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Hydrogen Exchange-Mass Spectrometry Analysis of β -Amyloid Peptide Structure[†]

Steven S.-S. Wang,^{‡,§} Scott A. Tobler,^{||,⊥} Theresa A. Good,^{‡,#} and Erik J. Fernandez^{*,||}

Department of Chemical Engineering, Texas A&M University, College Station, Texas 77843-3122, and
Department of Chemical Engineering, University of Virginia, Charlottesville, Virginia 22904-4741

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ABSTRACT: β -Amyloid peptide ($A\beta$) is the primary protein component of senile plaques in Alzheimer's disease and is believed to be responsible for the neurodegeneration associated with the disease. $A\beta$ has proven to be toxic only when aggregated; however, the structure of the aggregated species associated with toxicity is unknown. In the present study, we use hydrogen–deuterium isotope exchange (HX)-electrospray ionization mass spectrometry (MS) along with enzymatic digestion as a tool to examine at near residue level, the changes in $A\beta$ structure associated with aggregation to a fibril form. Our results show that the structure of $A\beta$ intermediate species formed early in the course of fibrillogenesis is dependent upon solvent conditions. Additionally, the HX-MS data of peptic $A\beta$ fragments suggest that the C-terminal segment of the peptide is approximately 35% protected from exchange in fibril-containing samples, relative to monomeric $A\beta$ species prepared in DMSO/H₂O. The N-terminus (residues 1–4) is completely unprotected from exchange, and the fragment containing residues 5–19 is over 50% protected from exchange in the fibril-containing samples. This work contributes to our understanding of $A\beta$ structure associated with aggregation and toxicity and further application of this approach may aid in the design of agents that intervene in the $A\beta$ aggregation processes associated with neurotoxicity.

Alzheimer's disease (AD) is the leading cause of dementia in the aging population, affecting over 6.9 million people worldwide annually (1). The defining feature of Alzheimer's disease is the formation and progressive deposition of insoluble amyloid fibrils within the cerebral cortex (2). The key constituent of these amyloid deposits has been identified as a 39–43 amino acid long polypeptide, β -amyloid ($A\beta$), which is derived primarily from the proteolytic cleavage of a much larger amyloid precursor protein (β -APP) (3).

In AD, the relationship between $A\beta$ peptide structure and toxicity has been demonstrated. In mixtures of aggregates containing fibrils, protofibrils, and low molecular weight intermediates/oligomers, $A\beta$ peptide has proven to be toxic to cultured neuronal cells (4–13). While there is some

disagreement on the exact structure of the toxic $A\beta$ species, most research indicates that toxicity is linked to one or more intermediates that are part of the aggregation pathway associated with amyloid formation. In this study we begin to address features of $A\beta$ molecular structure associated with the aggregation pathway and toxicity.

Understanding, at the residue-level, how $A\beta$ self-assembles will provide new targets for the development of aggregation modifiers that could potentially limit the toxicity of $A\beta$. In the work described here, we used hydrogen–deuterium isotope exchange (HX) coupled with electrospray ionization mass spectrometry (MS) to examine at near residue-level, the changes in $A\beta$ structure associated with aggregation to a fibril form.

EXPERIMENTAL PROCEDURES

Materials. Synthetic $A\beta$ (1–40) peptide was purchased from Biosource International (Camarillo, CA). Deuterium oxide (D₂O, 99.9% atom D) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). The pD of buffers made in D₂O were estimated from the measured pH using the equation: $pD = pH_{read} + 0.4$ (14). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

$A\beta$ Peptide Solution Preparation. Stock solutions of 10 mg/mL were prepared by dissolving the $A\beta$ peptides in 0.1% (v/v) trifluoroacetic acid (TFA) in water or 100% (v/v) dimethyl sulfoxide (DMSO). After incubating for 1 h at 25 °C, the peptide stock solutions were diluted in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.2) or deionized water to the concentrations used in the experiment.

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* Corresponding Author. Address: Department of Chemical Engineering, 102 Engineers' Way, P.O. Box 400741, University of Virginia, Charlottesville, VA 22904-4741. Tel: +1 434 924 1351. Fax: +1 434 982 2658. E-mail: erik@virginia.edu.

[‡] Texas A&M University.

[§] Current address: Department of Chemical Engineering, National Taiwan University, No.1, Sec. 4, Roosevelt Road, Taipei, Taiwan 106.

^{||} University of Virginia

[⊥] Current address: Wyeth BioPharma, 1 Burtt Road, Andover, MA 01810.

[#] Current address: Department of Chemical and Biochemical Engineering, University of Maryland, Baltimore County, 1000, Hilltop Circle, Baltimore, MD 21250.

¹ Abbreviations: $A\beta$, β -amyloid; AD, Alzheimer's disease; DMSO, dimethyl sulfoxide; HX, hydrogen–deuterium exchange; PBS, phosphate buffer saline; TFA, trifluoroacetic acid.

To obtain the monomeric conformation, the samples were used right after dissolution in DMSO or TFA solvents. These samples of A β prepared after only 1 h of incubation are referred to below as A β (T1h). To create fibrils, the samples were allowed to sit undisturbed for 3 days. Fibril-containing samples were centrifuged for 5 min at $14\,000 \times g$, and ~90% of the supernatant was removed and replaced to reduce the amount of soluble, potentially unaggregated A β analyzed. These fibril-containing samples aged for 3 days are referred to below as A β (T3day).

