Published in final edited form as:

Biotechnol Bioeng. 2009 September 1; 104(1): 181–192. doi:10.1002/bit.22367.

Kinetic Study of β -Amyloid residue accessibility using Reductive Alkylation and Mass Spectrometry

Irina Ramos¹, Dan Fabris², Wei Qi³, Erik J. Fernandez³, and Theresa A. Good¹

¹ Department of Chemical and Biochemical Engineering University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore MD, 21250

²Department of Chemistry and Biochemistry University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore MD, 21250

³ Department of Chemical Engineering, University of Virginia, 102 Engineers Way, Charlottesville, VA 22904

Abstract

Beta-amyloid peptide ($A\beta$) is the major protein constituent found in senile plaques in Alzheimer's disease (AD). It is believed that $A\beta$ plays a role in neurodegeneration associated with AD and that its toxicity is related to its structure or aggregation state. In this study, an approach based on chemical modification of primary amines and mass spectrometric (MS) detection was used to identify residues on $A\beta$ peptide that were exposed or buried upon changes in peptide structure associated with aggregation. Results indicate that the N *terminus* was the most accessible primary amine in the fibril, followed by lysine 28, then lysine 16. A kinetic analysis of the data was then performed to quantify differences in accessibility between these modification sites. We estimated apparent equilibrium unfolding constants for each modified site of the peptide, and determined that the unfolding constant for the N *terminus* was approximately 100 times greater than that for K28, which was about 6 times greater than that for K16.

Understanding $A\beta$ peptide structure at the residue level is a first step in designing novel therapies for prevention of $A\beta$ structural transitions and/or cell interactions associated with neurotoxicity in Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the aging population, affecting more than 4.5 million Americans (Alzheimer's Association). Beta-amyloid peptide ($A\beta$) is the major protein constituent found in senile plaques in AD patients' brain (Selkoe 1994). The amyloid hypothesis of AD states that the accumulation and deposition of fibrillar $A\beta$ is the primary driver of neurodegeneration and cognitive decline leading to dementia (Glenner and Wong 1984). It is widely believed that $A\beta$ toxicity is related to its structure or aggregation state, with accumulating evidence suggesting that the most neurotoxic species are soluble, nonfibrillar oligomers that are intermediates in the fibril formation process (Dahlgren et al. 2002; Hoshi et al. 2003; Lee et al. 2007). The exact structures of the toxic forms of the peptide and the mechanism by which they interact with cells to cause toxicity have not been elucidated. Alternative tools for elucidating the molecular structure of $A\beta$ in both toxic and non-toxic forms may be the key to unraveling the molecular basis of $A\beta$ toxicity, and to its prevention.

While there has been great interest in elucidating the structure of toxic $A\beta$ species, amyloid fibrils are non-crystalline and insoluble; therefore are not amenable to classic tools of structural biology, such as xray crystallography and solution NMR (Thompson 2003).

Significant progress has been achieved in determining the structure of A β fibrils (Iwata et al. 2001; Olofsson et al. 2006; Paravastu et al. 2006; Petkova et al. 2005; Wang et al. 2003; Williamson et al. 2006) and some A β intermediate structures (Chimon and Ishii 2005; Harper et al. 1999; Kheterpal et al. 2006; Kheterpal et al. 2003) using a combination of biophysical tools, such as solid state NMR, hydrogen-deuterium exchange-mass spectrometry, scanning mutagenesis, and molecular modeling. Results have been at times contradictory, due in part to different experimental conditions employed for obtaining and analyzing the fibrils and other A β structures. Working with A β 40 fibrils grown in phosphate-buffer saline under unstirred conditions, Wetzel *et al* (Shivaprasad and Wetzel 2006; Williams et al. 2004; Williams et al. 2006) obtained a model that includes two turns centered at residue pairs 22, 23 and 29, 30. In contrast, Petkova *et al* (2002) proposed a model in which residues 12–24 and 30–40 form β -strands that assemble into parallel β -sheets through intermolecular hydrogen bonding. Other key features shared by leading models include a disordered N- terminal segment (Paravastu et al. 2006; Petkova et al. 2005; Wang et al. 2003), a salt bridge between E23 and K28 (at least in some models (Baumketner et al. 2006; Tarus et al. 2006), and polymorphism in the fibril structure, which most likely reflects alternative packing of side chains at the interface between β -sheets (Paravastu et al. 2006; Petkova et al. 2005). After extensive investigations, the structure of the toxic A β species and the identity of the residues responsible for peptide toxicity are still controversial.

Growing experimental evidence suggests that parts of the A β structure may be relatively flexible and this characteristic may be associated with toxicity (Lee et al. 2007; Shivaprasad and Wetzel 2004). Structural flexibility is expected to induce changes in the fibril packing pattern, which may translate into subtle variations of side chains accessibility. Therefore, an approach capable of revealing whether a certain residue is buried or exposed, and the percentage of time that the residue is buried or exposed (or the fraction of the residue buried or exposed in a give structure) could provide valuable information about the mechanism of the interactions between peptides within a fibril, or between fibrils and other cellular components. The combination of chemical modification with mass spectrometric detection constitutes an excellent tool for monitoring the solvent accessibility of amino acid residues in proteins and biomolecular assemblies.

In light of the possible roles played by positively charged amino acids in A β toxicity, which include hypotheses that positively charged residues interact directly with the cell membrane (Terzi et al. 1994; Williamson et al. 2006; Yoshiike et al. 2007), or that positively charged amino acids participate in the formation of an A β membrane pore (Diaz et al. 2006; Pollard et al. 1995), we examine here the solvent accessibility of primary amines (K16, K28 and the N-terminus) as a function of peptide structure *in vitro*. A β 40 was subjected to reductive alkylation using formaldehyde, as described by (Iwata et al. 2001), after which limited trypsin proteolysis and ESI-MS/MS were used to identify modified residues. Subsequently, kinetic experiments were completed to evaluate the degree of accessibility exhibited by the targeted residues, which provided estimates of local unfolding equilibrium constants. The results afforded by the quantitative analysis of residue accessibility are discussed in the context of the current knowledge of A β interactions associated with aggregation and toxicity.

Material & Methods

Materials

β -Amyloid (A β 40: H₂N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-COOH) A β 11 and Interleukin-6-Receptor peptides were purchased from AnaSpec and employed without additional purification. Sinapinic Acid was purchased from Fisher. Sodium cyanoborohydride was purchase from Acros Organics. Trypsin was purchased from

Promega. C18 Zip Tips were purchased from Millipore. All the other chemicals were purchased from Sigma-Aldrich.

