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A Mass Spectrometric Approach for Characterization of Amyloid-β Aggregates and Identification of Their Post-Translational Modifications

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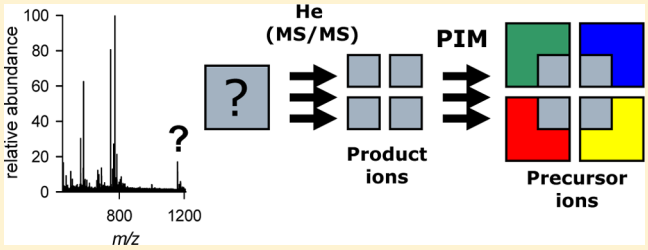
Supporting Information

ABSTRACT: Endogenous amyloid-β (Aβ) oligomeric aggregates have been proposed as toxic agents in Alzheimer's disease (AD). Knowledge of their structures not only may provide insight into the basis of their neurotoxicities but also may reveal new targets for therapeutic drugs and diagnostic tools. However, the low levels of these Aβ oligomers have impeded structural characterization. Evidence suggests that the endogenous oligomers are covalently modified in vivo. In this report, we demonstrate an established mass spectrometry (MS) methodology called *precursor ion mapping* (PIM) that potentially may be applied to endogenous oligomer characterization. First, we illustrate the use of this PIM technique with a synthetic Aβ(1−40) monomer sample that had been cross-linked with transglutaminase (TGase) and digested with pepsin. From PIM analysis of an Aβ(4−13) MS/MS fragment, precursor ions were identified that corresponded to peptic fragments of three TGase cross-linked species: Aβ(4−19)--(4−19), Aβ(4−19)--(20−34), and Aβ(1−19)--(20−34). Next, we demonstrate the applicability of the PIM technique to an endogenous Aβ sample that had been purified and concentrated by immunoaffinity chromatography. Without pepsin digestion, we successfully identified the full length and C-terminally truncated monomeric Aβ species 1−35 to 1−42, along with select methionine-oxidized counterparts. Because PIM focuses only on a subpopulation of ions, namely the related precursor ions, the resulting spectra are of increased specificity and sensitivity. Therefore, this methodology shows great promise for structural analysis and identification of post-translational modification(s) in endogenous Aβ oligomers.



Alzheimer's disease (AD) is a devastating neurodegenerative disorder that is characterized by the presence of senile amyloid plaques in the brains of deceased victims.[1] These plaques largely contain fibrils composed of 39- to 42-residue peptides denoted *amyloid-β* (Aβ). Aβ peptides are proteolytic products of membrane-bound amyloid precursor protein (APP). Although several proteases have been proposed to generate Aβ fragments of various lengths,[1−5] sequential cleavage of APP by first β- and then γ-secretase produces Aβ(1−40) and Aβ(1−42) peptides.[1] Both of these peptides are amyloidogenic because they can aggregate to form amyloid fibrils, but Aβ(1−42), with two additional C-terminal hydrophobic amino acid residues (I41 and A42), has a higher propensity to aggregate and is considered more toxic.[6,7] Depending on experimental conditions, Aβ peptides in vitro can assemble into aggregates with various structures, toxicities, and size distributions, from small oligomers[8−12] and intermediate protofibrils[13,14] to large fibrils.[15] Endogenous soluble Aβ aggregates including small oligomers are widely believed to initiate neurotoxicity in AD.[8−10] Oligomers that have been isolated from AD brains,[16,17] from brains of transgenic mice that overexpress APP,[9] and from conditioned media of CHO cells transfected with an APP-expressing gene (7PA2 cells)[10,18,19] have been reported to impair synaptic plasticity and disrupt memory. However, the very low levels of these endogenous oligomers (i.e., <1 nM[18]) have so far prevented any structural analysis by most biophysical methods.

Because of the low endogenous Aβ oligomer levels, several groups have attempted to generate structurally similar Aβ oligomers from synthetic Aβ peptides in vitro.[8,11,20−23] However, very few synthetic Aβ oligomers have been isolated as homogeneous species. We [21] and others [11] generated synthetic Aβ(1−42) oligomers in dilute SDS and, following removal of the SDS by dialysis, isolated them as stable 150 kDa[12,21] or 64 kDa species.[24] These oligomers partially dissociated into dimer, tetramer, and decamer bands during SDS-PAGE analysis[12,21] and completely dissociated into monomers upon boiling in 2% SDS/LDS loading buffer.[21] In contrast, the endogenous oligomer bands noted above are apparent in samples that have been boiled in SDS prior to SDS-PAGE. This higher structural stability of endogenous oligomers indicates that they have not yet been reproduced by synthetic oligomers and suggests the possibility that they are covalently

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modified in vivo.[25,26] As a result, the method of choice for structural analysis of endogenous Aβ oligomers must be sensitive enough to accommodate their low physiological levels and to detect any covalent modifications.