Others have indicated that A β peptide in 8 M urea pH 10 forms an unstructured monomer (15). To form an unstructured monomeric A β from urea, lyophilized A β peptide was solubilized using 8 M urea in 10 mM glycine–NaOH buffer, pH 10, at a concentration of 10 mg/mL. After incubating for 1 h at 25 °C, the peptide sample was diluted in 8 M urea to the concentration used in the experiment.

Structural Characterization of A β Monomer and Fibril Samples. Thioflavine T Fluorescence (ThT) Assay. A 40 μ L sample of unaggregated and aggregated A β samples was mixed with 960 μ L of 10 μ M thioflavine T (ThT) in phosphate buffer saline (PBS). Changes in ThT fluorescence, which would indicate the presence of amyloid fibrils (16), were measured by exciting samples at 437 nm and measuring emission at 485 nm using a PTI model spectrofluorimeter (Photon Technology International Inc., Monmouth Junction, NJ).

Congo Red Binding Assay. Congo red binding (CRB) was measured as described (17) with minor modification for use with a microplate reader. Samples of 25 μ L were mixed with 225 μ L of 20 μ M Congo red in PBS and incubated at room temperature for 30 min. Absorbance at 405 and 540 nm were determined using an Emax microplate reader (Molecular Devices, Sunnyvale, CA), and the amount of Congo red bound to amyloid fibrils (CRB) was determined by the equation $CRB \text{ (mol/L)} = A_{540}/47800 - A_{405}/38100$ (17).

Native PAGE. Unaggregated and aggregated A β (1–40) species dissolved in stock solutions of 0.1% TFA or 100% DMSO and diluted with PBS were mixed with native sample buffer (40% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 0.06 M Tris–HCl, pH 6.8). Samples were applied to precast 10–20% Tris–Tricine gels, and their electrophoretic separation was carried out in a Mini Protein II Electrophoresis system (Biorad, Hercules, CA). The gels were washed with deionized water twice and then stained with GelCode solution (Pierce, Rockford, IL).

Electron Microscopy (EM). A 5 μ L sample was fixed for 15 min in 0.25% glutaraldehyde in PBS, pH 7.4. The sample was then diluted 1:100 with deionized water; 2–5 μ L of diluted sample was placed on carbon-stabilized, Formvar-coated grids, which were treated with 0.1% poly-L-lysine in water (w/v) (Sigma Chemical Co., St. Louis, MO). Grids were negatively stained with 2% aqueous uranyl acetate (w/v) (Ted Pella Inc., Redding, CA) and then examined and photographed in a Zeiss 10C transmission electron microscope (Thornwood, NJ) at an accelerating voltage of 80 kV. A calibration grid (diffraction grating replica # 607, Electron Microscopy Sciences, Fort Washington, PA) was photographed at each session to verify magnification.

Hydrogen–Deuterium Exchange (HX) and Mass Spectrometric (MS) Analysis of Peptic Digest Peptides. The procedure for HX and MS analysis of peptic digest fragments

was modified from work reported previously (18). HX of fibril samples was initiated by adding 10 μ L of an aged [A β -(T3day)] 4 mg/mL A β solution to 40 μ L phosphate buffer in D₂O solution (20mM Na₂HPO₄, 20mM KH₂PO₄). This amount of peptide allowed for one HX sample; multiple HX samples could be obtained from the same 4 mg/mL solution. After the desired HX time (5, 30, 60 min) at 25 °C, the fibril-containing samples were dissolved for 30 s at 0 °C by the addition of 100 μ L of acetonitrile and 50 μ L of 0.5 M phosphate buffer (pH 2.5). Between 5 and 30 min an increase in exchange was certainly observed. Between 30 and 60 min, however, little or no change in protection was observed, indicating that the hydrogens protected at 30 min had rather long exchange times. This is consistent with the prior observation the multiple phases of exchange observed by Wetzel et al. in their prior whole-peptide analysis (19). Thus, we chose 30 min as an arbitrary time to discriminate strongly protected (e.g., long exchange times) versus weakly protected peptides.

The method of dissolving fibrils quickly under HX quench conditions was adapted from Kheterpal and co-workers (19). By this method, between 75% and 95% of fibril mass was recovered, as indicated by UV absorption and mass spectrometry signal intensity. A reduction in aggregate size was also seen by this method, as evidenced by the loss of the largest species on native PAGE (Figure 3B) and a reduction in hydrodynamic radius measured by dynamic light scattering by over a factor 2 (data not shown). However, acetonitrile fibril dissolution, while able to solubilize and dissociate the largest species in the fibril samples, was not sufficient to return all peptides to their unstructured monomer state.

The peptide was first digested for 90 s by the addition of pepsin (200 μ L, 0.4 mg/mL, 0.1 M phosphate, pH 2.5, 0 °C) and then further diluted with 1600 μ L of 0.1 M phosphate buffer (pH 2.5) 1 min before injection and reverse-phase chromatography (RPC)-MS analysis. HX and peptic digestion of A β (T1h) samples was performed analogous to aged, A β (T3day) samples except, that fresh A β solutions were used and diluted either in H₂O or PBS in H₂O. No centrifugation was performed for the A β (T1h) samples.

The digest mix was loaded into a 1 mL sample injection loop using a prechilled syringe and syringe filter (Millex-GV, 0.2 μ m; Millipore) and injected onto a stainless steel HPLC column containing Source RPC media (2.1 \times 30 mm, packed and donated by Amersham Biosciences). The injection loop and RPC column were kept in an ice bath. The solvents used here were 0.05% trifluoroacetic acid in H₂O (solvent A) and 0.05% trifluoroacetic acid in acetonitrile (solvent B). The peptic peptides were eluted from the column using a 6 min gradient 5–50% solvent B. The flow rate through the column was set to 0.25 mL/min, and approximately 25 μ L/min was split off and directed into an LCQ Duo ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). The rest of the flow was directed to a UV detector. Mass spectra were acquired in the full scan mode from 300 to 1500 *m/z*.