Protein sample preparation

Lyophilized A β 1–40 was first dissolved in HFIP, aliquoted out into small volumes, solvent completely evaporated, and the resultant A β films were stored at –80°C. At the time of use, A β films were freshly dissolved in DMSO at the concentration of 10mg/mL, then diluted to 100 μ M in water to make monomeric A β , or in phosphate buffered saline (PBS – 137 mM NaCl, 4.3mM Na₂HPO₄, 2.7 mM KCl, 1.4 mM KH₂PO₄ at pH7.4) to obtain oligomeric or fibril A β . The fibrils were prepared by rotating the solution (18 rpm) at 37°C for 24hr. This method of fibril preparation is comparable to that used by Petkova and coworkers (2002).

Reductive alkylation

NaBH₃CN, CH₂O and 100 μ M of monomer in deionized water or fibril A β in PBS were combined to yield a final concentration of 4.4mM NaBH₃CN, 22.2 μ M A β and a concentration of CH₂O ranging from 88.8 μ M to 1.1mM to start the chemical modification. The reaction was allowed to proceed for up to 3 hours at room temperature. Samples were taken at different times, fibril samples were dissolved, and all samples were desalted using C18 ZipTips. Control peptides were treated identically in water, except that no attempt was made to aggregate control peptides. All the solutions were quenched adding 4 times reaction volume of water. Fibrils were dissolved in a mixture of 80% acetonitrile, 0.1% TFA and the balance water. While not done in this study, in other studies we confirmed this method of fibril dissolution returned between 75 to 95% of mass of the original peptide (Wang et al. 2003, Kheterpal et al., 2000).

Peptide mapping

A 1:20 w/w enzyme to substrate ratio was used for peptide digestion. Before trypsin addition, each reaction mixture was submitted to centrifugation for 3 min at 12000 g. The supernatant was to enable the selective analysis of insoluble components (*i.e.*, fibrils). Digestion reactions were carried out at 37°C for 10 hours. Following digestion, fibril samples were dissolved in a mixture of 80% acetonitrile, 0.1% TFA, and water, as described earlier. Each sample was desalted with C18 ZipTips before analysis by MALDI-TOF mass spectrometry.

Liquid chromatography

Analyses by liquid-chromatography (LC) were conducted on-line using a Grace Vydac C18 reverse phase column (150 $\times$ 1mm, 5 μ m particle size). Solvent A was 0.1% aqueous TFA and solvent B was 0.1% TFA in acetonitrile. A linear gradient was run as follows: 0–5 min 15% B, 20 min 30% B, 40 min 50% B, 40.5–55 min 100% B, 55.5–65 min 15%B. The flow rate was kept constant at 1000 μ L/min; a 10 μ L sample loop was used and the injection volume was 10 μ L. All A β samples were first dissolved in a mixture of 80% acetonitrile, 0.1% TFA and the balance water to recover predominantly monomer A β prior to injection into the column. Between samples, 3 to 5 sequential 10 μ L injections of 10% ammonium hydroxide in water/acetonitrile (1:1 v/v) were done to further minimize carryover of A β peptides between injections (Oe et al. 2006). A HPLC pre-column filter (0.5 μ m SS, MicroSolv Technology Corp.) was connected before the column to avoid endogenous materials from entering and clogging the column or from being detected in the mass spectrometer.

Mass spectrometry

MALDI-TOF analyses of modified products were performed in positive ion mode on a Bruker Daltonics (Billerica, MA) Autoflex MALDI-TOF instrument. The matrix consisted of a saturated solution of sinapinic acid (SA) in 1:2 v/v acetonitrile:water. Equivalent volumes (0.5 μ L) of analyte and matrix solution were mixed onto the MALDI plate and allowed to dry at room temperature. Laser power was optimized on a sample-by-sample basis and was generally found to fall between 30 and 70% of the maximum power afforded by the instrument. Data interpretation was completed with the aid of PeptideMass software (ca.expasy.org Bioinformatics 2008), which was employed to predict the mass of theoretical products obtained by trypsin digestion of alkylated A β substrates.

ESI-MS analyses were performed on a Bruker Daltonics Esquire 3000 quadrupole ion trap equipped with an Agilent (Palo Alto, CA) 1100 liquid chromatograph. The column and methods used for LC were as described above. Nitrogen was used to assist with nebulization and desolvation. Typically, 10 μ L samples were loaded into the LC-MS system and a spray voltage of 1.00 kV was applied. Spectra were acquired in positive mode and processed using XMASS 7.0.2 (Bruker Daltonics, Billerica, MA). Scans were completed in a standard mode that allowed a detection range up to m/z 2000. Spectra were calibrated using 1 mg/mL aqueous solution of ribonuclease and insulin, which produced a series of peaks throughout the mass range of 1000–4000 m/z. Each analysis was performed at least three times and only representative spectra are shown. Tandem mass spectrometry (MS/MS) was carried out for one precursor ion at a time, releasing the precursor ion after 0.50 min. The precursor ions selected for activation are clearly indicated in the figure captions. Isolation widths for precursor masses and scan widths for product ions were set at 2Da. Spectra were interpreted by constructing a theoretical peak table with help from Protein Prospector (version 4.27.2, University California San Francisco, USA) (Chalkey et al., 2008).

Kinetic Analysis

To determine relative concentrations of each modified species, within the same spectra, the signal intensities of the ions of interest were measured and used to infer their abundance in each reaction mixture (Palmlblad and Cramer 2007; Randolph et al. 2005). From such measurements, the concentration of peptide modified with one, two, three, four, five or more methyl groups were estimated. While estimating relative abundance of different species from MALDI MS spectra can be problematic, by using a highly acidic matrix solution we ensured that the vast majority of primary, secondary, and tertiary amines were present in protonated form, thus minimizing possible charging differences. In this way, signal intensities provide reasonable estimates of the relative percentages of the different alkylated products in the samples. To mitigate the effects of inhomogeneous distribution of matrix and analyte molecules in the crystalline sample preparation that give rise to poor shot-to-shot and spot-to-spot reproducibility (Garden and Sweedler 2000), we used an integral measurement protocol that consisted of measuring different positions on a sample spot, which should result in more reproducible average signal intensity values (Kang et al. 2001). In addition, controls were performed to examine possible differences in elution of modified versus unmodified peptide from Zip Tips, and no differences in elution of the different methylated species or unmodified peptide were observed. The analysis of solutions obtained at different time intervals was repeated to improve the precision of these determinations. In this way, an overall uncertainty of less than 10% was estimated by comparing three spectra per sample, which exhibited intensity values of at least 1000 arbitrary units, or greater, and signal to noise ratios of 10, or greater. All concentrations employed in subsequent calculations carried comparable precision.

