To the Graduate Council

I am submitting herewith a thesis written by Brian Anthony Bledsoe entitled "Probing the Secondary Structure of Aβ (1-40) Fibrils " I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Ronald Wetzel

Ronald Wetzel, Major Professor

We have read this thesis

and recommend its acceptance

Accepted for the Council.

Associate Vice Chancellor and

Dean of the Graduate School

Probing the Secondary Structure of Aβ (1-40) Fibrils

A Thesis

Presented for the

Master of Science

Degree

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Brian Anthony Bledsoe

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Abstract

Limited information has been obtained on the secondary structure of amyloid fibrils In the absence of high resolution structural data, several groups have proposed models for amyloid fibril structure However, these models differ in hydrogen bonding patterns and residues that make up the rigid core of the fibril structure. Significant insight into fibril structure could be derived form a knowledge of the hydrogen bonding patterns within fibrils. Structural studies were performed on Aβ (1-40) fibrils using hydrogen exchange mass spectrometry as well as limited proteolysis under native conditions Data was obtained which illustrate differential amounts of exchange within the monomer sequence Results also suggest peptide bond exposure at specific residues within the N-terminal portion of Aβ incorporated into fibrils

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Background and Significance

The German psychiatrist Alois Alzheimer first described the disease which now bears his name in 1907 [1] At present, AD affects more than four million people in the United States alone [2] Over half of those over the age of 80 are afflicted with the disorder As human life expectancy increases, the effects of AD will continue to escalate unless methods are discovered to delay its onset or halt its progression

The primary pathological characteristic described by Alzheimer is the presence of senile plaques, composed of protein aggregates termed amyloid fibrils [3]. AD is one of several disease states termed amyloidoses in which fibril tissue deposits are found [4] Although fibrils from these differing amyloid deposits are generated from different peptides, they share a number of structural features. For example, fibril morphology is quite similar in EM [5, 6], and fibrils show similar responses to the fluorescent dye thioflavin T (ThT) [7, 8]. These structural similarities suggest related mechanisms of fibril formation among the amyloid diseases. Therefore, information derived from AD research may be applied to other similar diseases.

The primary component of AD associated amyloid fibrils is the Aβ peptide [9]. Aβ is a hydrophobic peptide, ranging in size from 39 to 43 amino acids in length, derived from β amyloid precursor protein (βAPP) An increase in brain Aβ levels correlates to the transition from a presymptomatic state to a

symptomatic state in the progression of AD [3]. Because of this correlation, it has been hypothesized that Aβ accumulation is the primary event in the onset of AD preceding neurological impairment and the development of neurofibrillary tangles, and recent data supports this idea [10]. The precursor protein βAPP [11, 12] is a 110-130 kDa membrane anchored glycoprotein expressed throughout the body The gene for βAPP is found on chromosome 21 spanning 400 kb of DNA and containing at least 19 exons The Aβ sequence lies between portions of exon 16 and 17 [13] and therefore cannot be generated by alternative splicing of the gene but requires proteolytic cleavage at both termini of the peptide. The specific function of the APP protein is unclear, however, several possibilities have been proposed that include functioning as a cell surface receptor, in cell to cell interactions, and in protease inhibition [14]

βAPP is processed through the secretory pathway during which it is most often cleaved at a region near the membrane within the Aβ sequence [15], thereby preventing formation of the Aβ peptide A membrane bound protease termed α secretase [16] is responsible for the cleavage at this site resulting in the release of a soluble peptide and leaving the carboxyl end of βAPP bound to the membrane. If α secretase does not cleave βAPP, the protein undergoes further processing [14] where it may be recycled to the membrane or degraded. It is likely that the Aβ peptide is generated during this extra processing step due to proteolytic cleavage by enzymes termed β secretase and γ secretase [12, 17]. Since Aβ

generation depends on the proteolytic cleavage of βAPP by these secretases, they are targets for therapeutic intervention

Several genetic studies have led to important discoveries and increased interest in AD research Genes responsible for early onset AD (before an average age of 60) are located on chromosomes 1 (presenilin 2), 14 (presenilin 1), 19 (ApoE), and 21 (βAPP). The first gene linked to AD was found to code for βAPP itself [9, 11]. A genetic defect leading to early onset AD was localized to chromosome 21 [9, 11, 18]. Studies involving patients with Down's syndrome (Trisomy 21) revealed the presence of senile plaques, implicating the genes on chromosome 21 play a role in amyloid fibril formation and AD pathology The presence of the precursor protein βAPP on chromosome 21 was linked to increased Aβ concentration and earlier plaque formation found in Down's syndrome patients

Mutations within the βAPP gene [9, 19] have also been linked to early onset forms of AD Mutations located near the sites of cleavage by α and β secretases lead to AD by enhancing the cleavage activity of β secretase to generate the Aβ peptide resulting in increased Aβ secretion. Mutations in βAPP account for 5-7 % of early onset AD. The average age of onset in βAPP mutations is 50-60 [20] Studies involving transgenic mice [21, 22] have confirmed βAPP involvement in early onset AD Cognitive impairment was observed after nine months accompanied by senile plaque formation in mice over

expressing mutated human βAPP. Three additional FAD (familial Alzheimer's disease) mutations have also been identified which lie within the Aβ sequence. The Dutch mutation was found in patients with hereditary cerebral hemorrhage due to extensive Aβ deposition in cerebral blood vessels. The Flemish mutation leads to Aβ deposition in cerebral blood vessels as in the Dutch mutation, but also leads to progressive dementia similar to AD. The Arctic mutation has been recently identified and unlike the other intra Aβ mutations, causes AD with no signs of stroke or vascular lesions [23] Together, these findings have helped demonstrate the pivotal role of βAPP and Aβ in AD pathogenesis.

The second and third genetic loci implicated in early onset AD involve the proteins presenilin 1 and presenilin 2 (PS1 and PS2) [2, 24, 25] Nearly 50 % of early onset AD cases have been associated with mutations in the presenilin genes Mutations in PS1 that lead to early onset AD are classified as autosomal dominant "causative" gene defects. More than 50 mutations have been identified in PS1, 2-3 in PS2 Patients with PS1 mutations exhibit significantly higher secretion levels of Aβ 1-42 (shown to be more prone to aggregation and to be the initially deposited amyloid peptide in AD) compared to those with sporadic non early onset AD [10]. The known sites of PS1 mutation are distributed throughout the gene, with half of these located in the transmembrane domain. Interestingly, although PS1 and PS2 are 65 % homologous, there is a clear difference in the mean age of onset. The mean age of onset in PS1 linked AD families is 45 years while the mean age of onset associated with PS2 mutations is 52 years. These

differences may be attributed to the mutations identified in PS1 being located within different codons of the PS2 gene.

Similar results were seen with transgenic mice expressing mutant PS1 [11] An increased concentration of βAPP mRNA, Aβ 1-42, and senile plaques were observed in the brain. Deletion of PS1 in mouse embryos was found to be lethal, however, both wild type and mutant PS1 could rescue the phenotype. These results demonstrate that PS1 mutations do not confer a loss but a gain in function PS1 deletions also lead to greatly reduced levels of γ secretase activity indicating PS1 plays a primary role in its activity. Further evidence suggests specific conserved transmembrane amino acids are critical for PS1 proteolysis and γ secretase activity Together these results suggest a possible role of PS1 as either a cofactor for γ secretase or as the γ secretase itself [25]

The fourth of the genetic loci implicated in early onset AD encodes apolipoprotein E (ApoE) [24, 26, 27]. ApoE has been termed a susceptibility gene To date no genetic mutations have been found in the gene encoding ApoE [2] The correlation to AD does not depend on genetic mutations but instead on genetic polymorphisms and the inheritance of one of three variants from each parent, the ε2, ε3, and ε4 alleles. Population studies [24, 26] have associated the ApoE4 isoform with a higher incidence and earlier age of onset of AD. In contrast, the E2 isoform seems to confer a protective advantage.

The role played by ApoE in conferring AD susceptibility is not clear. ApoE plays an important role in lipoprotein metabolism and cholesterol

homeostasis [28] In the peripheral and central nervous systems, ApoE secretion increases dramatically following neuronal injury. Because of the rapid response and increase in concentration, potential activities of ApoE may include involvement in the growth, repair, and maintenance of myelin and axonal membranes

One possibility for the involvement of ApoE in the progression of AD involves the ability binding to neuritic plaques [24, 26]. The three variants of ApoE vary in their affinity for binding Aβ amyloid Analysis of the interaction of the three variants with Aβ 1-40 fibrils illustrated that complex formation efficiency between ApoE and monomer follows E2>E3>>E4 [24]. Therefore, the ε4 allele may be a susceptibility factor to AD relative to the ε2 and ε3 alleles by virtue of increased levels of Aβ available for aggregation [20, 24]

Taken together, the alterations within the genes responsible for early onset AD (βAPP, PS1, PS2, and Apoε) coincide with modifications of the generation, aggregation, or clearance of Aβ that lead to amyloid fibril formation and deposition. Amyloid fibril formation thus appears to stand at the crossroads of many mechanisms of AD development. An understanding of the structure of Aβ fibrils could lead to new developments in drug design and therapeutic strategies

Research Methods

Aβ fibrils can be grown in vitro in aqueous solutions [29, 30]. Although peptides that aggregate into fibrils share traits in morphology, the structural roles

of specific amino acid residues in the Aβ sequence remain unclear. To date high resolution structural data has not been obtained in part because of the insoluble non crystalline nature of amyloid fibrils. Because of these characteristics, standard methods such as X-ray crystallography and NMR cannot be used to obtain high resolution molecular details of the structure. However, a number of lower resolution methods have provided valuable insights into fibril structure.

Amyloid fibrils are characterized by extreme insolubility under physiological conditions and are quantified by several methods. One such method to define fibrils is to measure fibril structure using the fluorescent dye ThT [7, 8]. ThT is useful in that it fluoresces upon binding to amyloid fibrils but not precursor polypeptides, monomer, or amorphous aggregates. Therefore, excitation of the fibril bound ThT is selective.

The physical properties of fibrils can be defined by electron microscopy (EM) [5, 31] EM has been used for characterization of the intermediate protofilament [32] and fibril ultra structure during fibrillogenesis at a resolution of 2 nm. Aβ amyloid fibrils were observed to be 6 to 10 nm in diameter and 5 to 160 nm in length These fibers were indistinguishable from those found in neuritic plaques.

Lansbury et al. have developed an application using Fourier transform infrared spectroscopy (FTIR) to illustrate the secondary structural differences between two peptides sharing characteristics of amyloid [33] It was demonstrated that amyloid fibrils producing indistinguishable IR spectra do not

necessarily have identical structure. The technique was also used to prove that amyloid does not routinely contain pure anti parallel β sheet structure

Although fibrils have this far proved refractory to high resolution X-ray crystallography studies (see below), X-ray fiber diffraction data has proven very useful in defining characteristics of amyloid fibrils [34]. Amyloid fibrils exhibit a distinct X-ray diffraction pattern. Early X-ray diffraction patterns of fibrils with 0.47-0 48 nm parallel reflections and 1 nm perpendicular reflections were characteristic of a cross β structure in which the peptide chain is organized into β sheets in which the hydrogen bonds are parallel to the fibril axis and the constituent β strands are perpendicular to the fibril axis These characteristics are similar among different amyloids suggesting that although formed from different proteins, the amyloids share structural similarity

Because of the insolubility and large size of fibrils, standard solution NMR cannot be efficiently used to obtain atomic resolution structural data on aggregated molecules such as amyloid fibrils [35]. However, solid state NMR (ssNMR) has been used to demonstrate that in terms of aggregation, shorter fragments behave much like the full length monomer Data from ssNMR seems to indicate an anti parallel structural motif for amyloid fibrils [36].