To identify the peptides obtained from this peptic digest, the above procedure was followed except that no deuterium was involved, and a 30 min gradient was used to elute the peptide. The LCQ Duo was set to acquire mass spectra in the “triple play” mode (full scan, zoom scan, MS/MS scan), and the SEQUEST software (U. Washington) provided by

ThermoFinnigan (San Jose, CA) was used to identify peptides against possible matches found in the A β (1–40) primary sequence.

The measured deuterium content in each peptide was corrected for back exchange that occurs during the digest and chromatography steps using eq 1 as previously described (20)

$$\frac{D}{N} = \frac{(m - m_{0\%})}{(m_{100\%} - m_{0\%})} \quad (1)$$

where D is the corrected deuterium content, N is the total number of exchangeable sites, m is the measured mass of the peptide in a labeling experiment, and $m_{0\%}$ and $m_{100\%}$ are the measured peptide masses from 0% and 100% deuteration controls, respectively. Since the monomeric A β shows much more exchange and is expected to be fully deuterated after 30 min (19), it was used to estimate and account for the back exchange during the analysis (20). Thus, the 100% deuteration control value was determined from the HX experiment of fresh A β (T1h), and 0% deuteration was determined from the average mass of the peptide with no artificial incorporation of deuterium. No significant incorporation of deuterium was expected during the redissolution and digest steps. The number of protected residues in the fibrils can be estimated using eq 2:

$$(\text{number of protected amides}) = N - D \quad (2)$$

HX-MS of Full-Length A β (1–40) Peptide. HX-MS was also performed on the full-length A β (1–40) peptide via a procedure described previously with some modifications (18). A β (1–40) (10 mg/mL) in 0.1% (v/v) TFA in water or 100% (v/v) DMSO was mixed with PBS or deionized water to the concentration of 4 mg/mL. Sample solutions were then diluted to 0.8 mg/mL in an appropriate D₂O buffer and kept at 25 °C. A 50 μ L sample of solution was removed at various time points and redissolved by the addition of 100 μ L of acetonitrile and 50 μ L of 0.5 M phosphate buffer (pH 2.5) for 30 s at 0 °C, and then further diluted with 1800 μ L of 0.1 M, pH 2.5 phosphate buffer 1 min at 0 °C before injection onto a column containing Source RPC media (2.1 \times 30 mm, Amersham Biosciences). The sample injection loop and RPC column were kept in an ice bath. The HPLC solvents used here were 0.05% trifluoroacetic acid in H₂O and 0.05% trifluoroacetic acid in acetonitrile. Running conditions for HPLC and LCQ Duo ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) were same as the ones used for peptic digest peptides.

RESULTS

We first needed to confirm that the fresh A β (T1h) and aged A β (T3day) samples contained the expected structures. As seen in Figure 1, ThT fluorescence or Congo red binding indicated there was no significant amyloid formation in fresh A β (T1h) samples prepared by dilution into water or PBS from stock solutions in DMSO or TFA. In contrast, aged A β (T3day) samples prepared in both stock solvents, and diluted into PBS, had considerable amyloid content as demonstrated by the increase in ThT fluorescence and Congo red binding above solvent control levels. In Figures 2 and 3, further evidence of A β sample structure is seen. Electron

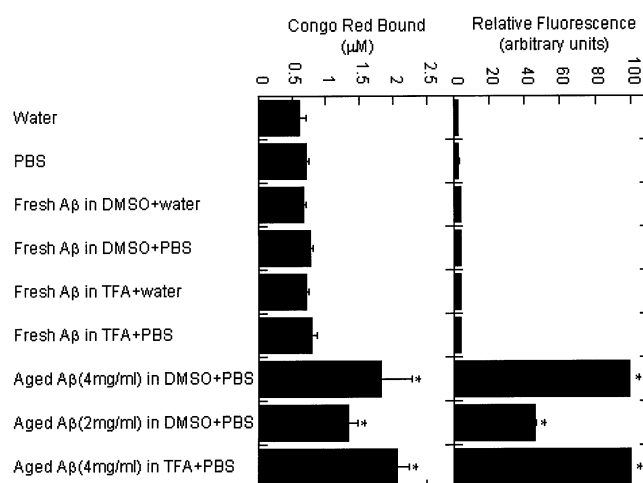


FIGURE 1: Amyloid fibril formation of A β (1–40) species in different aggregation states. The extent of amyloid fibril formation was semiquantified by (A) Thioflavine T binding and (B) Congo red binding. Fresh and aged A β (1–40) peptides were first dissolved in DMSO or TFA and then mixed with deionized water or PBS. Fresh samples (within 1 h of preparation) or samples aged for 3 days (fibrils) were then tested for amyloid formation. The mean \pm standard deviation of at least four determinations are presented. * indicates that measurement is significantly different than that for solvent alone ($p < 0.05$).