In order to estimate methylation rate constants, we initially performed the kinetic analysis of data obtained from the control peptide A β 1–11. Assuming that this peptide is fully exposed to solvent (A β_E) and that all sites are free to react with reagent (R), formaldehyde is expected to introduce one (A β_{M1}) or two (A β_{M2}) methyl groups per site:

The rate expressions and associated differential equations for the chemical modification reactions can then be readily expressed. For instance, equations (2)-(5) describe the modification of one amine on A β with depletion of reagent R:

$$\frac{dA\beta_E}{dt} = -k_1 \cdot A\beta_E \cdot R \quad (2)$$

$$\frac{dA\beta_{M1}}{dt} = k_1 \cdot A\beta_E \cdot R - k_2 \cdot A\beta_{M1} \cdot R \quad (3)$$

$$\frac{dA\beta_{M2}}{dt} = k_2 \cdot A\beta_{M1} \cdot R \quad (4)$$

$$\frac{dR}{dt} = -k_1 \cdot A\beta_E \cdot R - k_2 \cdot A\beta_{M1} \cdot R \quad (5)$$

The numerical solution to the differential equations for the chemical modification reactions were fit to the data obtained from all of the chemical modification reactions as a function of time using a nonlinear least squares regression algorithm developed in MATLAB® for this purpose. The parameters estimated from the fit of the solution to equations (2)-(5) to the relative concentrations of control peptide unmodified, singly modified and doubly modified were the chemical modification rate constants, k_1 and k_2 .

Comparing methylation rate constants in the absence and presence of steric protection allows for an estimation of equilibrium unfolding constants, which can be obtained from any of the different modification sites on the full A β sequence. According to a simple unfolding model, each primary amine in A β could transition from a buried (A β_B) to an exposed (A β_E) situation with equilibrium constant K_E :

Given that there are 3 available primary amines in the A β sequence for modification, 3 residue specific unfolding constants can be estimated (K_{E1} , K_{E2} , and K_{E3}). Differential equations (7)-(9) describe the relationship between the disappearance of a buried residue (A β_B^1 , A β_B^2 , and A β_B^3 at each of the 3 reactive sites), the equilibrium unfolding constants (K_{E1} , K_{E2} , and K_{E3}), the reverse rate of the unfolding reaction shown in equation (6), k_r , and the appearance of exposed residues (A β_B^1 , A β_B^2 , A β_B^3).

$$\frac{dA\beta_B^1}{dt} = -k_r (K_{E1} \cdot A\beta_B^1 - A\beta_E^1) \quad (7)$$

$$\frac{dA\beta_B^2}{dt} = -k_r (K_{E2} \cdot A\beta_B^2 - A\beta_E^2) \quad (8)$$

$$\frac{dA\beta_B^3}{dt} = -k_r (K_{E3} \cdot A\beta_B^3 - A\beta_E^3) \quad (9)$$

Each exposed site could then react with reagent R to form either singly- or doubly-methylated products ($A\beta_{M1}^i$ and $A\beta_{M2}^i$, respectively, where superscript *i* refers to one of the 3 reactive sites) analogous to those produced by the control peptide (*i.e.*, reaction (1)). Considering the negligible difference between the rate of first and second methylation manifested by the control peptide, we assumed $k_1 \approx k_2 = k_r$ to obtain differential equations analogous to equations (2)-(4) for the control for each of the 3 reactive sites (9 more differential equations). Finally, equation (10) is the differential equation describing the depletion of formaldehyde when 6 methyl groups are added to $A\beta$ at three different primary amines, where *k* is the rate of methylation estimated from the control peptide.

$$\frac{dR}{dt} = -\left(k \cdot A\beta_E^1 \cdot R + k \cdot A\beta_{M1}^1 \cdot R + k \cdot A\beta_E^2 \cdot R + k \cdot A\beta_{M1}^2 \cdot R + k \cdot A\beta_E^3 \cdot R + k \cdot A\beta_{M1}^3 \cdot R\right) \quad (10)$$

The numerical solution to the set of differential equations generated for the residue unfolding and methylation for the 3 reactive sites on $A\beta$ (equations (7)-(9), the 9 equations analogous to equations (2)-(4) for the control, and equation (10)) were then fit to the experimental data obtained from all modification reactions as a function of time using a non linear least squares algorithm developed in MATLAB. Although data were collected for each methyl group added to the peptide, we decided to focus only on those corresponding to the first, third, and fifth methyl group added to the peptide, which represent the first methyl added to the N-terminus, K28 and K16 in our model. MS/MS results, shown in Figure 7, support the assumption that amines are modified sequentially (N terminus is modified first prior to any modification of K28, which is fully modified prior to any modification of K16). All data were fit simultaneously using the rate constant for methyl addition, *k*, obtained from the fit of the control data, which provided three equilibrium unfolding constants, K_E , for the three primary amines in $A\beta$.

Results

The reaction of formaldehyde with amino groups, followed by reduction with NaBH_3CN , produces irreversible methylation products, which can reveal the extent of solvent accessibility of target functional groups within complex biomolecular structures (Iwata et al. 2001). With the goal of correlating the rate of methylation of susceptible sites with the rate of unfolding of $A\beta$ structures, we employed reductive alkylation with mass spectrometric detection to determine solvent accessibility of primary amines in toxic and non-toxic $A\beta$, corresponding to its fibril and monomeric forms. Figure 1 and 2 show representative MALDI-TOF spectra obtained from $A\beta$ fibrils at different reaction intervals and using increasing formaldehyde to $A\beta$ ratios (e.g., 4:1 and 7:1 $\text{CH}_3\text{O}:A\beta$, respectively). Multiple signals corresponding to alkylated $A\beta$ were readily detected with a 14 Da incremental mass characteristic of methyl groups. In control spectra obtained before probe application, we found evidence of oxidation of methionine 35, which resulted in a weak peak with 16 Da incremental mass. Addition of reducing agent (NaBH_3CN) in the absence of formaldehyde proved that such adduct was effectively reduced in the chemical modification environment and, thus, did not interfere with subsequent analysis (data not shown). A close examination of these data showed that the number of modifications clearly increased with time and

reached saturation more rapidly at the higher formaldehyde to A β ratio. In particular, the maximum number of modifications was recorded after 3 hours of reaction, and corresponded to the addition of three methyl groups per primary amine available on the peptide, for a total of nine (data not shown). At the highest concentrations of formaldehyde used (25:1 and 50:1 CH₃O:A β), within 3 hours, at least 95% of the peptide was fully modified. Reductive alkylation under the conditions used in this study did not appear, however, to lead to loss of fibril structure as observed via electron microscopy (data not shown). When the reaction was performed on the monomeric A β form at the 7:1 formaldehyde to A β ratio (Figure 3), the methylation patterns revealed a higher degree of accessibility by the susceptible sites than was observed in fibril samples. Within 60 minutes, even at lower concentrations of formaldehyde (7:1 CH₃O:A β), more than 95% of the peptide was fully modified.