In the absence of high resolution structural data, several groups have proposed models for amyloid fibril structure. These models differ substantially, perhaps in part because they are based on different, limited sets of low resolution structural data. One proposed model is based on synchrotron X-ray diffraction

patterns of six different amyloid fibrils and two other synthetic fibril preparations [37]. All eight preparations gave similar X-ray diffraction patterns confirming a common protofilament substructure regardless of the precursor protein. The model suggests 24 β strands are incorporated in each 11 5 nm repeating unit that twists around a common helical axis parallel to the axis of the protofilament with strands perpendicular to the axis of the protofilament. The helical axis structure of the protofilament enables hydrogen bonding between β strands that extend over the length of the fibril to form a rigid and stable structure

Novel graphical methods have also been used in conjunction with X-ray diffraction data to form a fibril assembly model [38]. In a conformation proposed for Aβ fibrils, β strands are wound into successive turns of a parallel β helix. Each β strand wound into the helix contains two internalized residues, while each turn of the helix contains six hydrophobic residues in the central lumen. This model allows a prediction to be made regarding the number and location of hydrophobic residues on the exterior of the helical cylinder, and in the loops of random coil, that may induce lateral aggregation of protofilaments The β helical structure may also explain the rounded morphology of protofilaments which have a relatively constant diameter regardless of the derivative peptide's length or sequence.

Experimental results have also been presented leading to a molecular level model of fibril formation [39]. Aβ fragments of varying size containing the 16-20 sequence, (an essential element for fibril formation) were systematically selected

and incubated for fibrillogenesis Fibril formation was evaluated by EM revealing the shortest fibril forming sequence to be 14-23 Substitution and deletion of this peptide from the full length monomer inhibited fibril formation completely. Molecular modeling of the 14-23 sequence in an antiparallel β sheet conformation displayed favorable hydrophobic interaction stabilized by salt bridges between charged residues. The 14-23 peptide was proposed to form the core of Aβ fibrils, with the hydrophobic C-terminus folding over the core structure.

Research Design

The models mentioned above differ with regard to hydrogen bonding patterns and residues that make up the rigid core of the fibril structure Significant insight into fibril structure could be derived from a knowledge of the hydrogen bonding patterns within fibrils As described below, hydrogen exchange mass spectrometry (HX-MS) can provide information on the number of residues within the Aβ peptide involved in intramolecular and intermolecular hydrogen bonding Information on the secondary structural role of a particular sequence element along a fibril incorporated peptide could be used to test model predictions and to assemble improved models of amyloid fibril structure

A promising technique to study protein structure involves hydrogen exchange (HX) [40]. Protons on the surface of the polypeptide exposed to deuterated solvent exchange quickly, protons either involved in intramolecular hydrogen bonding, or sterically shielded from deuterated solvent have a much

slower rate of exchange, taking up to several weeks [41]. These differences in exchange rates, giving information about the non covalent structure of the protein [42], can be detected by methods such as NMR [6] and MS [42-45].

In order to use HX for analysis of amide protons in amyloid fibrils, fibrils must be disaggregated to monomer before data collection. However, sample manipulation in protic solvent can lead to a time dependent loss of deuterium and, hence, of the exchange information [41] Speed of analysis is therefore critical. While MS is potentially rapid, collecting NMR data takes hours or days.

Although NMR can be used to measure isotopic exchange of individual amide protons in small proteins, the method is inefficient when used to analyze larger proteins [40]. Therefore sensitivity, protein solubility, and lengthy analysis time are common hurdles for NMR experimentation with HX samples For larger proteins not accessible to study by NMR methods, the use of MS as an HX probe for higher ordered structures has become increasingly popular. HX-MS has been used to study protein conformations [45, 46], protein-ligand interactions [47], and identification of structures of folded and unfolded intermediates [48] Since the difference in molecular weight of deuterium and hydrogen is one mass unit, the difference in molecular weight between deuterated and non deuterated samples is a direct measure of the number of deuteriums incorporated into the peptide.

A sample processing solvent containing water, acetonitrile, and formic acid has been identified that is compatible with MS detection of Aβ, rapidly dissolves fibrils, and has a pH of 2.5 which reduces the rate of exchange and thus

suppresses the loss of amide deuterium incorporation and therefore structural information during processing of the sample [41, 45]. A method was devised to minimize sample mixing time before injection into MS [41]. This was accomplished by injecting fibril samples directly into the MS through a mixing "T" where samples are mixed with sample processing solvent, are dissolved, and immediately injected into the MS in approximately 10 s. Exchange rates of amide protons in full length Aβ incorporated into fibrils have recently been determined using the HX-MS method [41] However, this method provides global information spanning the Aβ monomer sequence but does not allow for the determination of which regions within the protein are involved in hydrogen bonding or are inaccessible to the solvent The goal of the research described here is to use proteolytic enzymes to digest of Aβ (1-40) fibrils to localize HX information to specific regions of the sequence

Spatial resolution in the range of one to ten residues can be obtained by coupling MS with proteolytic digestion [42] and tandem MS (MS/MS) [49] followed by analysis of the peptide fragments The non specific protease pepsin was chosen to proteolyze samples prior to MS data collection because the enzyme is most efficient in the pH range of the sample processing solvent. For sample proteolysis, pepsin is included in the sample processing solvent so that fibril samples can be digested and disaggregated simultaneously prior to injection into the MS during a 10 s time frame.

In the experiments outlined in the following chapters, peptides are described by high performance liquid chromatography (HPLC), LC-MS, and electrospray ionization MS. Digestion conditions for pepsin are optimized to obtain the best fragment pattern for analysis These fragments can be analyzed individually using HX techniques with MS to localize information to more specific regions of the Aβ peptide sequence. Information derived from HX data of Aβ peptide fragments can then be used to test model predictions to rule out or confirm structural predictions and to develop new models

Another possible method to obtain secondary structural information on fibrils is by the comparative proteolysis of the Aβ 1-40 monomer and Aβ 1-40 fibrils Limited proteolysis using proteolytic enzymes has been used to evaluate the structural characteristics of proteins under physiologic conditions [50]. Information regarding structure-functional relationships [51], and conformations of protein folding intermediates [52] has been obtained.

Proteolytic enzymes act on a particular sequence element within a fibril incorporated peptide based on the accessibility of the cleavage site. In theory, differences in monomer and fibril fragment production are a direct consequence of the accessibility of proteolytic cleavage sites due to the secondary structure of fibrils Sites cleaved in the monomer but not in fibrils are assumed to be involved in intramolecular hydrogen bonding, positioned within or near the fibril core, or inaccessible to the enzyme due to conformational changes

Several proteolytic enzymes that digest most efficiently under physiological conditions are used to digest the monomer and fibrils. Two enzymes (trypsin and chymotrypsin) were chosen based on their ability to efficiently digest samples in a buffered solution containing Tris-HCl (pH 7 5) at 37° C Under these conditions, fibrils are digested before they are dissolved, instead of simultaneously as in HX-MS experiments. These enzymes typically produce peptides 5-25 amino acids in length that separate optimally via HPLC [53]

Because HX is not used in these experiments, speed of analysis is not a priority Therefore, digestion products can be analyzed by LC-MS following neutralization of the enzyme without a loss of information. Identification of these fragments reveals the locations of proteolytic cleavage within the monomer sequence A comparison can then be made between cleavage sites within the Aβ monomer and intact Aβ fibrils

The accessibility of proteolytic cleavage sites can be used to map residues to either the exposed surface of the fibril or near the rigid core structure of the molecule Data from these proteolysis experiments can be used to provide residue specific information, which can then be compared to existing models predicting fibril structure.

Chapter 2. Proteolysis of Aβ Monomer and Fibrils Using Pepsin

Introduction

Hydrogen exchange mass spectrometry (HX-MS) has been used to obtain global information on the total amount of deuterium exchanged in Aβ fibrils [41] This method has shown that approximately 60% of the backbone amides in the Aβ monomer that make up the fibrils are very resistant to exchange suggesting that these amides are buried within a rigid core structure However, this method does not provide any detailed information about which specific residues are protected or unprotected from exchange. One method to obtain such information is to proteolyze deuterated fibrils, inject them into the MS system, and measure the amount of deuterium exchanged in the smaller peptides.

Pepsin has been widely used to proteolyze hydrogen exchanged samples prior to injection into the MS [40] The enzyme activity of pepsin is highest between pH 2 and 3, which is the pH range used for HX-MS experiments on Aβ fibrils [53]. In HX-MS experiments a mixture of acetonitrile, water, and formic acid is used to dissolve fibrils within 10 s at room temperature before injection into MS However, it is unclear whether pepsin would work under these conditions In the experiments presented here Aβ monomer and Aβ fibrils have been used to optimize the conditions for proteolysis using pepsin. These

experiments have demonstrated the feasibility of using pepsin to digest fibrils in conjunction with HX-MS to obtain detailed structural information.

Materials and Methods

Protein Preparation and Fibril Synthesis

Aβ (1-40) monomer (Keck Biotechnology Center, Yale University, New Haven, CT) was used in all of the experiments presented here. Dry Aβ monomer was pretreated to remove any aggregates as described by Zagorski and colleagues [54] Briefly, Aβ was first dissolved in TFA at 1 μg/μl and sonicated for 15 min before TFA was evaporated off under argon. The above procedure was repeated twice. The monomer was then dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Acros, Fisher Scientific, Pittsburgh, PA) to a final concentration of 1 μg/μl and incubated at 37° C for 1 hour before being dried under argon. The monomer was again dissolved in HFIP at 1 μg/μl This processed monomer was then used to synthesize fibrils as described below and was also used to optimize proteolysis conditions for pepsin. A general overview of monomer preparation is presented in Figure 2.1

Aβ fibrils were generated from pretreated Aβ (1-40) monomer for use in proteolysis experiments. After the organic solvent was evaporated off under argon, the protein was dissolved in H_20 to 1 μg/μl. An equal volume of 0.1% sodium azide in phosphate buffered saline (PBS) (20mM phosphate, 276mM sodium chloride, 5 4mM potassium chloride) (pH 7.4) was added to the monomer