Mass spectrometry (MS) is a feasible technique for analyzing minute amounts of structurally modified Aβ. In particular, electrospray ionization (ESI) MS has the MS/MS capability necessary to detect and characterize unknown structural changes, and it is a "soft" technique that does not induce the random fragmentation sometimes observed with MALDI-TOF MS.[27] One of the most useful aspects of ESI-MS in the analysis of biological samples is the ability to reveal post-translational modification(s) by means of an established technique known as *precursor ion mapping* (PIM).[28−32] Conventionally, PIM experiments are performed on a triple quadrupole mass spectrometer. Ions following ionization are scanned in the first quadrupole and fragmented to product ions by collision-induced-dissociation (CID) in the second quadrupole. The third quadrupole is set to monitor only a desired product ion and record only those ions in the first quadrupole which give rise to this product ion. Our LTQ Velos ESI mass spectrometer with dual-pressure linear ion trap technology also has this PIM capability. While the high pressure cell can trap, fragment, and isolate ions of interest, the subsequent low pressure cell can increase resolution in mass analysis by allowing faster scanning. Post-translational modifications in proteins routinely characterized by PIM include phosphorylation,[30] glycosylation,[31] and oxidation.[29] In addition, PIM has been utilized in detection or identification of oxidized phospholipids[32] and protein/peptide metabolites (i.e., in studies of drug metabolism).[28] Therefore, ESI-MS is a well-suited methodology for structural analysis of endogenous Aβ oligomers regardless of the types of post-translational modifications present.

In this report, we first demonstrate the utility of the PIM technique to identify covalent modifications of the Aβ peptide structure using a transglutaminase (TGase) cross-linked Aβ monomer sample as a model system. Following successful identification of precursor peptic fragments of TGase cross-linked and noncross-linked Aβ species, we extended the application of PIM to an analysis of an endogenous Aβ sample that had been purified and concentrated by immunoaffinity chromatography. In addition to full length and C-terminally truncated Aβ peptides, PIM analysis of this sample at a submicromolar concentration allowed detection of methionine-oxidized variants. Results from this endogenous Aβ sample show that the ESI PIM-MS technique has both the sensitivity and specificity required for determining covalent modifications of endogenous Aβ oligomers.

EXPERIMENTAL PROCEDURES

Chemicals and Aβ Preparations. All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Aβ(1−40) was synthesized according to standard Fmoc solid phase synthesis by the Peptide Synthesis Facility at the Mayo Clinic (Rochester, MN). The purity of synthesized materials was determined by MALDI-MS to be >95%. Lyophilized Aβ(1−40) peptide, stored desiccated at −80 °C, was purified by SEC as previously described.[21] Briefly, monomers were isolated by dissolving 2.5−3.0 mg of crude Aβ in 35 mM NaOH and fractionating on a Superdex 75 HR 10/30 column (Amersham Pharmacia, Piscataway, NJ) equilibrated with 20 mM Tris-HCl (pH 8.0) at a flow rate of 0.5 mL/min. Collected fractions were quantified by UV absorbance at 276 nm (i.e., ε = 1450 M^{-1} cm^{-1} [14]).

TGase Cross-Linking and Pepsin Digestion. TGase cross-linking of Aβ(1−40) monomers and subsequent pepsin digestion were performed according to previously optimized protocols.[12] Briefly, SEC-purified Aβ(1−40) monomer (100 μM) was initially incubated with 100 μg/mL of TGase (from guinea pig liver, 2 units/mg, Sigma-Aldrich, St. Louis, MO) in 10−50 mM Tris-HCl (pH 8.0) with 5 mM $CaCl_2$ for 30 min at 37 °C. Immediately following incubation, the cross-linked sample was subjected to pepsin digestion. First, the pH of cross-linked Aβ sample was adjusted to 2 with trifluoroacetic acid. Then, 10 μg/mL of porcine pepsin was added and the sample was incubated for 2 h at 37 °C with periodic brief shaking. The pepsin-digested cross-linked Aβ sample was either immediately analyzed by ESI-MS or stored frozen at −20 °C.