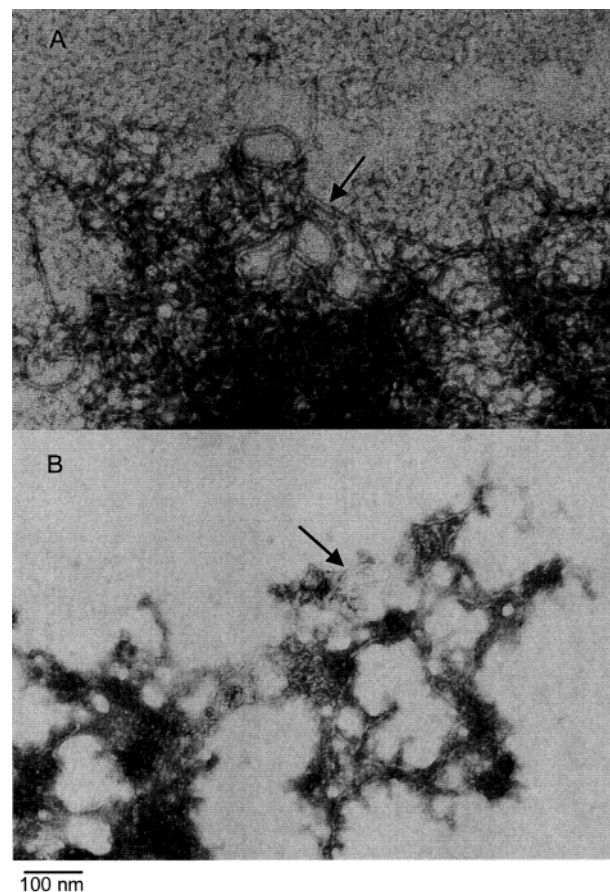


FIGURE 2: Electron micrographs of negatively stained A β (1–40) peptide prepared from different solvents. (A) Aged A β prepared in DMSO/PBS; (B) aged A β prepared in TFA/PBS. Arrows indicate A β fibrils.

micrographs confirmed the presence of amyloid fibrils in aged A β (T3day) samples of A β prepared from both stock solutions (Figure 2). No fibrils or other aggregated species

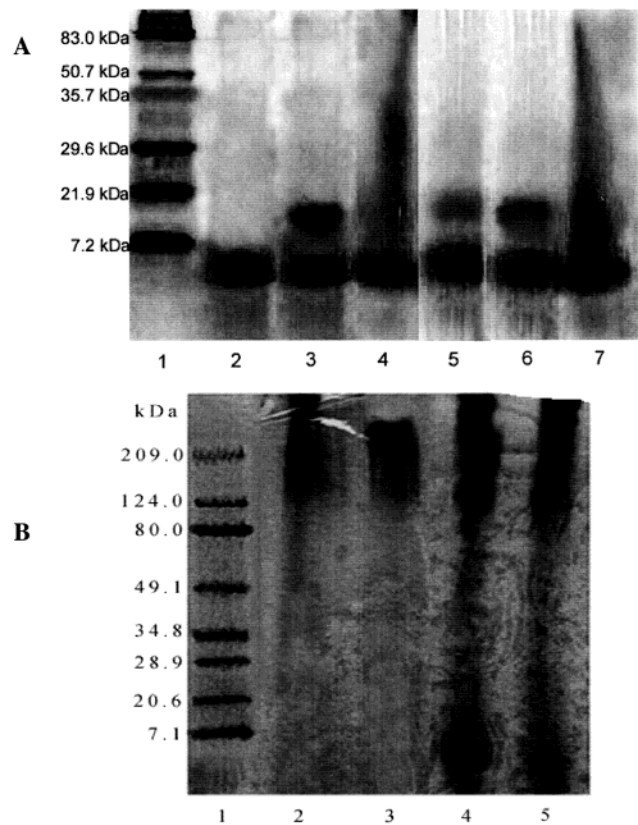


FIGURE 3: Representative native polyacrylamide gel electrophoresis of Aβ(1–40) peptides in different aggregation states. (A) Low molecular weight fractions: lane 1, molecular weight markers (aprotinin, 7.2 kDa; lysozyme, 21.9 kDa; soybean trypsin inhibitor, 29.6 kDa; carbonic anhydrase, 35.7 kDa; Ovalbumin, 50.7 kDa; bovine serum albumin, 83.0 kDa); lane 2, fresh Aβ in DMSO/H₂O; lane 3, fresh Aβ in DMSO/PBS; lane 4, aged Aβ in DMSO/PBS; lane 5, fresh Aβ in TFA/H₂O; lane 6, fresh Aβ in TFA/PBS; lane 7, aged Aβ in TFA/PBS. (B) High molecular weight fractions: lane 1, molecular weight markers; lane 2, aged Aβ in DMSO/PBS after centrifugation and supernatant removal; lane 3, aged Aβ in DMSO/PBS after centrifugation, dissolved in acetonitrile/PBS; lane 4, aged Aβ in DMSO/PBS prior to centrifugation and supernatant removal; lane 5, aged Aβ in TFA/PBS prior to centrifugation and supernatant removal.

were found in fresh Aβ(T1h) samples, regardless of solvents used (micrographs not shown). Native PAGE gels confirmed the presence of large aggregates in aged Aβ(T3day) samples that are missing in fresh Aβ(T1h) samples (Figure 3A,B). In addition, aged Aβ(T3day) samples contained a variety of small Aβ oligomers (molecular weight below 80 kDa) which could be removed upon centrifugation (Figure 3B). No attempt was made to quantify relative proportions of different Aβ oligomer, protofibril, or fibril species.