Control experiments were conducted using the shorter A β peptide (1–11) to assess the susceptibility of the N-terminus and other possible residues under the selected experimental conditions. In this case, only two methyl groups were added to the peptide (Figure 4), suggesting that only the N-terminus was effectively modified. When we completed a kinetic analysis by fitting the relative concentrations of each species as a function of time to different kinetic models (Figure 5), we found that the rate of addition of the second methyl group to the mono-methylated N-terminus was indistinguishable from that of the addition of the first methyl group to the free amine. Indeed, fitting two rate constants to the kinetic data did not provide a better correlation than fitting only one rate constant (*i.e.*, the difference in the sum of the squares of the residuals from both fits were within the variance of the data). The fact that this finding contrasts with earlier reports on methylation rates, in which the rate of addition of the second methyl group was observed to be faster than the rate of addition of the first methyl group (Jentoft and Dearborn 1980; Means and Feeney 1995) may be a direct reflection of the selected reaction conditions. In our case, a single rate constant for N-terminus methylation was estimated to be 18 M⁻¹s⁻¹ ($r=0.91$, see *Material and Methods*), which is consistent with analogous values reported for methylation reactions of amino groups (Jentoft and Dearborn 1980). No evidence was found to support the addition of a third methyl group to the N-terminus during the reaction period considered. Within experimental error, the reaction had gone to completion within 60 minutes.

We subsequently employed the value of methylation rate exhibited by N-terminus to describe also the addition of a methyl group to any exposed lysine, regardless of its position in the A β fibril. When any effects of local environment are neglected, less basic amines (with lower pK_A's) are generally more reactive towards reductive alkylation (Means and Feeney 1995, Garcia-Borrón et al., 1987), therefore the lysine's modification rate should be somewhat lower than that of the N-terminus. Approximating the rates of K16 and K28 methylation to that of the N-terminus might lead to a slight underestimation of the corresponding equilibrium unfolding constants. This approximation, however, does not compromise the validity of our findings, which is based on the difference between estimated unfolding constants, rather than on their absolute values. For this reason, the rate constant obtained here for the N-terminus was employed in subsequent calculations involving the remaining primary amines present in full-length peptide.

Two methods were used to map the position of modified residues on A β fibrils during the course of reaction, a classic bottom-up approach based on trypsin proteolysis, and LC-ESI-MS using MS/MS to cleave the peptide backbone and identify fragments within the MS instrument. At pre-determined intervals, aliquots were removed from the reaction mixture, excess reductive alkylation reagents were removed, and peptides were either subjected to tryptic digestion or LC-ESI-MS. While both methods provided similar information, only the LC-ESI-MS results are shown. The unmodified A β fibril that was present before the reaction had started was detected in the +4 and +5 charge (Figure 6a); once MS/MS was

performed to the $[M+H^{5+}]^{+5}$ precursor ion several fragments (Figure 6b) were identified and used for comparison to the results obtained after reaction. After 15 min of reaction, an aliquot was taken and MS/MS analysis was done. Two different species were identified: A β with 4 methyl groups (Figure 7a) and with 6 methyl groups (data not shown). MS/MS was performed to the precursor ion with +5 charge and the fragments obtained were carefully analyzed. The fragments detected from the A β fibril population with 4 methyl groups indicated that the N-terminus was fully modified [$b9^{+}(+2CH_3)$], K16 was unmodified [$b23^{3+}(+2CH_3)$] and K28 was fully modified [$y25^{3+}(+3CH_3)$] (Figure 7b). The other population that was detected with 6 methyl groups fragmented in such a way as to indicate that K28 was fully modified and K16 was present in several modification stages. These results convincingly demonstrate that K28 is fully modified prior to K16 modification in the A β fibril.

In order to obtain insights into A β fibril accessibility at the single residue level, we completed a kinetic analysis of the extent of alkylation of A β -sites as a function of time and formaldehyde concentration (Figure 8). The solution of the system of differential equations describing the unfolding and methylation of each primary amine on A β were fit to the data provided from chemical modification experiments (prior to tryptic digestion or MS/MS) in order to obtain equilibrium unfolding constants for the three modification sites, K_{E-N} , K_{E-16} , and K_{E-28} (see *Materials and Methods*). Estimated values of the equilibrium unfolding constants were 33, 0.050 and 0.30, respectively. The correlation coefficient for the fit, or r , was estimated to be 0.87. The fit of the model for the modification reactions to the data is quite good given that only 3 estimable parameters were used. These observations support the initial assumption that the two first methyl groups added to the A β fibril were added at the N-terminus and provided information about N-terminus solvent accessibility, the following two methyl groups were related to K28, and the remained methyl groups provided solvent accessibility information related to K16. Other models were explored in which methyl groups were not added sequentially to each of the amines, but without improved results. These results provide, for the first time, a quantitative measure of the differences in solvent accessibility of the N-terminus, K16 and K28.

Discussion

The aggregation state of A β has been linked to its toxicity, thus the structure of different aggregated species has been the object of extensive investigation. Revealing possible variations in the residues exposed on the surface of aggregated peptide may provide important insights into the mechanism of aggregation and possible interactions between aggregated peptide and cellular components. Thus far, progress has been made in elucidating fibril structures, whereas solving the structure of toxic A β species of intermediate size, such as oligomers or protofibrils, has lagged behind. Towards understanding A β interactions with cellular components, early work suggested that A β interacted with cell membranes via electrostatic interactions (Hertel et al. 1997; Terzi et al. 1994). More recent work has suggested that positively charged amino acids, including K16, K28, H13 and H14, may be involved in specific interactions with surface glycolipids or glycoproteins (Avdulov et al. 1997; Chimon and Ishii 2005; Choo-Smith et al. 1997; Wang et al. 2003) and in the formation of an A β pore (Diaz et al. 2006), and could therefore play an important role in determining overall toxicity (Williamson et al. 2006). For these reasons, we have specifically focused on positively charged amino acids in monomer and fibril as a first step toward understanding how A β aggregates and interacts with cells.

