomerization of Aβ40 monomers by binding to hydrophobic regions and Lys residues bearing positive charges. The strongest DSS binding site is proposed to be the central hydrophobic regions and positively charged Lys6 residues of two Aβ40 monomers, one each from the inner and outer shells (Fig. 6C). Other plausible DSS binding sites are indicated. New NMR techniques like inverse relaxation-optimized spectroscopy (60) and cross relaxation-induced polarization transfer (61) yield good quality spectra of protein complexes up to 900 kDa at high magnetic field strength; these methods will be applied to study the high resolution structures of free and DSS-bound β-balls.

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Alzheimer's Aβ40 Studied by NMR at Low pH Reveals That Sodium 4,4-Dimethyl-4-silapentane-1-sulfonate (DSS) Binds and Promotes β-Ball Oligomerization

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