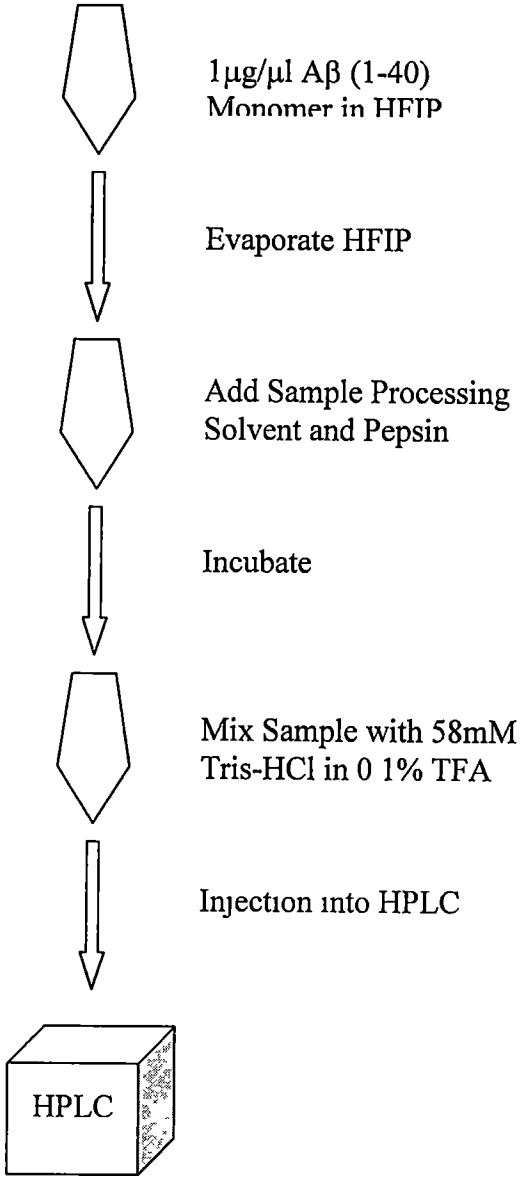

Figure 2 1 General Protocol for Monomer Sample Preparation Processed Aβ monomer was digested with pepsin for various incubation times before injection into HPLC to optimize proteolysis conditions for use with HX-MS experiments.

to a final concentration of 0.5 μg/μl, followed by ultracentrifugation for 17 hours at 20,000 rpm and 4° C to remove any remaining aggregates The monomer was then "seeded" by adding fibrils from a previous stock at 1·1000 (w/w) to initiate fibril formation Aliquots were incubated undisturbed at 37° C for 4-6 days. Fibril growth was monitored by thioflavin T (ThT) fluorescence [7] Fibril quality was also evaluated by electron microscopy An electron micrograph of fibrils is presented in Figure 2 2

Protease Digestion

For protease digestion experiments, HFIP was evaporated off of under Argon and Aβ monomer was dissolved in sample processing solvent containing 50% acetonitrile (ACN) (Fisher), 50% H_20 (Fisher), and 0.2% formic acid (HCOOH) (Sigma) to a final concentration of 0.75μg/μl. Pepsin (Sigma, St. Louis, MO) was then added to the Aβ monomer sample followed by varying times of incubation The monomer was then diluted to a concentration of 0 125 μg/μl in 58 mM Tris-HCl (Fisher) and 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL) in H_20 (pH 7.0) for injection into HPLC The concentration of pepsin, incubation time, and incubation temperature were varied in order to optimize proteolysis conditions for digestion of Aβ.

To prepare fibril samples for proteolysis, fibrils were collected by centrifugation for 15 min at 14,000 rpm and washed with filtered H_20 Fibrils were again collected by centrifugation followed by the addition of sample

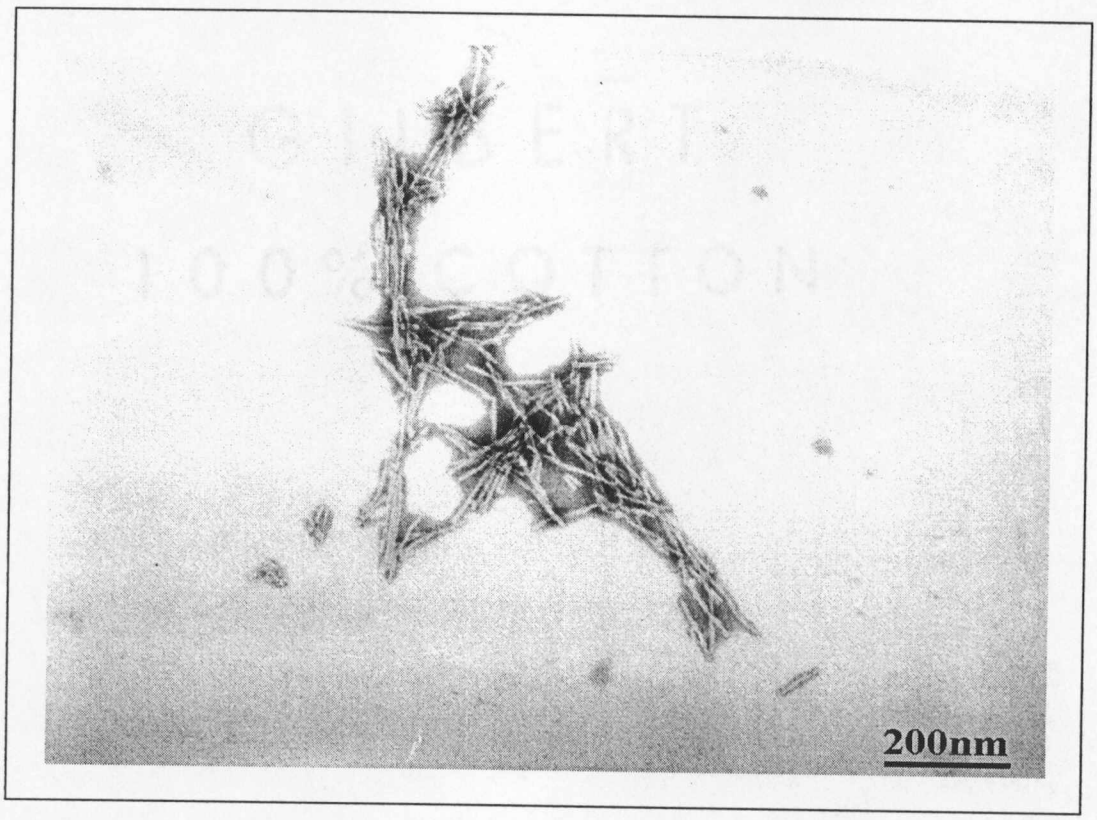

Figure 2.2 Electron Micrograph of Aβ (1-40) Fibrils. Fibrils were evaluated by electron microscopy at a magnification of 50,000 X. Data provided by the UT Electron Microscopy Center.

processing solvent and pepsin The concentration of fibrils and pepsin in these samples was 0.75 μg/μl and 0.5 μg/μl respectively. After incubation, the samples were diluted to a concentration of 0.125 μg/μl in 58 mM Tris-HCl and 0.1% TFA (pH 7.0) for injection into HPLC Fibrils were also digested under dilute conditions to more closely approximate the conditions used with MS Fibrils and pepsin were diluted 6-fold to concentrations of 0.125 μg/μl and 0 08 μg/μl respectively Fibril samples were injected into HPLC after solvent addition.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) Series 1100 Quaternary pump system (Hewlett Packard, Palo Alto, CA) was used with a 3 X 150 mm C3 column (Hewlett Packard) with Zorbax stable bond packing to fractionate peptides after proteolysis with pepsin The solvent flow rate through the system was 1 ml/min The temperature was controlled at 25° C. Solvent A was 0 05% TFA in H_20, solvent B was 0.05% TFA in ACN. Samples were injected at 1% B and peptides were fractionated with a gradient from 1% to 51% B over 25 minutes at 2% per minute In chromatograms from HPLC experiments (Figure 2.3), the area under each peak represents the concentration of the peptides that were fractionated. The relative percentage of each peptide identified by LCMS (described below), was calculated from the ratio of the peak area vs the summed areas of all peaks

Liquid Chromatography-Mass Spectrometry

A continuous flow microblotter capillary HPLC-electrospray ionization mass spectrometer (ESI-MS) (PE-SCIEX, Perkin Elmer, Foster City, CA) with a 0 3 X 150 mm microbore C4 column (LC Packings, San Francisco, CA) was used for fractionation of peptic digest products and measurement of their molecular weights for fragment identification The solvent flow rate was 5µl/min Solvent A was 0 1% TFA in H_20, solvent B was 0.085% TFA in ACN The system was equilibrated at 5% B for 30 mins followed by sample injection at 5% B over 90 mins Peptides were fractionated with a gradient from 5% to 65% B over 120 mins at 0.5% per minute. After passing through the LC section of the system, the sample solution was injected into a single quadrupole ESI-MS Experiments were performed in the positive ion mode with a capillary voltage of 4800V and an alternating mode orifice voltage varying from 21V to 126V Ring voltage was also varied from 220V to 350V The source temperature was 25° C. Spectra were analyzed using the Biomultiview program provided by the system manufacturer (PE-SCI-EX, Perkin Elmer).

Results and Discussion

In HX-MS experiments performed on Aβ fibrils, samples are injected from one arm of a mixing "T," and sample processing solvent containing 50% ACN, 50% H_20, and 0.2% HCOOH is injected from the second arm of the "T" [41]. Aβ fibrils are disaggregated into Aβ monomer when mixed with the solvent

in the "T" and are directly injected into the MS through the third arm. The entire process takes approximately 10 s Therefore, to conduct proteolysis experiments prior to MS, pepsin must be included in the sample processing solvent and should be able to digest samples under these conditions For the optimization of pepsin under these conditions, the extent of monomer and fibril digestion was monitored by HPLC in the experiments presented below. The primary digestion products obtained with HPLC were fractionated and identified using LC-MS and the identity, sequence, and masses of these peptides are presented in Table 2 1.

The proteolytic activity of pepsin was tested by digesting an Aβ monomer sample at room temperature for 1 hour. An enzyme concentration of 0.05 μg/μl was used to digest the monomer and results were compared to samples incubated at 37° C for 1 hour. As presented in Table 2.2, the digestion products obtained from samples incubated at room temperature with 0.05 μg/μl pepsin for 1 hour were similar to those obtained from samples incubated at 37° C. However, digestion did appear to be less efficient at room temperature. The amount of monomer left undigested after 1 hour at room temperature was increased about 2-fold from the amount present after 1 hour at 37° C. This 2-fold increase in monomer was reflected by a decrease from 19% to 9% in the amount of the 1-19 fragment Also at room temperature, 19% of the total sample was comprised of the 20-40 peptide, while at 37° C, this peptide was further digested to smaller fragments such as the 20-34 peptide. The two primary fragments produced (1-19 and 20-40) span the entire monomeric sequence and can be compared to give us

Table 2 1 Peptide Fragments Identified Using LC-MS

Mass	Peptide Sequence	Segment
2314.50	DAEFRHDSGYEVHHQKLVF	D1 - F19
1490.68	FAEDVGSNKGAIIGL	F20 - L34
1999.22	FRHDSGYEVHHQKLVF	F4 - F19
2033 37	FAEDVGSNKGAIIGLMVGGVV	F20 - V40

Table 2 2 A Comparison of Aβ (1-40) Monomer Digestion Using 0.05 μg/μl Pepsin and Incubating for 1 hour at 37° C and Room Temperature

Peptides Identified After Digestion	% of Sample[a] After 1 hr Incubation at 37° C	% of Sample[a] After 1 hr Incubation at Room Temperature
1-40	9	19
1-19	61	51
20-40	10	19
20-34	14	4

[a] Percent remaining after digestion with pepsin

more information on how the two ends of the molecule differ in the amount of HX for increased structural resolution. In theory, these peptides could also be further fragmented using tandem MS (MS/MS) (described in Ch 1) to obtain higher structural resolution