Chemical modification of specific amino acid residues has been used to probe the solvent accessibility of protein side chains (Alcalde et al. 1999; Sharp et al. 2003; Yem et al. 1992; Suckau et al. 1992). Modification rates are determined by the intrinsic chemical reactivity of

target functional groups, as well as by their solvent accessibility. In our case, reductive alkylation was employed to monitor the accessibility of primary amines in different A β structures. The experiments have shown that the monomeric form of A β is clearly less structured than the fibrils (Figures 1, 2 and 3). This was substantiated by the observation that even at low concentration of formaldehyde all monomer sites were labeled with two methyl groups (six total) after 3 hours reaction, whereas fibrils exhibited a distribution of products with four to six added methyls. More specifically, peptide mapping of modify substrates revealed that the N-terminus is more exposed than K28, while K16 is the least exposed among the three sites (Figure 6 and 7). The existence of two populations, where different numbers of methyl groups were added, is evidence of heterogeneity in A β fibril structure. These results are only partially consistent with those reported by Iwata *et al* (2001), who used reductive alkylation to demonstrate that the N-terminus, K16, and K28 were equally accessible when the protein was free in solution, but reported that K28, rather than K16, was protected in the fibril form. Leading fibril models show both K16 and K28 in accessible positions (Shivaprasad and Wetzel 2006; Williams et al. 2004; Williams et al. 2006), or have K16 exposed and K28 engaged in a salt-bridge interaction in at least some of the fibril structures (Paravastu et al. 2006; Petkova et al. 2005). It should be noted, however, that fibrils were prepared under quiescent conditions in one case (Paravastu et al. 2006; Petkova et al. 2005), whereas they were formed by gently mixing A β during aggregation in the other (Shivaprasad and Wetzel 2006; Williams et al. 2004; Williams et al. 2006).

These results reaffirm the notion that fibril structure is affected by a high degree of polymorphism, which hampers the ability of clearly correlating a certain structure with toxicity (Paravastu et al. 2006; Petkova et al. 2005). Although models are necessarily binary in nature (*e.g.*, a residue is either buried or exposed), the peptide structure is expected to be more dynamic in solution, with plasticity assuming a critical importance in A β toxicity (Lee et al. 2007; Williams et al. 2006). For example, A β peptide must undergo some conformational rearrangement to enable its participation into an A β pore in the cell membrane. Our kinetic data provide a measure of the relative flexibility of the regions including the target amino groups, which can be expressed in terms of equilibrium unfolding constants.

In this work, we report for the first time, the quantitative examination of local or residue specific equilibrium unfolding constants for A β fibrils that we obtained using chemical modification coupled with MALDI-MS. While MALDI-MS is frequently used for identification, and not for quantitative analysis, a number of examples of quantitative analysis using MALDI have been reported (Bungert et al. 2004; Chan et al. 2004; Gusev et al. 1996; Tost et al. 2003). In addition, there have been recent reports of chemical modification coupled with MALDI-MS, ESI-MS, and LC-MS to infer both global and regional (domain specific) protein stability of other proteins (Thirumangalathu et al. 2007; West et al. 2008) demonstrating the power of such methods at quantitatively probing protein structure.

We constructed our analysis and modeled our data based on several assumptions about equilibrium unfolding of lysine side chains in the A β fibril. We have electron micrographs suggesting that the A β fibrils do not unfold during reductive alkylation (data not shown). However, we show that at 3 hours and 1.1 mM formaldehyde concentration, we achieve complete modification of all amines in the A β fibril samples (Figure 8C). These data suggest that side chains unpack or unfold regionally during reductive alkylation and that we are assessing local equilibrium unfolding constants, not a global unfolding constant for A β fibrils. In an unrelated study, we have estimated global equilibrium unfolding constants for A β fibrils extrapolated from structure changes in denaturant to be on the order of 0.008

(unpublished data). The global equilibrium unfolding constant is an order of magnitude smaller than any local equilibrium unfolding constants estimated in this study.

We assumed that unfolding of a side chain was in rapid equilibrium in order to perform our quantitative analysis. However, our equilibrium assumption holds only if the rate of refolding is much faster than the rate of chemical reaction. In our case, we measured the rate of chemical reaction for the addition of a methyl group to an amine to be $18 \text{ M}^{-1} \text{ s}^{-1}$. Given the range of formaldehyde concentrations used in our studies, the equivalent first order rate constant for reaction is approximately 0.02 to 0.004 s^{-1} . While we did not measure the rate of refolding of the lysine side chains, others have measured protein domain refolding constants to be on the order of 0.002 s^{-1} to 2 s^{-1} (Graczer et al., 2009, Blancas-Mejia et al., 2009, Schlepckow et al, 2008, for example). If we take the local refolding of the lysine side chains within the amyloid fibril to be on the order of 0.2 s^{-1} , the more commonly reported refolding rate constant, then our equilibrium assumption is reasonable, especially at low formaldehyde concentrations. If, however, the local rate of refolding of lysine side chains in an A β fibril are much slower than 0.2 s^{-1} , then our measurements more accurately represent a ratio of rate constants defined by equation 11, where k_u is the local rate of unfolding, k_r is the rate of refolding, k is the rate of reaction of the amine with formaldehyde, R is the concentration of formaldehyde, and K_E^{app} is the apparent equilibrium unfolding constant.

$$K_E^{app} = \frac{k_u}{k_f + k_r} \bullet R \quad (11)$$

This apparent equilibrium unfolding constant still provides a useful measure of local differences in A β structure.

Finally, we assumed in our analysis that modification of one lysine did not affect the reactivity of other lysines, and that the intrinsic reactivities of lysines and the N terminus were the same. Most reports of reductive methylation of proteins indicate that modification of a lysine results in very minor changes in both protein structure and residue pKA (Zhang and Vogel, 1993, Means and Feeney 1995, Taylor and Webb, 2001), suggesting that modification of one lysine would not effect modification of another residue. In general, the rate of addition of methyl groups we measured in the control peptide are comparable to what others have measured both for free terminal amines and solvent exposed lysines (Means and Feeney, 1995, Garcia-Borron et al., 1987). However, if one of the lysines in the A β fibril had an abnormally low pKA, we might expect higher than normal lysine reactivity, and thus an erroneously high prediction of an apparent equilibrium unfolding constant (Garcia-Borron et al., 1987). To our knowledge there have been no experimental measurements of the pKA's of K16 and K28 in the A β fibril, but from simulation, neither is expected to be abnormally low (Tarus et al., 2006). Thus, we expect that these assumptions, while necessary for determining quantitative values for unfolding constants, will not compromise the conclusions we draw from this study.