The native PAGE gels also revealed significant differences in the structure of fresh Aβ(T1h) samples prepared from stock solutions in DMSO and TFA (Figure 3A). The fresh Aβ(T1h) samples from DMSO stock solution diluted into water migrated as a single species with the approximate molecular weight of an Aβ monomer, while fresh Aβ(T1h) samples from DMSO stock diluted into PBS migrated as at least two species, most likely a monomer and small oligomer such as a dimer. Fresh Aβ(T1h) samples from TFA stock diluted in either water or PBS migrated as two species as well.

Table 1: Observed Mass of Fresh (Monomer) and Aged (Fibril) Aβ Peptides Prepared from Different Stock Solvents after 30 Minutes of HX

sample	no. of runs	observed molecular mass, Da	mass increase from proto-nated Aβ, Da	% exchange relative to fresh Aβ in DMSO/water
fresh Aβ in DMSO/H ₂ O	3	4356.0 ± 0.02	25.0	100.0
Aβ in 8 M urea	3	4355.8 ± 0.03	24.8	99.2
fresh Aβ in DMSO/PBS	3	4355.2 ± 0.05	24.2	96.8
		4344.8 ± 0.06	13.8	55.2
aged Aβ in DMSO/PBS	3	4354 ± 0.07	23.0	92.0
		4343.6 ± 0.03	12.6	50.4
fresh Aβ in TFA/H ₂ O	3	4352.4 ± 0.2	21.4	85.6
		4342.0 ± 0.3	11.0	44.0
fresh Aβ in TFA/PBS	3	4353.2 ± 0.1	22.2	88.8
		4342.8 ± 0.2	11.8	47.2
aged Aβ in TFA/PBS	3	4352.8 ± 0.1	21.8	87.2
		4343.2 ± 0.1	12.2	48.4

Typical mass spectra for the +4 charge state of full-length peptides prepared under all conditions (DMSO and TFA stock solutions, fresh, diluted in water or in PBS, and aged) after hydrogen exchange are shown in Figure 4. When starting from a fresh DMSO stock solution diluted in water, Aβ(T1h) subjected to 30 min hydrogen exchange yielded a mass spectrum with only one narrow peak (Figure 4A) with a molecular mass of 4356.0 ± 0.02 Da (Table 1). The mass spectrum of fresh Aβ from 8 M urea after 30 min hydrogen exchange was identical to the peptide from DMSO stock solution (spectrum not shown). This implies that fresh Aβ-(T1h) from a DMSO solvent can be taken as a true, unstructured monomer. Upon dilution of the fresh Aβ(T1h) peptide from DMSO stock into PBS, two peaks in the mass spectra were observed, one with a mass similar to that of the DMSO monomer and a second species which was more resistant to exchange (4344.8 ± 0.06 Da). On the basis of structural data (Congo red binding, ThT fluorescence, native PAGE and electron microscopy), the species resistant to exchange is most likely a small structured Aβ oligomer, and not a large fibril or protofibril. Mass spectra of Aβ(T3day) prepared from DMSO stock, after hydrogen exchange, again showed two peaks at 4354 ± 0.07 and 4343.6 ± 0.03 Da, respectively. The former was near the mass of the deuterated monomer from DMSO stock solution, and the latter near the mass of the more protected small oligomer species.

Aβ peptide prepared from a TFA stock solution, fresh or aged, diluted into water or diluted into PBS, resulted in two distinct species detected by HX-MS, one with a mass of approximately 4352 Da and one with a mass of around 4342 Da. In addition, broader mass peaks were observed in the spectra of the peptide from the TFA stock solution. The relative size of the peaks changed with aging and fibril formation (Figure 4D–4F). The heavier, less protected species, was the more soluble of the species in the aggregated mixtures. Upon centrifugation and removal of supernatant, all low molecular weight oligomers were removed, as evidenced by native PAGE (Figure 3B), along with the majority of the mass in the heavier species detected via mass spectrometry. A summary of species observed in the different fresh Aβ(T1h) and aged, fibril-containing Aβ(T3day) samples is presented in Table 1 along with percentage exchange observed in each sample relative to the DMSO monomer. Approximately 50% of the peptide was protected from