Enzyme concentrations were varied next to analyze the effect on Aβ monomer digestion for further optimization of proteolysis conditions. To optimize the concentration of pepsin used for proteolysis of Aβ monomer, samples were digested at room temperature for 10 min with the concentration of pepsin varying from 0 05 μg/μl to 2 5 μg/μl. A comparison of monomer digestion using varied concentrations of pepsin is presented in Table 2 3 The amount of digestion increased as the enzyme concentration was increased The two primary products observed from digestion using 0 05 μg/μl and 0 5 μg/μl pepsin were the 1-19 and 20-40 fragments that cover the sequence of the monomer and are the same as those observed for longer digestion times (Table 2 2). When the pepsin concentration was increased to 2.5 μg/μl, digestion was extensive and 53% of the sample had been digested into much smaller unidentified fragments The concentrations and sizes of these peptides would be insufficient to obtain useful information by HX-MS or to be used for further fragmentation by MS/MS. At an enzyme concentration of 0.5 μg/μl, almost all of the full-length monomer was digested, proteolysis was efficient and peptides were produced that covered the length of the monomer For these reasons an enzyme

Table 2.3 A Comparison of Aβ (1-40) Monomer Digestion Using Pepsin Concentrations of 0 05 μg/μl, 0 5 μg/μl, and 2 5 μg/μl and Incubation for 10 mins

Peptides Identified After Digestion	% of Sample[a] After Incubation for 10 mins with 0 05μg/μl Pepsin	% of Sample[a] After Incubation for 10 mins with 0 5 μg/μl Pepsin	% of Sample[a] After Incubation for 10 mins with 2 5 μg/μl Pepsin
1-40	50	12	6
1-19	27	51	16
4-19	2	3	7
20-40	13	21	0
20-34	2	3	18

[a] Percent remaining after digestion with pepsin.

concentration of 0.5 μg/μl was used in the next set of experiments to monitor pepsin digestion at lower incubation times

Using pepsin at a concentration of 0.5 μg/μl, incubation times were varied to see if similar digestion could be obtained within the 10 s time interval for MS experiments To digest the monomer at room temperature for incubation times ranging from 10 s to 1 hour 0.5 μg/μl pepsin was used. As illustrated in Table 2 4, the extent of digestion increased at extended incubation times. Nearly all of the monomer had been digested after 10 s The two major fragments at this incubation time were the 1-19 and 20-40 peptides also observed in earlier experiments. Approximately 70% of the total sample injected was comprised of these peptides These fragments were further digested as was demonstrated by an increase in the presence of smaller peptides such as 4-19 and 20-34 at an incubation time of 1 hour Interestingly, there was no appreciable difference in the amount of digestion after 10 mins compared to 10 s

Taken together, these experiments demonstrate the feasibility of using pepsin in digestion experiments with HX-MS The enzyme works in the organic solvent used with MS (sample processing solvent) and can digest the sample in 10 s at room temperature, a typical chromatogram is presented in Figure 2.3.

Proteolysis experiments were extended to Aβ fibrils next to ensure that they could be dissolved and proteolyzed with pepsin within 10 s at room temperature The final concentration of fibrils in sample processing solvent was 0 75 μg/μl and after 10 s, samples were diluted with 58 mM Tris-HCl in 0 1%

Table 2 4 A Comparison of Aβ (1-40) Monomer Digestion at Incubation Times of 1 hour, 10 mins, and 10 s Using 0 5 μg/μl Pepsin

Peptides Identified After Digestion	% of Sample[a] After Incubation for 1 hr	% of Sample[a] After Incubation for 10 mins	% of Sample[a] After Incubation for 10 s
1-40	2	12	5
1-19	27	51	58
4-19	32	6	3
20-40	0	21	17
20-34	18	3	9

[a] Percent remaining after digestion with pepsin.

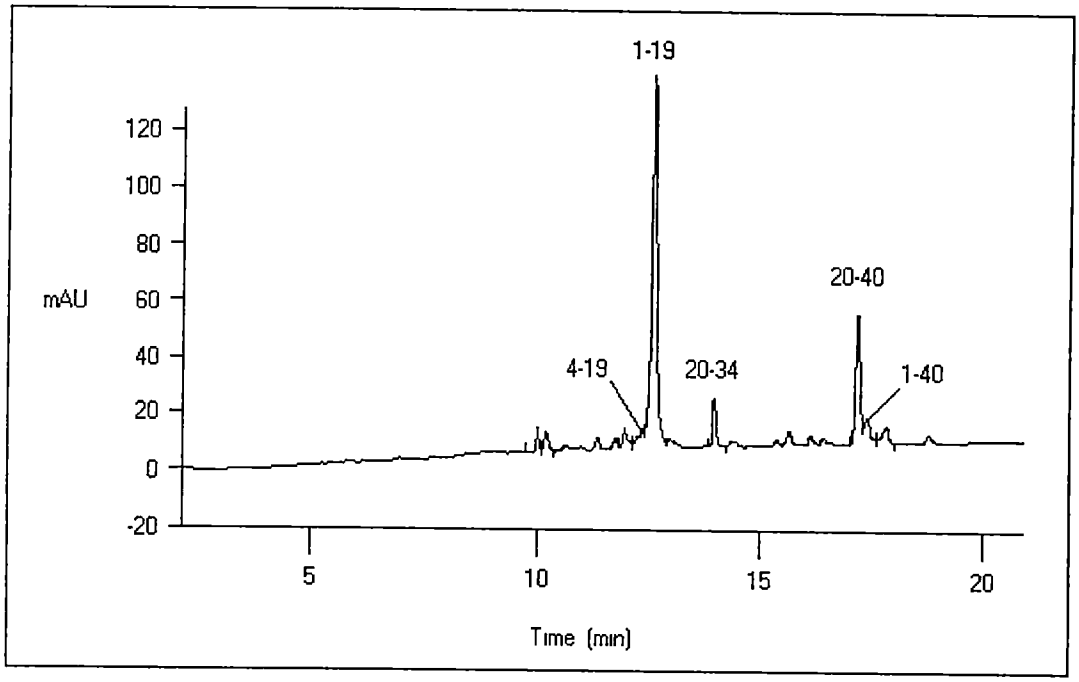

Figure 2 3 HPLC Chromatogram of Aβ (1-40) Digestion Proteolysis conditions for pepsin were optimized to digest the monomer within 10 s at room temperature using an enzyme concentration of 0.5 μg/μl

TFA in H_20 before injection into HPLC as presented in Table 2.4 This solvent was used for compatibility with the HPLC system and to increase the pH of proteolyzed samples to neutralize pepsin after 10 s so that no further digestion would take place Under these conditions, fibril digestion was similar to digestion of the monomer. However, in fibrils, the 1-19 peptide was further digested to smaller fragments including the 4-19 fragment This allows for the possibility of studying this fragment using HX-MS and tandem MS, as well as the two primary fragments observed (1-19 and 20-40) to gain increased structural information about these segments of the monomer sequence. Therefore, fibrils can be digested in 10 s at room temperature using pepsin at a concentration of 0.5 μg/μl for online experimentation with MS.

Since fibrils would be diluted in the "T" with the sample processing solvent, experiments were carried out to digest Aβ fibrils under dilute conditions to further match the conditions used for sample processing prior to MS. Fibrils and pepsin were diluted to final concentrations of 0 125 μg/μl and 0.08 μg/μl respectively, with the addition of sample processing solvent Samples were injected into HPLC after solvent addition. The solvent containing Tris-HCl was not added before injection to neutralize pepsin. As a result, the enzyme may have continued to digest the sample as it passed through the column The injection process took approximately 1.5 mins.

As illustrated in table 2.5, under these conditions an increase from 4% to 15% in residual monomer was observed compared to the previous fibril sample

Table 2 5 A Comparison of Aβ (1-40) Monomer and Fibril Digestion Using Pepsin

Peptides Identified After Digestion	% of Monomer Sample[a]	% of Fibril Sample[a]	% of Fibril Sample, Dilute Conditions[b]
1-40	5	4	15
1-19	58	40	46
4-19	3	16	10
20-40	17	16	9
20-34	9	15	12

[a] These monomer and fibril samples were digested with pepsin at a concentration of 0 5 μg/μl for 10 s

[b] Under dilute conditions fibrils and pepsin were diluted 6-fold to concentrations of 0 125 μg/μl and 0.8 μg/μl respectively.

digested at higher fibril and pepsin concentrations. A decreased amount of the 20-40 fragment was also observed at 9% compared to 16% in fibrils digested under concentrated conditions Interestingly, with further digestion of the 20-40 fragment to produce the 20-34 peptide the difference in digestion between fibrils under dilute and concentrated conditions was decreased at 12% and 15% respectively One explanation could be that fibrils were being digested during injection into HPLC The injection process took approximately 1.5 mins This would essentially correspond to an incubation period 1.5 mins that may have allowed for increased digestion of fibrils in contrast to samples digested under concentrated conditions where pepsin was neutralized after 10 s. However, no further digestion was observed between monomer digested at 10 mins from 10 s (Table 2 4) These experiments demonstrated that the concentration of pepsin does not need to be increased to compensate for a 6-fold dilution This diluted concentration of pepsin will be used in the sample processing solvent for future experiments with the ESI-MS system

Conclusion

Aβ (1-40) monomer and fibrils were digested with 0 5 μg/μl pepsin at room temperature for 10 s, the conditions required for experiments directly online with MS The digestion products were fractionated by LC-MS and evaluated based on their mass to charge ratios for determination of molecular weight as illustrated in Table 2.1. During experimentation online with MS, these peptides can be directly identified based on their mass to charge ratios. Therefore, when

HX-MS is performed, it should be possible to calculate the degree of deuterium incorporation by the peptides identified earlier with LC-MS

Chapter 3. Measuring Hydrogen Exchange of Proteolyzed Aβ Monomer and Fibrils Using Electrospray Ionization Mass Spectrometry

Introduction

Hydrogen Exchange Mass Spectrometry (HX-MS) has been successfully applied to several proteins in order to probe highly ordered, hydrogen bonded secondary structures [40, 43-45, 47, 48]. Standard techniques such as X-ray crystallography and NMR have been unsuccessful in obtaining this information because of the insoluble nature of the Aβ fibrils.

In previous HX-MS experiments, a solvent containing H_2O, ACN, and HCOOH was identified that dissolves at least 50 % of the Aβ fibrils before injection into MS [41]. MS was then used to detect differences in molecular weight between protonated and deuterated samples which were used to calculate extents of exchange. In theory, these differences between Aβ monomer and Aβ fibrils are due to the secondary structure of the fibrils in which protons involved in intramolecular hydrogen bonding (or are sterically shielded from deuterated solvent) have a much slower rate of exchange. Approximately 60 % of the backbone amide bonds in fibrils were found not to exchange even after prolonged incubation in deuterated buffer [41]. However, this data could not be used to determine which backbone amides along the primary sequence had been protected

and which were exchanged. In theory, to localize which backbone amides do not undergo HX to specific regions of the monomer sequence, samples can be proteolyzed with digestive enzymes to produce peptide fragments that can be analyzed collectively in the MS. Pepsin can digest fibrils while they are simultaneously disaggregated in sample processing solvent during the 10 s time interval required for MS experiments as demonstrated in Chapter 2.

Materials and Methods

Monomer and Fibril Sample Preparation

Aβ (1-40) monomer dissolved in HFIP at a concentration of 1 μg/μl was used in the experiments presented here. To prepare monomer samples for MS analysis, HFIP was evaporated off followed by the addition of 2.5 mM Tris-HCl (pH 7.5) or D_2O based 2.5 mM Tris buffer (pD 7.5) (D_2O supplied by Sigma, St Louis, MO) to a final monomer concentration of 0.25 μg/μl. This monomer was also used to synthesize fibrils, as described in Ch. 2. To prepare fibril samples, 200 μl of fibrils at a concentration of 0.5 μg/μl were centrifuged for 25 mins at 14,000 rpm. The supernatant was removed and the fibrils were washed with 300 μl of filtered H_2O. The sample was mixed and centrifuged for 25 mins at 14,000 rpm before the H_2O was removed. Either 2.5 mM Tris-HCl or 2 5 mM deuterated Tris buffer was then added to a final fibril concentration of 0.25 μg/μl. Deuterated solvents were added under argon to prevent the solvent deuterium from exchanging with atmospheric H_2O vapor After the addition of deuterated

buffer, samples were incubated overnight at room temperature to allow exchange to take place.