We report that the apparent equilibrium unfolding constants for K16 and K28 are, respectively, 500 and 100 times smaller than the unfolding constant estimated for the N-terminus. Our data are consistent with the observation that the N-terminal region is disordered and completely solvent-exposed (Kheterpal et al. 2001; O'Nuallain and Wetzel 2002; Wang et al. 2003). Our results are not consistent with leading models where K16 is always more exposed than K28, which is affected by the possible formation of a salt bridge at that location (Petkova et al. 2002). The calculated constants show that the solvent environments of K16 and K28 are much more similar to each other than to the solvent environment of N-terminus. A ~6 fold difference in equilibrium unfolding constant between K28 and K16 suggests that the K28 is somewhat more solvent exposed than K16; however,

both residues, with equilibrium unfolding constants of 0.050 (K16) and 0.30 (K28), have a higher probability of being buried within the fibril, or within a fibril bundle, than they do of being exposed to solvent. An equilibrium unfolding constant of 0.3 (K28) suggests that 23% of residues can be solvent exposed at any given time, while an equilibrium unfolding constant of 0.05 (K16) would suggest that only 5% of residues are exposed. Future work will have to address whether this level of exposure is sufficient for interactions with cellular components that play a role in the mechanism of A β toxicity.

In conclusion, our analysis provides a different perspective on peptide plasticity and suggests a strategy for investigating how subtle differences in residues accessibility may affect interactions with the cell surface and therefore A β toxicity. Using residue-specific equilibrium unfolding constants provide a quantitative tool to examine how changes in aggregation conditions leads to changes in macroscopic structure (fibril versus oligomer), and molecular level changes in structure and structural plasticity. Using analogous methods, we hope to obtain quantitative measurements of residue solvent accessibility of other aggregated A β structures such as oligomers that are associated with toxicity.

Acknowledgments

We would like to thank Dr. Alexei Gapeev, from the Chemistry Department at UMBC, for his help with all Mass Spectrometry techniques. Financial support for this work was provided by NIH (R01 NS042686 to TAG and EJF) and NSF (CHE-0439067 to DF).

Abbreviations and Textual Footnotes

Aβ	β -Amyloid
AD	Alzheimer's disease
Da	Dalton (mass/mole unit)
ESI	Electrospray Ionization
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization – Time of Flight
MS	Mass Spectrometry
SA	Sanipinic Acid

References

- Alzheimer's Association.
- Alcalde M, Plou FJ, Andersen C, Martin MT, Pedersen S, Ballesteros A. Chemical modification of lysine side chains of cyclodextrin glycosyltransferase from *Thermoanaerobacter* causes a shift from cyclodextrin glycosyltransferase to α -amylase specificity. *FEBS Lett.* 1999; 445(2–3):333–7. [PubMed: 10094484]
- Avdulov NA, Chochina SV, Igbavboa U, Warden CS, Vassiliev AV, Wood WG. Lipid binding to amyloid beta-peptide aggregates: preferential binding of cholesterol as compared with phosphatidylcholine and fatty acids. *J Neurochem.* 1997; 69(4):1746–52. [PubMed: 9326304]
- Baumketner A, Bernstein SL, Wyttenbach T, Lazo ND, Teplow DB, Bowers MT, Shea JE. Structure of the 21–30 fragment of amyloid beta-protein. *Protein Sci.* 2006; 15(6):1239–47. [PubMed: 16731963]
- Bioinformatics SIO. ExPASy, peptide-mass tools. 2008.
- Blancas-Mejia LM, Tellez LA, del Pozo-Yauner L, Becerril B, Sanchez-Ruiz JM, Fernandez-Velasco DA. Thermodynamic and kinetic characterization of a germ line human lambda6 light-chain protein: the relation between unfolding and fibrillogenesis. *J Mol Biol.* 2009; 386(4):1153–66. [PubMed: 19154739]

- Bungert D, Heinzle E, Tholey A. Quantitative matrix-assisted laser desorption/ionization mass spectrometry for the determination of enzyme activities. *Anal Biochem.* 2004; 326(2):167–75. [PubMed: 15003557]
- Chalkley RJ, Baker PR, Medzihradszky KF, Lynn AJ, Burlingame AL. In-depth analysis of tandem mass spectrometry data from disparate instrument types. *Mol Cell Proteomics.* 2008; 7(12):2386–98. [PubMed: 18653769]
- Chan R, Chen V, Bucknall MP. Quantitative analysis of membrane fouling by protein mixtures using MALDI-MS. *Biotechnol Bioeng.* 2004; 85(2):190–201. [PubMed: 14705002]
- Chimon S, Ishii Y. Capturing intermediate structures of Alzheimer's beta-amyloid, Abeta(1–40), by solid-state NMR spectroscopy. *J Am Chem Soc.* 2005; 127(39):13472–3. [PubMed: 16190691]
- Choo-Smith LP, Garzon-Rodriguez W, Glabe CG, Surewicz WK. Acceleration of amyloid fibril formation by specific binding of Abeta-(1–40) peptide to ganglioside-containing membrane vesicles. *J Biol Chem.* 1997; 272(37):22987–90. [PubMed: 9287293]
- Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem.* 2002; 277(35):32046–53. [PubMed: 12058030]
- Diaz JC, Linnehan J, Pollard H, Arispe N. Histidines 13 and 14 in the Abeta sequence are targets for inhibition of Alzheimer's disease Abeta ion channel and cytotoxicity. *Biol Res.* 2006; 39(3):447–60. [PubMed: 17106577]
- Garcia-Borron JC, Bieber AL, Martinez-Carrion M. Reductive methylation as a tool for the identification of the amino groups in alpha-bungarotoxin interacting with nicotinic acetylcholine receptor. *Biochemistry.* 1987; 26(14):4295–303. [PubMed: 3663591]
- Garden RW, Sweedler JV. Heterogeneity within MALDI Samples As Revealed by Mass Spectrometric Imaging. *Anal. Chem.* 2000; 72(1):30–36. [PubMed: 10655631]
- Glennner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun.* 1984; 120(3):885–90. [PubMed: 6375662]
- Gráczner E, Varga A, Melnik B, Semisotnov G, Závodszky P, Vas M. Symmetrical refolding of protein domains and subunits: example of the dimeric two-domain 3-isopropylmalate dehydrogenases. *Biochemistry.* 2009; 48(5):1123–34. [PubMed: 19154118]
- Gusev AI, Wilkinson WR, Proctor A, Hercules DM. Direct quantitative analysis of peptides using matrix assisted laser desorption ionization. *Anal Bioanal Chem.* 1996; 354(4):455–63. [PubMed: 15048433]
- Harper JD, Wong SS, Lieber CM, Lansbury PT Jr. Assembly of A beta amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease. *Biochemistry.* 1999; 38(28):8972–80. [PubMed: 10413470]
- Hertel C, Terzi E, Hauser N, Jakob-Rotne R, Seelig J, Kemp JA. Inhibition of the electrostatic interaction between beta-amyloid peptide and membranes prevents beta-amyloid-induced toxicity. *Proc Natl Acad Sci U S A.* 1997; 94(17):9412–6. [PubMed: 9256496]
- Hoshi M, Sato M, Matsumoto S, Noguchi A, Yasutake K, Yoshida N, Sato K. Spherical aggregates of beta-amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3beta. *Proc Natl Acad Sci U S A.* 2003; 100(11):6370–5. [PubMed: 12750461]
- Iwata K, Eyles SJ, Lee JP. Exposing asymmetry between monomers in Alzheimer's amyloid fibrils via reductive alkylation of lysine residues. *J Am Chem Soc.* 2001; 123(27):6728–9. [PubMed: 11439074]
- Jentoft N, Dearborn DG. Protein labeling by reductive methylation with sodium cyanoborohydride: effect of cyanide and metal ions on the reaction. *Anal Biochem.* 1980; 106(1):186–90. [PubMed: 7416460]
- Kang M-J, Tholey A, Heinzle E. Application of automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the measurement of enzyme activities. *Rapid Communications in Mass Spectrometry.* 2001; 15(15):1327–1333. [PubMed: 11466793]
- Kheterpal I, Chen M, Cook KD, Wetzel R. Structural differences in Abeta amyloid protofibrils and fibrils mapped by hydrogen exchange--mass spectrometry with on-line proteolytic fragmentation. *J Mol Biol.* 2006; 361(4):785–95. [PubMed: 16875699]