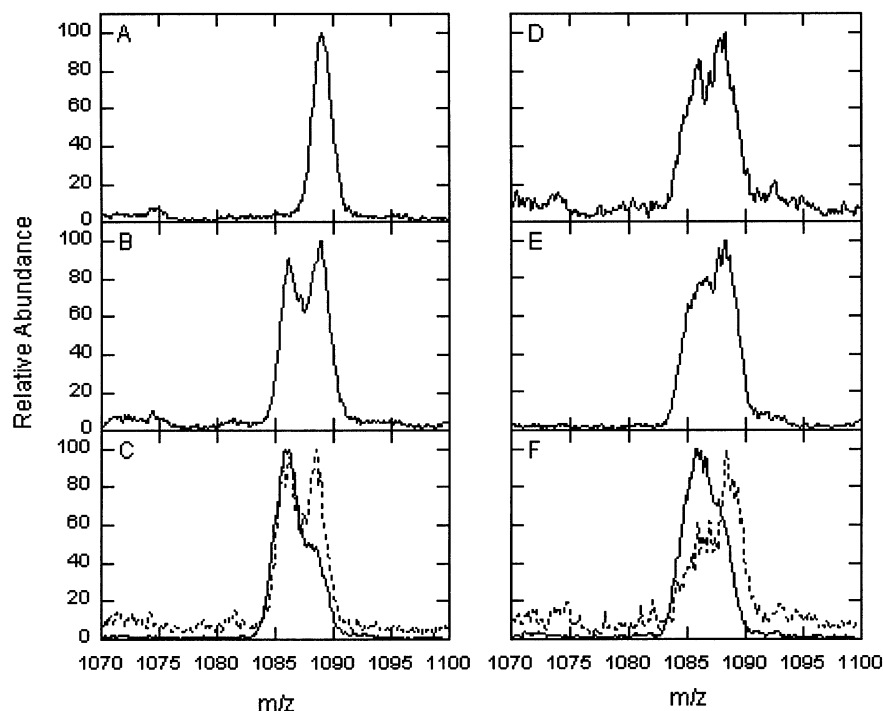


FIGURE 4: Representative mass spectra of full-length A β (1–40) peptides in the +4 charge state after 30 min hydrogen exchange. A β samples were prepared in six different conditions: (A) fresh A β in DMSO/H₂O; (B) fresh A β in DMSO/PBS; (C) aged or fibrillar A β in DMSO/PBS; (D) fresh A β in TFA/H₂O; (E) fresh A β in TFA/PBS; (F) aged or fibrillar A β in TFA/PBS. Mass spectra of aged A β precipitates after centrifugation (solid line) and aged A β sample mixture (without centrifugation) (dashed line) prior to hydrogen exchange are shown in panels C and F.

Table 2: Protection of Peptide Fragments from A β Fibrils after 30 Minutes of HX

peptide	amino acids	no. of protected amides (total #) ^a
P1	1–20	5.7 \pm 1.6 (19)
P2	1–19	5.4 \pm 1.8 (18)
P3	4–19	8.1 \pm 1.9 (15)
P4	20–40	8.3 \pm 2.0 (20)
P5	20–33	4.9 \pm 1.4 (13)
P6	20–34	5.6 \pm 1.7 (14)
P7	20–35	6.3 \pm 1.0 (15)
P8	20–37	6.8 \pm 2.0 (17)
P9	34–40	2.2 \pm 0.6 (6)
P10	35–40	1.7 \pm 0.5 (5)

^a N-terminal amino groups of peptides exchange rapidly under all conditions. Thus, the deuterium labeling of peptides will be sensitive mainly to the number of remaining peptide backbone amide groups (total #).

exchange in the fibril-containing A β (T3day) samples. In addition, even in fresh A β (T1h) samples prepared from TFA where no fibrils were detected or in the fresh samples from DMSO diluted into PBS, there was still a relatively protected species formed almost immediately.

We then performed experiments in which we subjected the A β peptide either in monomeric A β (T1h) or fibril-containing A β (T3day) (both from DMSO stock solutions) to hydrogen exchange, followed with proteolytic digestion and MS analysis. This allowed us to map which portions of the A β peptide were protected from exchange during fibril formation. More than 60 different peptide fragments were produced in significant amounts. On the basis of MS/MS analysis using the SEQUEST software, 10 peptide fragments, listed in Table 2, were identified with confidence and with sufficient signal intensity for hydrogen exchange analysis.

Representative mass spectra in Figure 5 show differences in exchange for three of the peptide fragments (1–20, 20–35, and 35–40) between the fresh A β (T1h) (solid lines) and fibril-containing A β (T3day) samples (dotted lines). In all cases, broad envelopes of peaks were observed due to the natural isotope distribution as well as deuterium exchange. Mass spectra for fresh and fibril-containing states were significantly different from each other in the N-terminal peptide (1–20) and the peptide spanning residues 20–35, indicating that substantial portions of these sequences were protected from solvent in the fibril state. The C-terminal peptide (35–40) showed a lower protection from exchange in the fibril sample relative to the monomeric state, as indicated by the greater degree overlap of the monomer and fibril mass spectra.

Table 2 shows the estimated number of protected amides in the aged, fibril-containing A β (T3day) samples relative to the fresh A β (T1h) DMSO samples for each of the peptic fragments. The results in Table 2 indicate clear differences in protection between fresh and aged samples, consistent with considerable protection in the fibril. Because several peptides in Table 2 overlap, labeling in smaller segments of A β could be estimated from differences in labeling between peptides. Table 3 shows calculated differences. The differences between mass spectra of peptide 2 (1–19) and peptide 3 (4–19) were used to estimate the solvent accessibility to the peptide N-terminus (residues 2–3), which in this case was almost entirely unprotected. Peptide 10 and the difference between peptides 7 and 8 indicated that the C-terminal group of residues (36–37 or 35–40) showed little protection. On the average, much of the rest of the peptide was protected in the fibrillar state, although to varying extents. For example, 5–19 was approximately 54% protected, 21–33 was ap-

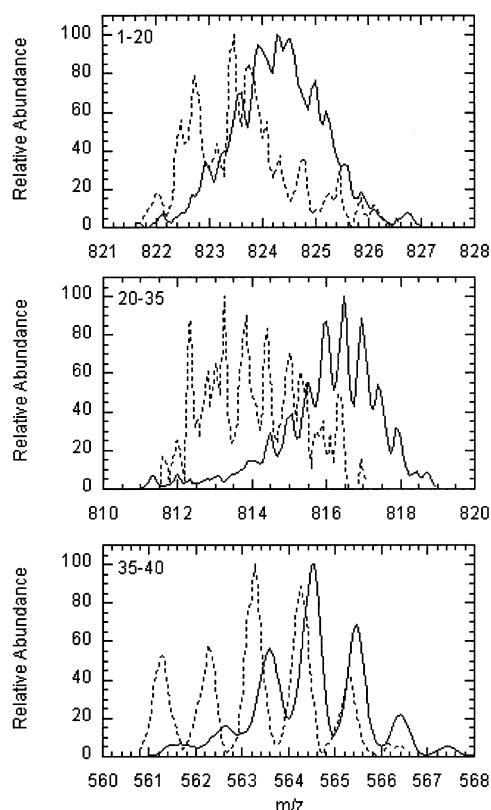


FIGURE 5: Mass spectra for three representative peptic peptides (segments 1–20, 20–35, 35–40) from A β (1–40) in the +3, +2, and +1 charge states, respectively, after 30 min of hydrogen exchange. Fresh and aged (or fibrillar) A β samples were prepared in DMSO/H₂O and DMSO/PBS, respectively. Spectra for both the fresh A β sample (solid lines) and aged (or fibrillar) A β sample (dashed lines) are shown and are normalized by area in each plot.