Elecrospray Ionization Mass Spectrometry

Aβ monomer and fibril samples, and sample processing solvent (50 % H_2O, 50 % ACN, and 0.2 % HCOOH) were directed through two 100 μm diameter fused silica capillaries (Polymicro, Phoenix, AZ) at flow rates of 2 μl/min and 18 μl/min respectively. Samples were mixed with solvent in a 0.25 mm diameter "T" union (Valco Instruments Co., Houston, TX). Both Aβ monomer and fibrils were injected into the "T" at a concentration of 0.25 μg/μl in 2.5 mM Tris buffer. To proteolyze these samples, pepsin was included in the sample processing solvent at a concentration of 0.08 μg/μl (as described in Ch. 2) to digest the monomer before MS analysis.

The sample/solvent mixture was injected from the "T" into a Quattro II triple quadrupole electrospray ionization mass spectrometer (ESI-MS) (Micromass, Manchester, U.K.) used here to analyze the amount of HX in Aβ monomer and fibrils. Experiments were performed in the positive ion mode with a capillary voltage of 3000 V and a cone voltage of 20 V. Sample solutions were injected using a Harvard model 22 syringe pump (South Natick, MA) while solvents were injected using a Harvard model 11 syringe pump. The source temperature was maintained at 80° C. All spectra were acquired in multi-channel accumulation mode with a mass range of 700 to 1200 Da/sec.

Data Reduction

The masses of peptides analyzed with ESI-MS are derived from fragment data based on mass to charge ratios (M/Z). To calculate mass, therefore, the data from the mass spectra must be adjusted for the charge state (or number of excess protons) of the analyte. The equation Mp=(M/Z)(C)-C, where Mp is the mass of the peptide in question, M/Z is the mass to charge ratio of the analyte derived from MS analysis, and C is the charge state, was used to derive mass.

When deuterated sample is mixed with sample processing solvent in the "T," approximately 18 % of the mixture is D_2O [41]. As a result, 18 % of the labile side chain hydrogens are expected to remain deuterated at equilibrium. Therefore, to correct for the difference in weight contributed by the remaining deuterated side chains, the number of deuteriums expected to remain on 18 % of the available side chains was subtracted from the total number of deuteriums incorporated into the sample.

In experiments with the monomer, the percentage of backbone amide back exchange of identified fragments was then calculated by the ratio of exchanged amides to the total number of amides available This estimate of back exchange extent was then used to correct values for the fibril derived fragments by a standard method [55] The following equation is routinely used to correct for backbone amide back exchange, $D=[(m-m_0)/(m_{100}-m_0)]xN$ where D is the corrected deuterium content in the backbone amides, m is the observed mass of a

partially deuterated analyte, m_0 is the mass obtained for non deuterated peptides, m_{100} is the observed mass of fully deuterated peptides, and N is the number of backbone amides in the peptide. The m_{100} value has been previously described using Aβ (1-40) monomer [41].

Results and Discussion

In the experiments presented here, ESI-MS was used to determine the amount of HX in fragments of Aβ monomer and fibrils after digestion with pepsin. Protonated and deuterated monomer samples were compared, and the ability of pepsin to proteolyze these samples directly online with the MS system was evaluated. A general illustration of the "T" and MS components is presented in Figure 3.1 Initial MS experiments were performed using Aβ monomer. The measured mass of the Aβ (1-40) monomer was 4329.15 Da which compared well with the calculated mass of 4329.86 Da and is in agreement with results obtained from previous MS experiments with monomer [41]. A spectrum representative of the Aβ (1-40) monomer is presented in Figure 3.2 (A).

Deuterated monomer was injected into the MS system next at an identical concentration for comparison to non deuterated monomer. The Aβ monomer was incubated overnight in D_2O based Tris buffer to allow the forward exchange of amide and labile side chain hydrogens to occur. The amides in Aβ monomer are fully deuterated after this overnight incubation period [41]. When deuterated samples were injected into the "T," they were mixed with sample processing

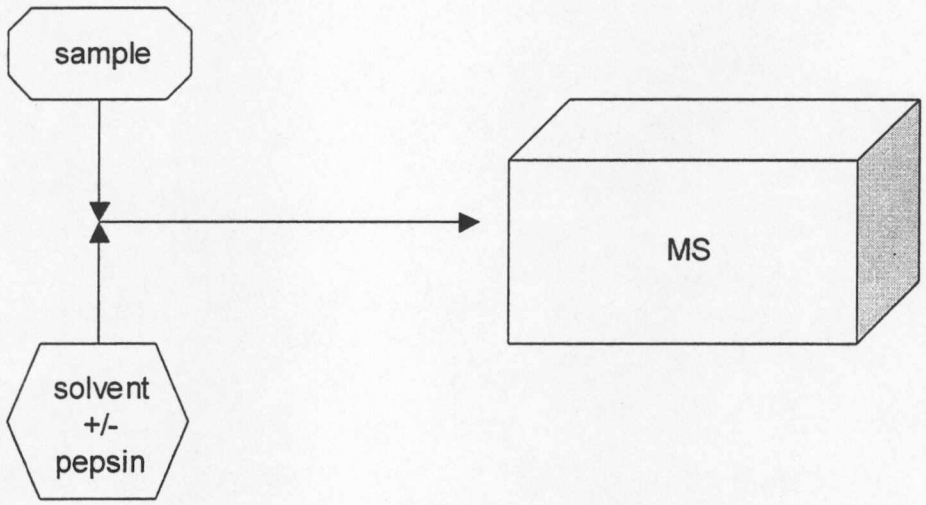

Figure 3.1 Sample and Solvent Injection into MS. Sample solutions are mixed with sample processing solvent in the "T" before injection into the ESI-MS system. For proteolysis, pepsin was included in the sample processing solvent to digest samples in the "T" before injection into MS.

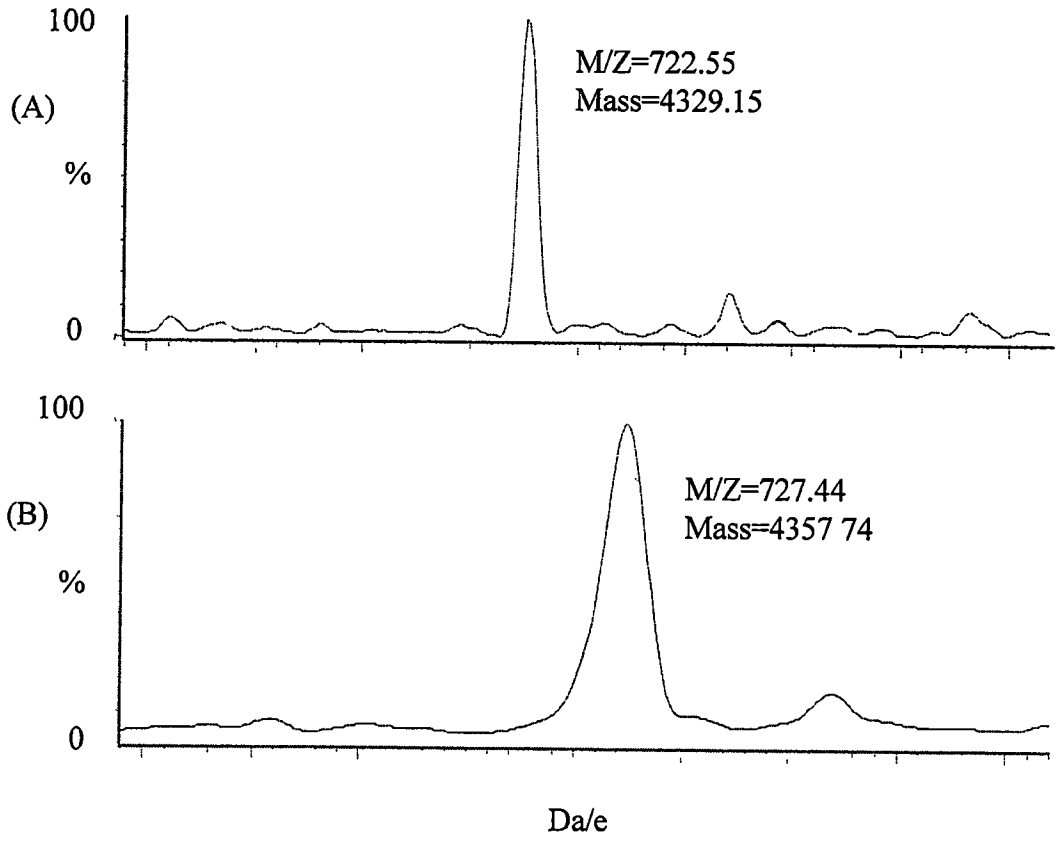

Figure 3 2 HX-MS of Aβ Monomer A signal of Aβ (1-40) monomer is presented in (A). The deuterated monomer presented in (B) demonstrates a mass increase by the peak shift to the right of the spectrum.

solvent. This mixture has a final D_2O concentration of approximately 18 %. Therefore 18 % of the labile side chain hydrogens will remain deuterated As described in Materials and Methods, back exchange was corrected for deuterated side chains. Some of the amide protons also back exchanged in this solvent. The percentage of amide back exchange was calculated as described in Materials and Methods. Deuterated Aβ (1-40) monomer had an observed mass of 4357.74 Da. The spectrum in Figure 3.2 (B) illustrates a peak shift to the right, representative of the 29 Da increase in mass from the non deuterated sample (Fig. 3.2 (A))

ESI-MS was also used to analyze fragments of protonated Aβ monomer after digestion with pepsin. Pepsin was included in the sample processing solvent at a concentration of 0.08 μg/μl to digest monomer samples as they were mixed with solvent in the "T" before injection into MS. Proteolysis was complete so that no residual monomer remained. Five peptide fragments were obtained from proteolysis of the monomer: 3 of these fragments were identified using LC-MS (Ch. 2), these were the 1-19, 20-34, and 20-40 fragments. Two new peptides with measured masses of 1680 Da and 561 Da were identified as 17-32 and 35-40 fragments respectively. These peptides were identified by comparing their observed masses to the calculated masses of the possible peptide sequences. The calculated masses of these peptides were within 1 mass unit of their observed masses. The spectrum presented in Figure 3.3 (A) illustrates the signals obtained from the proteolysis of Aβ monomer.