- Kheterpal I, Lashuel HA, Hartley DM, Walz T, Lansbury PT Jr, Wetzel R. Abeta protofibrils possess a stable core structure resistant to hydrogen exchange. *Biochemistry*. 2003; 42(48):14092–8. [PubMed: 14640676]
- Kheterpal I, Williams A, Murphy C, Bledsoe B, Wetzel R. Structural features of the Abeta amyloid fibril elucidated by limited proteolysis. *Biochemistry*. 2001; 40(39):11757–67. [PubMed: 11570876]
- Kheterpal I, Zhou S, Cook KD, Wetzel R. Abeta amyloid fibrils possess a core structure highly resistant to hydrogen exchange. *Proc Natl Acad Sci U S A*. 2000; 97(25):13597–601. [PubMed: 11087832]
- Lee S, Fernandez EJ, Good TA. Role of aggregation conditions in structure, stability, and toxicity of intermediates in the Abeta fibril formation pathway. *Protein Sci*. 2007; 16(4):723–32. [PubMed: 17327396]
- Means GE, Feeney RE. Reductive alkylation of proteins. *Anal Biochem*. 1995; 224(1):1–16. [PubMed: 7710054]
- O'Nuallain B, Wetzel R. Conformational Abs recognizing a generic amyloid fibril epitope. *Proc Natl Acad Sci U S A*. 2002; 99(3):1485–90. [PubMed: 11818542]
- Oe T, Ackermann BL, Inoue K, Berna MJ, Garner CO, Gelfanova V, Dean RA, Siemers ER, Holtzman DM, Farlow MR, others. Quantitative analysis of amyloid beta peptides in cerebrospinal fluid of Alzheimer's disease patients by immunoaffinity purification and stable isotope dilution liquid chromatography/negative electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2006; 20(24):3723–35. [PubMed: 17117458]
- Olofsson A, Sauer-Eriksson AE, Ohman A. The solvent protection of alzheimer amyloid-beta-(1–42) fibrils as determined by solution NMR spectroscopy. *J Biol Chem*. 2006; 281(1):477–83. [PubMed: 16215229]
- Palmblad M, Cramer R. Liquid matrix deposition on conductive hydrophobic surfaces for tuning and quantitation in UV-MALDI mass spectrometry. *J Am Soc Mass Spectrom*. 2007; 18(4):693–7. [PubMed: 17223354]
- Paravastu AK, Petkova AT, Tycko R. Polymorphic fibril formation by residues 10–40 of the Alzheimer's beta-amyloid peptide. *Biophys J*. 2006; 90(12):4618–29. [PubMed: 16565054]
- Petkova AT, Ishii Y, Balbach JJ, Antzutkin ON, Leapman RD, Delaglio F, Tycko R. A structural model for Alzheimer's beta -amyloid fibrils based on experimental constraints from solid state NMR. *Proc Natl Acad Sci U S A*. 2002; 99(26):16742–7. [PubMed: 12481027]
- Petkova AT, Leapman RD, Guo Z, Yau WM, Mattson MP, Tycko R. Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. *Science*. 2005; 307(5707):262–5. [PubMed: 15653506]
- Pollard HB, Arispe N, Rojas E. Ion channel hypothesis for Alzheimer amyloid peptide neurotoxicity. *Cell Mol Neurobiol*. 1995; 15(5):513–26. [PubMed: 8719038]
- Randolph TW, Mitchell BL, McLerran DF, Lampe PD, Feng Z. Quantifying peptide signal in MALDITOF mass spectrometry data. *Mol Cell Proteomics*. 2005; 4(12):1990–9. [PubMed: 16195224]
- Schlepckow K, Wirmer J, Bachmann A, Kiefhaber T, Schwalbe H. Conserved folding pathways of alpha-lactalbumin and lysozyme revealed by kinetic CD, fluorescence, NMR, and interrupted refolding experiments. *J Mol Biol*. 2008; 378(3):686–98. [PubMed: 18377934]
- Selkoe DJ. Alzheimer's disease: a central role for amyloid. *J Neuropathol Exp Neurol*. 1994; 53(5):438–47. [PubMed: 8083687]
- Sharp JS, Becker JM, Hettich RL. Protein surface mapping by chemical oxidation: structural analysis by mass spectrometry. *Anal Biochem*. 2003; 313(2):216–25. [PubMed: 12605858]
- Shivaprasad S, Wetzel R. An intersheet packing interaction in A beta fibrils mapped by disulfide cross-linking. *Biochemistry*. 2004; 43(49):15310–7. [PubMed: 15581343]
- Shivaprasad S, Wetzel R. Analysis of amyloid fibril structure by scanning cysteine mutagenesis. *Methods Enzymol*. 2006; 413:182–98. [PubMed: 17046397]
- Suckau D, Mak M, Przybylski M. Protein surface topology-probing by selective chemical modification and mass spectrometric peptide mapping. *Proc Natl Acad Sci U S A*. 1992; 89(12):5630–4. [PubMed: 1608973]