Table 3: Protection of Regions within A β Fibrils after 30 Minutes of HX Calculated from Differences between Labeling of Overlapping Peptides

overlapping peptides ^a	nonoverlapping backbone peptide amides ^b	no. of protected amides (total #) ^c
P2–P3	2–4	–2.7 (3)
P1–P2	20	0.3 (1)
P8–P5	34–37	1.9 (4)
P6–P5	34	0.7 (1)
P7–P6	35	0.7 (1)
P9–P10	35	0.5 (1)
P8–P7	36–37	0.5 (2)
P4–P8	38–40	1.5 (3)

^a The two overlapping peptides used to calculate a difference in labeling. ^b N-terminal and side chain amino groups of peptides exchange rapidly under all conditions. Thus, the difference between labeling of the two peptides will reveal the labeling of the nonoverlapping backbone amides in the longer peptide. These are the residues shown. ^c Calculations were based on the data in Table 2. The total number of peptide backbone amide groups will be one less than the length of the peptide, as indicated in Table 2.

proximately 38% protected, and 36–40 was approximately 34% protected. Of course, these percentages cannot be interpreted as uniform within a given segment.

DISCUSSION

A variety of approaches have been used by previous investigators to examine A β structure upon fibril formation and the mechanism of A β fibril formation (21–27). Given the importance of A β aggregation and fibril formation in

the mechanism of neurotoxicity associated with Alzheimer's disease (21, 22, 28), understanding the mechanism by which A β self-assembles into fibril and protofibril structures at the residue level will provide valuable molecular level clues in the design of strategies to prevent A β aggregation and/or neurotoxicity.

Using HX-MS, we have examined the portions of the peptide backbone that are protected from hydrogen exchange during self-assembly into fibrils at both the full-length peptide level and at near the residue-level. Other investigators have used HX along with NMR to map residue-level aggregation of A β , other amyloids, and other proteins (24, 29, 30). Although HX-NMR method does map at truly a residue level, the HX-MS method has a number of advantages regarding its speed, simplicity, sensitivity, and much higher molecular weight limits. More importantly, HX-MS reveals the distribution of species (monomers, oligomers, and fibrils) with various masses, which is a critical issue here.

For the full-length peptide (Table 1, Figure 4), we found that approximately 50% of the peptide was protected from exchange in fibril-containing samples relative to A β monomers. In addition, even in fresh A β (T1h) peptide solutions that have been shown by others to be nontoxic (6) and have no detectable fibril or amyloid content (Figures 1 and 2), there existed some fraction of the peptide that had similar degree of protection as the full-length peptide. On the basis of native PAGE of the fresh A β (T1h) samples diluted into PBS (Figure 3), we assume that this species, which is resistant to hydrogen exchange, is a small A β oligomer such as a dimer. The rapid formation of a dimer upon dilution into PBS has been seen by others (15). Alternatively, it is possible that the species we observe both via native PAGE and mass spectrometry, though too small to observe via TEM and not of sufficient extended structure to bind Congo red or ThT, could be a larger, weakly associated oligomer such as an A β micelle. A β micelles have been reported to form immediately upon dissolution and be in rapid equilibrium with A β monomer and small oligomer (31). Micelles would likely disassemble during electrophoresis and may not have stable or extended β -sheet structures, but might still be considerably protected from solvent.

Close comparison of the mass spectra after hydrogen exchange of fresh A β (T1h) samples from DMSO and TFA stock solutions (Figure 4A,B,D,E) indicate that the relatively unprotected "monomeric" species seen in both solvents was not the same. On the basis of our comparison of fresh A β (T1h) samples prepared in DMSO and 8 M urea and the results of others (15), we believe that our fresh A β (T1h) peptide prepared from DMSO stock solution is an unstructured/unfolded monomer. The higher mass species seen in fresh A β (T1h) samples from TFA stock, diluted into water, had less mass than the DMSO monomer and a broader mass peak, indicating that the TFA monomer is more structured than the DMSO monomer. This is consistent with earlier studies that indicated that A β (A β 1–39, to be more specific) in TFA/water solvent had approximately 30% β -sheet while in DMSO the peptide was relatively unstructured (32).

In all aged A β (T3day) peptide samples (Figure 4C,F), two distinct mass peaks could be seen. One is relatively unprotected from exchange, analogous to monomers prepared either in DMSO or 8 M urea. The other is approximately 50% protected from exchange, which we assume represents



FIGURE 6: Schematic of peptide showing relative protection of exchange. The white bar indicates 0% protection (complete solvent accessibility), light gray and gray bars indicate <35% and 35%~50% protection, respectively (moderate solvent accessibility), and the black bar indicates >50% protection (low solvent accessibility). The percent protection, calculated by the ratio between the number of protected amides and total number of amides in Tables 2 and 3, is indicated beneath the sequence.

the protected structure present in A β fibrils. Recent experiments from Wetzel and co-workers, in which fragmentation analysis was not performed, are consistent with this overall degree of protection (19). On the basis of both our native PAGE and EM analysis of aged A β (T3day) samples, these samples contain fibrils along with a variety of other high molecular weight species, possibly both ordered and unordered. The broadness of the mass peaks in these samples is probably indicative of the heterogeneity in the sample.