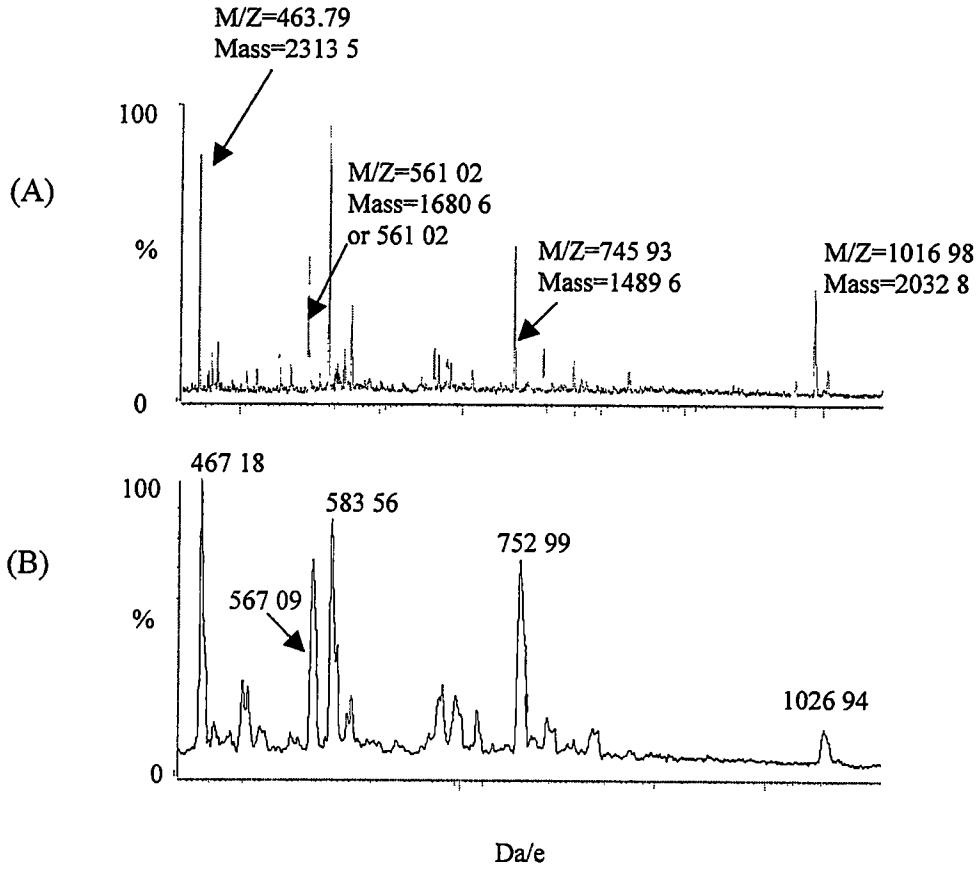

Figure 3.3 Proteolyzed Aβ Monomer Signals from protonated monomer digestion are presented in (A) The M/Z signal at 561.02 can be representative of either the 35-40 fragment at a charge state of +1 (Mass=561.02) , or 17-32 at a charge state of +3 (Mass=1680.6). Corresponding signals from deuterated monomer digestion are presented in (B) Both protonated and deuterated monomer were digested with pepsin before injection into MS.

Deuterated monomer was mixed with sample processing solvent containing pepsin at a concentration of 0.08 μg/μl and injected into the MS system. The corresponding deuterated signals were matched to those obtained from protonated Aβ monomer A shift in the location of the peaks to the right of the spectrum demonstrates the incorporation of deuterium into the fragments as presented in Figure 3 3 (B). As explained in the Introduction, analysis of these fragments was used to increase the resolution of the back exchange information obtained for the entire monomer. HX-MS results obtained by comparing digested Aβ monomer fragments to corresponding deuterated fragments are presented in Table 3.1.

The number of backbone amides exchanged was identified by the difference in molecular weight between deuterated and non deuterated samples, and was corrected for side chain deuterium incorporation. The extent of back exchange was then calculated for each fragment, and as illustrated in Table 3.1, we found that, significantly, the extent of backbone amide hydrogen back exchange in the Aβ (1-40) monomer differs between the two ends of the molecule. The hydrophilic amino terminal half has a much greater rate of back exchange than the hydrophobic carboxyl terminal half. Back exchange in the 1-19 fragment had occurred in 23.3 % of the amides before injection into MS, whereas only 7 2 % of the amides in the 20-40 fragment were back exchanged under the same conditions. Smaller fragments from the 20-40 peptide were analyzed to obtain more precise information on the amides that do exchange. For

Table 3.1 HX-MS of Aβ (1-40) Monomer. Both protonated and deuterated Aβ monomer were digested and peptide fragments were compared for increased resolution of HX

Fragments Identified After Digestion with Pepsin	Observed Molecular Weight, Protonated Aβ	Mass Increase Between Deuterated and Protonated Aβ	Corrected Mass for Side Chain Deuterium Incorporation	Percent of Backbone Amides Back Exchanged
1-19	2313 5	17.4	13.8	23.3
20-40	2032.8	20.26	18 46	7.7
17-32	1680.0	18.6	16.8	0
20-34	1489.6	14.56	12.76	8.9
35-40	561 02	6.2	5.66	0

example, the 17-32 and 35-40 peptides appear not to undergo back exchange, while the 20-34 peptide did back exchange by 8.9 %. The amount of back exchange in the 20-40 and 20-34 peptides of 7.2% and 8.9 % respectively, indicates that 1-2 amides within the 20-40 sequence have back exchanged and are located within the 20-34 segment. When the 20-40 and 20-34 peptides are compared with the 17-32 and 35-40 peptides, the only possibilities for back exchange are the backbone amides of residues 33 and 34 (glycine and leucine).

There are 5 glycine residues in the 20-40 fragment and 1 glycine in the 1-19 fragment and 2 leucine residues located at positions 17 and 34 in the 1-40 monomer sequence. Because of the predominance of glycine in the C-terminal portion of the molecule, it seems less likely for the glycine at position 33 to have undergone back exchange while the other glycine residues remained deuterated. No back exchange was observed in the 17-32 fragment. Therefore leucine at position 17 did not back exchange. The implicated back exchanged backbone amides at positions 33 and 34 may be explained by the effect of the surrounding hydrophobic amino acids. There were no smaller fragments of the 1-19 peptide observed to obtain more specific information on this segment. These results demonstrate that the primary sequence of the Aβ peptide plays a role in the rates of exchange of individual backbone amide hydrogens within the Aβ (1-40) monomer. This will have to be taken into account in the analysis of exchange data from fibril digestion experiments. In most cases [55], global HX information derived from the intact protein is used to find an exchange rate that is averaged

over all peptide backbone amide bonds. These averaged values were used in previous HX experiments to correct the deuterium content in fibrils [41].

Next, fibrils generated from Aβ (1-40) monomer, as described in chapter 2, were used in MS experiments for analysis of HX. Fibrils were compared to monomer to observe differences in proteolysis and deuterium incorporation Protonated fibrils were first injected into the MS system at a concentration of 0.25 μg/μl. Fibrils were mixed with the sample processing solvent in the "T" where they were dissolved into monomeric Aβ before analysis with MS. The measured mass, therefore, was equal to that of the monomer at 4329 15 Da. A representative spectrum is presented in Figure 3.4 (A).

Deuterated samples were analyzed next with MS to determine the amount of HX in non proteolyzed fibrils Fibrils at a concentration of 0.25 μg/μl were incubated overnight at room temperature in deuterated Tris buffer for forward exchange to take place. Deuterated fibrils analyzed with ESI-MS had an observed mass of 4344.87 Da. This result compares favorably with the observed mass of 4345.7 over protonated samples in previous experiments [41]. Deuterated fibril samples demonstrated a mass increase of 16.55 Da compared with a 28 59 Da increase between deuterated and protonated monomer. A spectrum representative of deuterated fibrils is presented in Figure 3.4 (B).

Pepsin was used in the following experiments to proteolyze fibrils in the "T" for analysis of individual fragments. Protonated fibrils were injected into the "T" and mixed with sample processing solvent containing pepsin at a

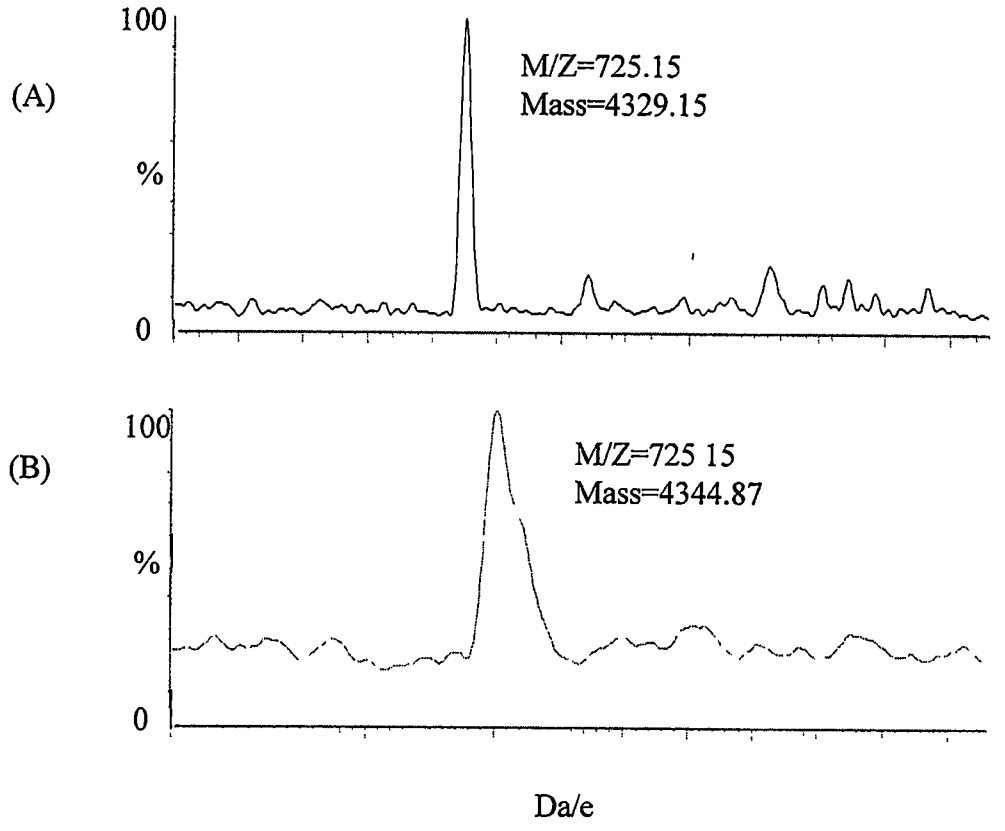

Figure 3.4 HX-MS of Aβ Fibrils A M/Z signal spectrum of Aβ (1-40) fibrils is presented in (A). Deuterated monomer in (B) demonstrates the incorporation of deuterium leading to an increase in mass and a shift in the peaks position to the right.

concentration of 0.08 μg/μl. In this solvent mixture, fibrils are simultaneously dissolved and digested in the "T" prior to injection into the MS system. Upon analysis of these fibrils, differences compared to proteolyzed monomer were apparent Spectra comparing monomer and fibril digestion for protonated Aβ are presented in Figure 3.5 (A) and (B) respectively.

We found that although the same M/Z peaks appear in the proteolysis of both monomer and fibrils, the ratios of these peaks are different. For example, the signal of the mass to charge (M/Z) peak of 463.79, identified as the 1-19 fragment, is approximately 15 % greater in monomer. Likewise, the signal at 561.2, identified as either the 17-32 or the 35-40 fragment was 50 % greater in monomer. The signal at 745.88, identified as the 20-34 peptide, was approximately 60 % greater in monomer In fact, the signal at 745 is so low in fibril digestion samples that it almost completely blends with the background noise. Also of note, the signal at 799.16, which was not identified, was 50 % greater in fibrils, whereas the signal in monomer is blended into the background noise Other signal peaks not identified either by LC-MS or by sequence analyses also show differences in digestion ratios. These differences suggest preferential digestion occurs at sites in fibrils, presumably because of the secondary structural differences from the monomer.