- Tarus B, Straub JE, Thirumalai D. Dynamics of Asp23-Lys28 salt-bridge formation in Abeta10–35 monomers. *J Am Chem Soc.* 2006; 128(50):16159–68. [PubMed: 17165769]
- Taylor IA, Webb M. Chemical modification of lysine by reductive methylation. A probe for residues involved in DNA binding. *Methods Mol Biol.* 2001; 148:301–14. [PubMed: 11357593]
- Terzi E, Holzemann G, Seelig J. Alzheimer beta-amyloid peptide 25–35: electrostatic interactions with phospholipid membranes. *Biochemistry.* 1994; 33(23):7434–41. [PubMed: 8003508]
- Thirumangalathu R, Krishnan S, Bondarenko P, Speed-Ricci M, Randolph TW, Carpenter JF, Brems DN. Oxidation of methionine residues in recombinant human interleukin-1 receptor antagonist: implications of conformational stability on protein oxidation kinetics. *Biochemistry.* 2007; 46(21): 6213–24. [PubMed: 17480058]
- Thompson LK. Unraveling the secrets of Alzheimer's beta-amyloid fibrils. *Proc Natl Acad Sci U S A.* 2003; 100(2):383–5. [PubMed: 12525689]
- Tost J, Schatz P, Schuster M, Berlin K, Gut IG. Analysis and accurate quantification of CpG methylation by MALDI mass spectrometry. *Nucleic Acids Res.* 2003; 31(9):e50. [PubMed: 12711695]
- Wang SS, Tobler SA, Good TA, Fernandez EJ. Hydrogen exchange-mass spectrometry analysis of beta-amyloid peptide structure. *Biochemistry.* 2003; 42(31):9507–14. [PubMed: 12899638]
- West GM, Tang L, Fitzgerald MC. Thermodynamic analysis of protein stability and ligand binding using a chemical modification- and mass spectrometry-based strategy. *Anal Chem.* 2008; 80(11): 4175–85. [PubMed: 18457414]
- Williams AD, Portelius E, Kheterpal I, Guo JT, Cook KD, Xu Y, Wetzel R. Mapping abeta amyloid fibril secondary structure using scanning proline mutagenesis. *J Mol Biol.* 2004; 335(3):833–42. [PubMed: 14687578]
- Williams AD, Shivaprasad S, Wetzel R. Alanine scanning mutagenesis of Abeta(1–40) amyloid fibril stability. *J Mol Biol.* 2006; 357(4):1283–94. [PubMed: 16476445]
- Williamson MP, Suzuki Y, Bourne NT, Asakura T. Binding of amyloid beta-peptide to ganglioside micelles is dependent on histidine-13. *Biochem J.* 2006; 397(3):483–90. [PubMed: 16626304]
- Yem AW, Epps DE, Mathews WR, Guido DM, Richard KA, Staite ND, Deibel MR Jr. Site-specific chemical modification of interleukin-1 beta by acrylodan at cysteine 8 and lysine 103. *J Biol Chem.* 1992; 267(5):3122–8. [PubMed: 1531337]
- Yoshiike Y, Akagi T, Takashima A. Surface Structure of Amyloid- β Fibrils Contributes to Cytotoxicity. *Biochemistry.* 2007; 46(34):9805–9812. [PubMed: 17676931]
- Zhang D, Gill LA, Cooks RG. Deamination of protonated amines to yield protonated imines - Fundamentals of Ion Trap Mass Spectrometry. *Journal of the American Society for Mass Spectrometry.* 1998; 9(11):1146–1157.
- Zhang W, Culley DE, Gritsenko MA, Moore RJ, Nie L, Scholten JC, Petritis K, Strittmatter EF, Camp DG 2nd, Smith RD, others. LC-MS/MS based proteomic analysis and functional inference of hypothetical proteins in *Desulfovibrio vulgaris*. *Biochem Biophys Res Commun.* 2006; 349(4): 1412–9. [PubMed: 16982031]
- Zhang M, Vogel HJ. Determination of the side chain pKa values of the lysine residues in calmodulin. *J. Biol. Chem.* 1993; 268(30):22420–8. [PubMed: 8226750]

Figure 1.

(a) Representative mass spectra of reaction mixture after reductive alkylation of Aβ fibrils as a function of time. Results are for 4:1 mole ratio CH₃O:Aβ fibrils rotated for 24 hours (100 μM Aβ in PBS). Peak mass and expected number of methyl groups added to Aβ is indicated. Under the reaction conditions used in this experiment the reaction, at 180 min, the reaction still had not gone to completion (unmodified Aβ was still remaining). (b) Mass spectra after 30 minutes and 3 hours of reaction. The expected 14 Da increment between peaks corresponds to the addition of a single methyl group.

Figure 2. Representative mass spectra of reductive alkylation of Aβ fibril reaction mixture as a function of time. 7:1 mole ratio CH₃O:Aβ fibrils rotated for 24 hours (100μM Aβ in PBS).

Figure 3.

Representative mass spectra of reductive alkylation reaction mixture of A β monomer as a function of time for 7:1 mole ratio CH₃O:A β monomer (100 μ M A β in DWI).

Figure 4. Representative mass spectra of reductive alkylation reaction with the control peptide, A β 1–11 in DIW. Mole ratio of CH₂O:A β was 7:1. Only 2 methyl groups were added to N-terminus in 30 minutes.

Figure 5.

Fit of chemical modification of control data to obtain rate constant for modification.

Experimental data are shown as closed symbols, model fits are shown as open symbols (circles: relative concentration of unmodified peptide; diamonds: concentration of peptide with one methyl group added; triangles: concentration of peptide completely modified by addition of two methyl groups). Estimated uncertainty of each concentration was less than 10%. Correlation coefficient for fit, r , was 0.91. There were no significant differences in correlation when one versus two rate constants were fit to the data.

Figure 6. Mass spectrum of unmodified A β fibril (a) full MS spectrum (b) MS/MS spectrum of isolated precursor ion 867.0 Da [M+5H⁺]⁵⁺.

Figure 7.

Mass spectrum of A β fibril after 15 minutes of reaction (a) full MS spectra. The +4 and +5 parent ions have masses 14 and 11.5 greater than the masses of the +4 and +5 parent ions in the unmodified peptide, consistent with the addition of 4 methyl groups. (b), MS/MS spectrum of isolated precursor ion 878.0 Da [M+4CH₃+5H⁺]⁵⁺, with peaks labeled with mass of ion, identity of fragment, and number of methyl groups added to the fragment.

Figure 8.

Representative fits of the experimental A β fibril chemical modification data. Data for all the modification reactions are shown as closed symbols, model fits are shown as open symbols (circles: one or more methyl groups added; squares: three or methyl groups added; triangles: five or more methyl groups added). The solid, dotted, and dashed lines represent model fits for the first modification, third modification, or fifth modification. The concentrations of formaldehyde used in each experiment were nominally (A) 150 μ M; (B) 280 μ M; (C) and 1100 μ M. Data from chemical modification reactions at 3 more concentrations of formaldehyde are not shown. Correlation coefficient, r , for fit of all 6 sets of concentration data simultaneously, was 0.87.