We proteolytically cleaved A β fresh and fibril-containing samples after hydrogen exchange in order to identify which segments of the full-length peptide are accessible to exchange and which are relatively protected. As seen in Figure 5 and Tables 2 and 3, the overall degree of protection observed in the proteolytic fragmentation analysis was consistent with the full-length peptide analysis. However, the proteolytic fragmentation results revealed significant variations in protection from exchange in different regions of the full-length A β peptide.

Figure 6 shows a schematic of the peptide regions accessible and protected from exchange. The region from residues 5–19 was about 54% protected from exchange, which is among the strongest protection we observed in the fibril-containing samples. A variety of evidence from others, based on substitutions made in synthetic fragments of the A β sequence, indicate that residues 17–21 are important for β -sheet formation (33–35) and that some portion of the peptide between residues 11 and 16 is probably important for conformational stability necessary for aggregation (34, 36, 37). Reductive alkylation and limited proteolysis data suggest that residues 16–19 are more protected in the fibril than in the monomer (38, 39). Regions of the peptide necessary for aggregation would be expected to be protected from exchange (protected from solvent) in the fibril.

The most pronounced feature of the exchange pattern was the lack of protection at the N-terminus of the peptide. This is consistent with data obtained via reductive alkylation (38), limited proteolysis (39), and other methods (40–42), suggesting the N-terminus of the peptide does not participate in fibril formation.

In contrast, the C-terminus of the peptide was approximately 35% protected from exchange. This portion of the peptide contains the most hydrophobic residues and has been postulated to be important in fibril formation (43–45). Reductive alkylation and limited proteolysis data suggest that portions of the C-terminus (residues 28 and 35–36) are significantly protected in the fibril relative to the monomer (38, 39). However, data from fluorescence quenching and electron paramagnetic resonance spectroscopy indicate that portions of the C terminus have considerable mobility

(residues 38–40 (27)) and that solvent accessibility may increase upon conversion from dimer to fibril (residues 34, 40 (23)).

Thus, on the basis of what has been previously reported, the relative solvent accessibility of the peptide C-terminus is not surprising. While we report an “average” solvent accessibility over the entire C-terminus, local protection of certain residues may vary significantly within the segment. In addition, there may be some structure in the C-terminus sufficient to hinder proteolysis or reductive alkylation but insufficient to exclude water molecules from participating in hydrogen exchange. A weakly structured hydrophobic C-terminus may be important for peptide-cell interactions associated with peptide toxicity.

The hydrogen–deuterium exchange patterns observed here are also generally consistent with a study of A β fibril structure very complementary to this one by Wetzel and co-workers using limited proteolysis (19). The two techniques are related in that hydrogen–deuterium exchange requires solvent accessibility, and peptide bond fission requires peptide backbone flexibility. Proteolytic cleavage of a target peptide indicates that several residues near the cleavage site are flexible and able to access and adapt to the shape of the protease active site (46–48). In their study, Wetzel and co-workers found the N-terminus to be readily cleaved than sites at the C-terminal end of the peptide. Interior residues (e.g., residues 16, 17, and 19) were also found to be relatively inaccessible in fibril preparations relative to monomeric A β . Interestingly, they found residues 10 and 11 to be susceptible to cleavage, while the peptide containing these residues was rather well protected from exchange. This may well be consistent with our data, as we do not expect uniform protection from exchange over an entire peptide fragment.

A number of three-dimensional models of A β structure have recently been proposed (refs 25 and 26 and others). Data presented here would pose the following constraints on such models: the 4 N terminal residues, residue 20, and some of the C terminal residues between 35 and 40 would need to be relatively solvent-accessible. In the model proposed by Perutz and co-workers (25), A β fibrils may actually consist of hollow nanotubes of approximately 20 residues in diameter, with at least 37 residues are needed to form a stable cylindrical structure. If A β (1–40) is assumed to form the shorter stable structure, then the N-terminus of the peptide could be solvent-accessible in this model. Given that the structure proposed by Perutz is water-filled and that A β fibrils are not crystalline, there are likely small gaps and imperfections in the structure which would allow water exchange and the overall degree of hydrogen exchange we observe here. In the model proposed by Petkova and co-workers, A β fibrils are formed via in register parallel alignment of β -strands, in which the N terminus of the A β peptide is solvent-accessible, but the C-terminus is highly confined (26). Certainly, more detailed investigations of exchange along the A β peptide will be required to determine whether exchange patterns are consistent with the Perutz structure or others proposed. Such data as we would generate could be used in the refinement of three-dimensional models of A β .

In conclusion, utilizing HX-MS combined with enzymatic digestion, we were able to determine, at near residue level, which parts of the A β molecule are protected from solvent

during aggregation and probably participate in aggregation from a nontoxic to toxic species. In addition, the technique provides information on the dynamics of amyloid fibril formation. At very short times after dissolution of A β into physiological buffers, a fraction of the peptide adopts a conformation similar in protection as the fully formed fibril. This work contributes to our better understanding of fibril formation process associated with AD neurotoxicity and further serves as an exploratory guide for rational drug design.

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