Next, deuterated fibrils were mixed with H_2O based sample processing solvent containing pepsin to determine if the M/Z peaks observed with protonated fibril digestion could be matched with deuterated counterparts to calculate HX

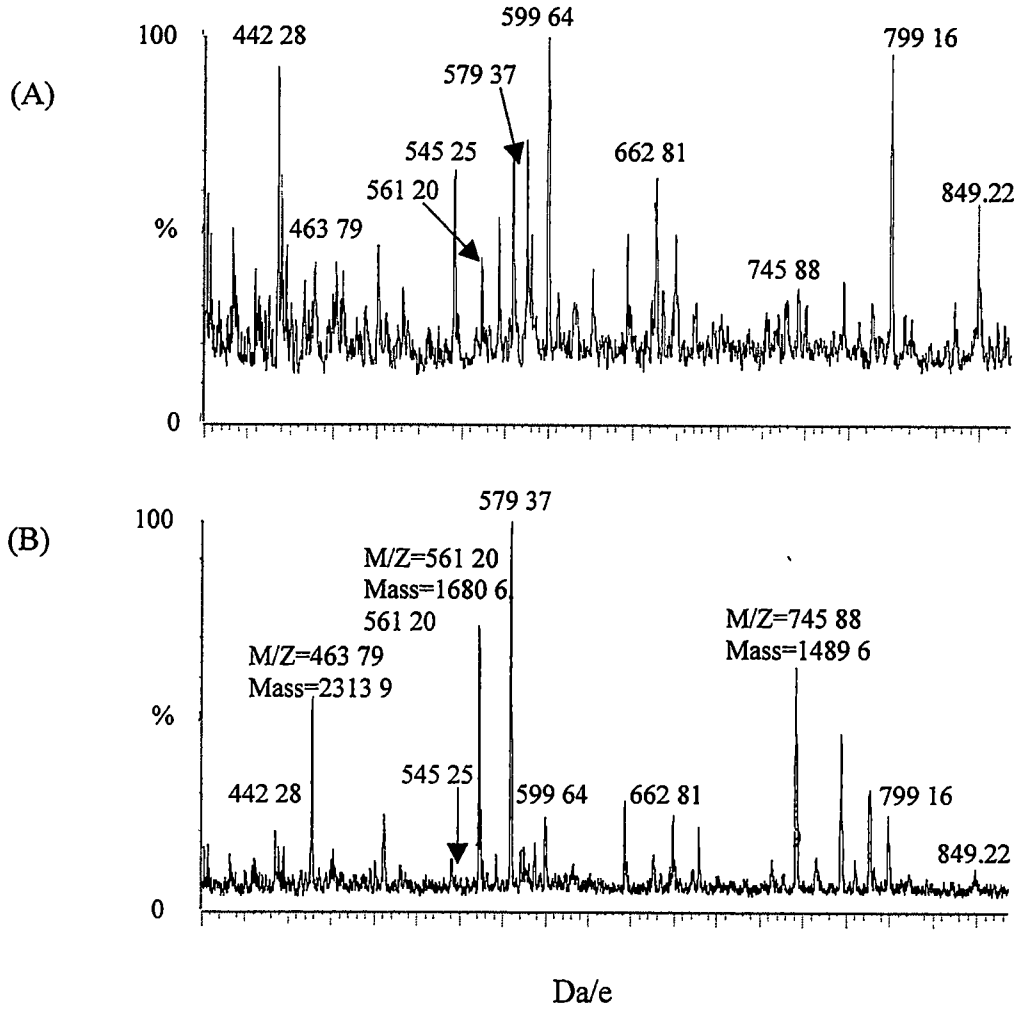

Figure 3.5 Fibril Digestion vs. Monomer Digestion Protonated fibril digestion presented in spectrum (A) occurred at different ratios than protonated monomer digested with pepsin (B). These differences in proteolysis demonstrate preferential digestion by pepsin

Deuterated fibrils, at a concentration of 0.25 μg/μl were mixed with sample processing solvent containing pepsin to produce deuterated fragments that could be used in a comparison with protonated fibril digestion. It may be possible to use this information to localize HX to specific sections of the 1-40 sequence of monomer incorporated into fibrils. However, with deuterated fibril digestion, the signal obtained was very low compared to the background noise. As was observed with protonated and deuterated fibril spectra (Figure 3.4 (A) and (B) respectively) without proteolysis, the background noise for deuterated samples was approximately 15 % higher while the signal strength remained the same. The increased level of background noise combined with much lower signal strength makes it technically difficult to identify and analyze masses of the expected fragments.

The identity of the fragments obtained by proteolysis must be known so that the number of backbone amide bonds and side chain hydrogens can be used to calculate the extent of HX in these fragments. Of the peaks that were observed in deuterated fibril digests, no signal could be positively matched with a counterpart in protonated fibril digest samples. Overall, little information could be derived from these fibril digests Spectra representative of protonated and deuterated fibril digestion are presented in Figure 3.6 (A) and (B) respectively.

The deuterated fibril sample also appears to be more completely digested than protonated fibrils. Signal peaks were observed at 1030 55 and 1001.75,

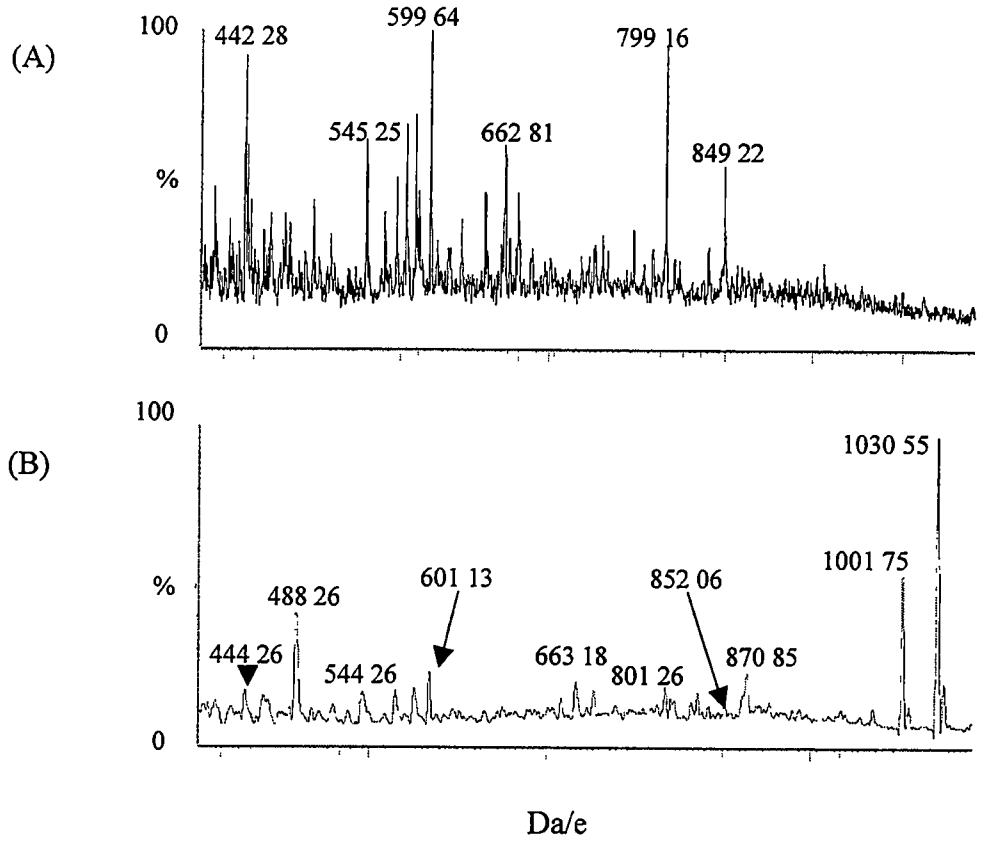

Figure 3 6 Proteolyzed Fibrils. Signals from protonated fibril digestion are presented in (A) Signals from Deuterated fibril digestion are presented in (B). The signals of deuterated fibril fragments are much lower than in the protonated sample.

which were not present in earlier experiments and do not represent residual monomer. The origin of these peaks is not known.

Conclusion

Due to the technical difficulties in the HX-MS analysis of Aβ (1-40) fibrils, high resolution structural data was not obtained. One reason for the low signal to noise may be an increase in the digestion of the deuterated fibrils. One possible explanation is that increased digestion could be due to isotope effects on Aβ binding to pepsin and/or Aβ cleavage by pepsin. A practical approach to future HX-MS experiments with fibrils may be to lower the concentration of pepsin used in the experiments so that larger fragments of the monomer sequence could be obtained and more easily identified.

These possible observed differences in digestion sensitivities of protonated vs. deuterated fibrils might also be explained by differences with the MS system. For the previous experiments with fibrils, the conditions of the MS system were different because of a damaged pump. One effect of this pump failure was that the inlet pressure of the system was much higher than in previous experiments with monomer which may have affected fibril data.

Despite the inability of these experiments to pinpoint areas of protection within fibril bound Aβ, valuable data was obtained from analysis of HX in the monomer. Surprisingly, there were differential backbone exchange rates between the N terminal and C terminal halves of the peptide Although back exchange

rates are usually averaged over the whole molecule [55], we see, significantly, that these differences in exchange rates within the molecule must be taken into account for the accurate correction of HX data in protein fragmentation HX-MS.

Chapter 4. Comparative Proteolysis of Aβ Monomer and Aβ Fibrils at Physiologic pH

Introduction

Besides hydrogen exchange, another way to obtain secondary structure information is to use proteolytic enzymes to explore the susceptibility of specific portions of amino acid sequence to cleavage [50]. The proteolysis experiments presented here are conducted at physiologic pH, since we are interested in the fibril structure under native conditions. Since HX is not a factor in these experiments, sample processing times are not a priority and digestion products can be evaluated using LC-MS.

In the following experiments, proteolytic enzymes are used to evaluate the secondary structural characteristics of Aβ (1-40) fibrils This method is based on the hypothesis that differences between monomer and fibril digestion are due to the highly ordered secondary structure of the fibrils. By comparing digestion products from monomer and fibril samples, specific information regarding which residues are inaccessible to the enzyme may be obtained.

Materials and Methods

Aβ (1-40) monomer was used in all of the experiments presented here The monomer was pretreated, stored at 1 μg/μl in HFIP, and used to

generate Aβ fibrils as described in Ch. 2. The enzymes used in proteolysis experiments included trypsin (Promega, Madison, WI), and chymotrypsin (Sigma).

To prepare monomer samples for proteolysis using these enzymes, HFIP was evaporated off under argon The monomer was then dissolved in a buffered solution containing 50 mM Tris-HCl (pH 7.5) to a concentration of 0.75 μg/μl. To prepare fibrils for proteolysis, fibrils were collected in microcentrifuge tubes by centrifugation for 15 mins at 14,000 rpm and washed with filtered H_20 Fibrils were again collected by centrifugation followed by the addition of buffered solution to a concentration of 0.75 μg/μl. The proteolytic enzyme was immediately added to this mixture of sample and buffer.

Protease digestion of monomer and fibril samples was initiated by the addition of either trypsin or chymotrypsin at a concentration of 0.1 μg/μl The samples were gently mixed for approximately 10 s after enzyme addition followed by incubation for 1 min, 15 mins, or 30 mins at room temperature, or 60 mins at 37° C. Enzyme activity was then neutralized by the addition of 0.1% TFA (pH 2.0) for injection into HPLC. The primary digestion products were identified using LC-MS. The relative percentage of each peptide identified by LC-MS was calculated from the ratio of the peak area vs. the summed areas of all peaks as in Ch. 2. The HPLC and LC-MS systems are described in Ch. 2, Materials and Methods.

Results and Discussion

A buffered solution containing 50 mM Tris-HCl (pH 7.5) was used in the digestion experiments presented here. In this solution, fibrils are not dissolved during digestion (as in HX-MS) because of the higher pH. However, fibrils must be dissolved before injection into HPLC. The addition of 0.1% TFA at pH 2.0, serves to both neutralize the enzyme and dissolve the fibrils prior to analysis

The extent of monomer and fibril digestion can be monitored by HPLC. The primary digestion products obtained with HPLC were fractionated and identified using LC-MS. The identified fragments, their sequences, and masses are presented in Table 4.1 (A) for trypsin digests and (B) for chymotrypsin digests.

A trypsin concentration of 0.1μg/μl was used to digest the monomer for incubation times of 1 min, 15 mins, and 30 mins at room temperature, as well as 60 mins at 37° C. As presented in Table 4.2 (A) the extent of Aβ monomer digestion increased as the incubation period was lengthened. In the first minute of digestion, at least 68 % of the monomer had been cleaved into smaller fragments This decrease in monomer was reflected by increases in the relative amounts of other identified fragments such as 29-40, 6-16, and 1-5. However after 1 min, the relative amount of the 17-28 fragment decreased from 8 % to 1.1 %, nearly 8 fold.

Aβ fibrils were proteolyzed with trypsin next to compare digestion products and digestion efficiency to the monomer. Aβ (1-40) fibrils were digested with trypsin under identical conditions to the monomer. Fibril digestion

Table 4.1 Fragment Identity From Proteolysis. The primary fragments obtained from the proteolytic digestion of Aβ monomer and fibrils were identified using LC-MS. The identity of these fragments, sequence, and observed masses are illustrated for trypsin digestion (A) and chymotrypsin digestion (B).

A

Fragment Identity	**Sequence**	**Observed Mass**
6-40	HDSGYEVHHQKLVFFA EDVGSNKGAIIGLMVGG VV	3711.21
29-40	GAIIGLMVGGVV	1084.97
17-28	LVFFAEDVGSNK	1325.04
6-16	HDSGYEVHHQK	1336.38
1-5	DAEFR	636.66

B

Fragment Identity	**Sequence**	**Observed Mass**
20-35	FAEDVGSNKGAIIGLM	1621.66
21-35	AEDVGSNKGAIIGLM	1490.02
20-34	FAEDVGSNKGAIIGL	1491.02
21-34	AEDVGSNKGAIIGL	1343.29
35-40	MVGGVV	560.26

Table 4.2 Comparative Digestion with Trypsin. Aβ monomer and fibrils were digested for varying incubation times. The percentage[a] of each identified fragment from digestion of Aβ monomer (A) and fibrils (B) for each of these incubation times are presented below.

A

	1-40, 6-40	**29-40**	**17-28**	**6-16**	**1-5**
1 min	32	22	8	23.7	11.5
15 mins	30.7	25	1.1	26.8	13.5
30 mins	30	26	1.4	26	12.7
[b]60 mins	18.8	29	1.1	30	12.7

B

	1-40, 6-40	**29-40**	**17-28**	**6-16**	**1-5**
1 min	92	0	0	0	4
15 mins	83	1 7	0	3.6	7.5
30 mins	84	1 8	0	3.4	7 7
[b]60 mins	80	3.2	0	5 3	8.9

[a] Peak area of fragment in relative to total peak area for all fragments

[b]Incubations for 60 mins were at 37° C

with trypsin was much less extensive than was observed with the monomer as illustrated in Table 4.2 (B), an indication of fibril's resistance to proteolysis. After 1 min, only 8 percent of the full length monomer had been cleaved. The 17-28 fragment was not observed in fibril digests at any time point, but this could be due to the detection limits of the HPLC The 29-40 fragment was not present after 1 min, but made up 1.8 % of the sample after 30 mins and 3.2 % after 60 mins at 37° C. The amount of this fragment after monomer digestion for 60 mins at 37° C is approximately 10 fold greater than occurred during fibril digestion. These results suggest a physical barrier for the enzyme at the cut site located between residues 28 and 29. Other fragments obtained from fibril digestion were analyzed for similar results.

The 1-5 fragment made up 4 % of the sample after 1 min and increased to 9 % after 60 mins at 37° C. The 1-5 sequence contains 4 peptide bonds and should theoretically make up approximately 10 % of the peptide bond absorbance in HPLC. Significantly, after 1 min the theoretical maximum possible area contributed by this peptide exceeds 100 %. Therefore, the 5-6 peptide bond is exposed in the fibril structure. The fragment identified as 6-16 is not present after 1 min of fibril digestion with trypsin. However, this fragment was observed at 3 5 % after 30 mins. The relative amount of the 6-16 fragment was 7.5 fold higher in monomer digestion than fibril digestion at both 15 and 30 min time points. This difference in the extent of digestion suggests a structural barrier in fibrils which the enzyme must overcome in order to produce the 6-16 fragment. Considering

the exposure of the 5-6 peptide bond and the absence of the 17-28 fragment in fibril digestion, these results suggest that this barrier to digestion involves the C terminal end of this fragment between residues 16 and 17.

Chymotrypsin was used at a concentration of 0.1 μg/μl to digest the monomer for various incubation times at room temperature and 37° C as discussed in Materials and Methods. As presented in Table 4 3 (A), the extent of Aβ monomer digestion increased as the incubation time was lengthened. Many smaller fragments that were not identified were present and together made up approximately 30 % of the total sample. In the first minute of digestion, 77 % of the monomer is cleaved. The relative amount of full length Aβ (1-40) decreased to 10 % after 30 mins and to 3 5 % after 60 mins at 37° C. The next fragment, identified as 11-40, made up 29 % of the sample after 1 min, slightly more than the full length monomer. The relative amount of the other fragments, which were identified: the 20-34, 21-34, 21-35, and 35-40 peptides followed the same trend and continued to increase with digestion time.

Aβ (1-40) fibrils were digested next under identical conditions to the monomer. As presented in Table 4.3 (B), the extent of Aβ fibril digestion was much less extensive than was observed with the monomer After 1 min, 95 % of the Aβ (1-40) in fibrils remained undigested Surprisingly, it appears that digestion of Aβ (1-40) in fibrils by chymotrypsin is biphasic, since cleavage is more than 50 % complete after 15 mins but not completely digested after 60 mins. The relative amount of residual monomer further decreased to 39 2 % after 30

Table 4.3 Comparative Digestion with Chymotrypsin. Aβ monomer and fibrils were digested for varying incubation times. The percentage[a] of each identified fragment from digestion of Aβ monomer (A) and fibrils (B) for each of these incubation times are presented below

A

	1-40	**11-40**	**20-35**	**21-35**	**20-34**	**21-34**	**35-40**
1 min	23	29.3	1.8	0	3.9	4.9	0
15 mins	13 1	19 5	6.7	4.8	7	10	5.3
30 mins	10	14 6	7.9	7 6	8.8	11.7	7.9
[b]60 mins	3 5	20.4	6 4	9.6	10.2	12	11

B

	1-40	**11-40**	**20-35**	**21-35**	**20-34**	**21-34**	**35-40**
1 min	95	0	0	0	0	2.2	0
15 mins	42	37	0	0	1.2	8	2
30 mins	39 2	37	2 2	1.9	1.9	8.9	3.3
[b]60 mins	38	19	4.4	5 8	5.1	13 3	7.4

[a] Peak area of fragment relative to total peak area for all fragments

[b] Incubations for 60 mins were at 37° C

mins and 38 % after 60 mins at 37° C. One explanation for this could be the presence of two classes of Aβ in fibrils. One class could be susceptible to cleavage while the other class forms a highly protected structure. Another possibility could be the presence of two types of aggregate in the sample. One of these aggregates may be more susceptible to cleavage than the other. However, the loss of enzyme activity over 60 mins cannot be discounted as another possibility. The only fragment observed after 1 min was 21-34, which made up 2.2 % of the total sample. The relative amount of the 11-40 fragment made up 37 % of the total sample at the 15 min time point, after which it held constant for 15 mins before decaying to 19 % at the 60 min time point. Interestingly, after 15 mins the amount of the 11-40 fragment was approximately 2 fold higher in fibril digest samples than observed in monomer digestion. This difference increased to 2.5 fold after 30 mins. These results seem to indicate the 11-40 sequence is offered some degree of protection between 15 and 30 mins of digestion with chymotrypsin This suggests the 11-40 fragment remains part of the fibril structure after cleavage between residues 10 and 11 while the 1-10 fragment is released into solution Since the relative amounts of fragments composing the "20-35 family" begin to increase between 15 and 30 mins, it is probable that these fragments are derived from the 11-40 fragment. In fact, probably none of these smaller fragments are derived from cleavage of full length Aβ bound to fibrils.

Analysis of the 35-40 fragment illustrated a 2.5 fold increase in monomer digestion samples compared to fibril digestion after 30 mins. In fact, this increase

in the 35-40 fragment appears to be coordinated with the increase in the fragments of the "20-35 family."

Fibrils in solution have been shown to dissociate into monomer to reach an equilibrium of 95 fibrils : 5 monomeric Aβ. However, the rate of the reaction for fibril dissociation to reach this equilibrium is not known. It is possible that fragments representing very small percentages of total sample, or are only 5-10 % of their theoretical possible area, could be explained by cleavage of the monomer from this fibril dissociation. For this reason, there is a certain amount of error introduced into the data collected from fibril digestion; therefore, data collected in the first few minutes of the digestion reaction may be the most important. As explained in Materials and Methods, the proteolytic enzyme was added immediately to the fibril sample to avoid the presence of monomer in equilibrium with fibrils before digestion began.

Many of the differences in relative amounts of peptide observed between monomer and fibril digestion samples after 30 mins, were less significant after the incubation period was extended to 60 mins at 37° C. It is possible that after prolonged incubation, the fibril structure begins to break down due to increasing proteolysis making the specific cleavage sites for the enzyme more accessible.

Conclusion

It is apparent that the high order structure of Aβ fibrils prevents the extensive proteolysis as seen with the monomer. Explanations for decreased

proteolysis at specific sites within the monomer sequence incorporated into fibrils may include intramolecular hydrogen bonding, positioning of specific amino acids within or near the fibril core, or inaccessibility of the enzyme due to conformational changes [50]. Although high resolution structural information cannot be obtained by this method, the susceptibility of specific amino acids to proteolytic cleavage can be mapped.

There are several important conclusions about the Aβ fibril structure that can be made from these experiments. For example, it appears that, significantly, the peptide bond between residues 5 and 6 is exposed in the fibril structure since this fragment appears in the first minute of digestion where it makes up more than 100 % of the calculated theoretically possible area. It also appears that physical barriers to enzyme digestion exist at cleavage sites between residues 16 and 17 and also between residues 28 and 29. Also of importance, we see evidence that suggests cleavage between residues 10 and 11 leaves the 11-40 fragment bound as part of the fibril structure and somewhat protected from further digestion. Surprisingly, digestion of Aβ (1-40) in fibrils appears to be biphasic where, in chymotryptic digests, approximately 60 % of the 1-40 Aβ was digested in the first 15 mins with the relative amount decreasing only 4 % over the next 45 mins.

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