Immunoaffinity Purification of Conditioned Media from the 7PA2 CHO Cells. 7PA2 cells were grown to near confluence in 225 mL cell culture flasks and then conditioned for ∼20 h in serum-free OptiMEM (Life Technologies Corp., Grand Island, NY). The conditioned media (300 mL) was spun at 1600*g* to pellet cells and concentrated 10-fold using Amicon Ultracel 3 kDa centrifugal filters. The concentrated conditioned media was subjected to immunoaffinity chromatography on Sepharose CL-4B resin (1 mL) that had been cyanogen-bromide cross-linked to mAb MM27−33.1.1, an anti-Aβ IgG1k which specifically recognizes the N-terminus of Aβ and does not recognize APP. Antibody concentration on the resin was confirmed to be ∼0.7 mg/mL by UV absorbance of the resin in a 1 mm path length cuvette. The sample was circulated through the resin overnight at 0.15 mL/min and 4 °C, and the resin was washed with 8−10 column volumes (CVs) of 0.1 M sodium phosphate, pH 7, followed by 8−10 CVs of ultrapure water. Bound species were eluted from the antibody with 0.1 M NH_4OH at a rate of 33 μL/min and collected in 0.5 mL fractions. The fractions were dried overnight on a Speed-Vac and stored at −20 °C until use.

MALDI-TOF Mass Spectrometric Analysis. A Bio-Rad ProteinChip System series 4000 (Enterprise Edition) mass spectrometer was used in MALDI-TOF mode. Samples were mixed in equal volume with the energy absorbing matrix (sinapinic acid (Acros) (25 mg/mL) in 50% acetonitrile plus 0.5% trifluoroacetic acid), and 1 μL (corresponding to 1−5 pmol Aβ) was spotted on a ProteinChip Gold Array (A-H Format). Laser intensity was adjusted to 3000 nJ.

ESI Mass Spectrometric Analysis. An aliquot of the pepsin-digested sample was diluted to 1 μM with a mixture of 50% acetonitrile/methanol:50% water:0.1% formic acid for ESI-MS analysis in a positive ion mode on a Thermo-Scientific LTQ Velos ion trap mass spectrometer (San Jose, CA) equipped with an Advion Nanomate 100 nanospray ion source (Ithaca, NY). Samples (10−20 μL) were infused via the Nanomate at a rate of 200 nL/min. Full MS spectra scanned over the range of m/z 500−2000. Ultrazoom spectra scanned a selected m/z at a much slower rate, and the higher sampling intervals achieved greater resolution and resulted in an unambiguous isotopic separation that allowed charge state assignment. A typical ultrazoom analysis scanned over a narrow range of m/z 8 centered on the ion of interest, in a Profile Data acquisition mode. Ultrazoom analyses can be done on MS ions in a selected ion monitoring (SIM) mode or on MS/MS ions in a selected reaction monitoring (SRM) mode. Tandem MS/MS spectra were also monitored over the same m/z range by

collision-induced dissociation (CID) of individual precursor ions with isolation width of m/z 1, normalized collision energy of 35 V, activation Q value of 0.25, and activation time of 10 ms at 3 microscans per scan. Precursor ion mapping on a select MS/MS product ion was performed using the parent ion map function listed under Instrument Setup option of the Thermo Xcalibur 2.1 software. PIM experiments were acquired from m/z 450−2000 with isolation width of m/z 1.5, normalized collision energy of 35 V, activation Q value of 0.25, and activation time of 10 ms at m/z 1 parent mass step per scan. Recorded data were processed and analyzed in Thermo Xcalibur 2.1 software. Tandem MS or MS/MS fragment ions were assigned based on masses generated by the MS-Product predictor tool of ProteinProspector (http://prospector.ucsf.edu/prospector/mshome.htm) from UCSF (San Francisco, CA).

RESULTS

TGase Cross-Linking and Pepsin Digestion of Aβ. To develop tools to ultimately explore endogenous oligomer structure, we first examined synthetic Aβ that had been converted to covalently cross-linked oligomers. TGase was chosen as a model for cross-linking because of its highly selective sites of action. However, it is worth noting that an elevated level of TGase activity has been reported in AD brains,[25,26,33] supporting the potential contribution of this covalent modification to the structural stability of endogenous Aβ oligomers. Although the presence of specific TGase cross-linked Aβ aggregates in AD brain has not been reported, immunochemical analyses did indicate colocalization of Aβ in senile plaques with both TGase2 and cross-links specifically generated by TGase.[33] TGase is a calcium-dependent enzyme that can catalyze formation of a covalent bond between a γ carboxamide moiety of glutamine and a free ε amino group of lysine in peptides.[34] The Aβ primary sequence contains one glutamine (i.e., Q15) and two lysine residues (i.e., K16 and K28) that are susceptible to TGase cross-linking (Figure 1).[12,35] Indeed, a number of studies have shown TGase cross-linking of APP as well as of Aβ fragments retaining these amino acid residues.[36−38]

To confirm cross-linking by TGase and identify the cross-linked Aβ residues, we previously conducted proteolytic fragmentation of Aβ(1−42) before and after treatment with TGase followed by MALDI-TOF MS.[12] We verified a report[39] that pepsin cleaves Aβ preferentially between F19 and F20, between L34 and M35, and between E3 and F4 (Figure 1). The three most prominent peptic fragments observed by MALDI-TOF MS were Aβ(1−19) ($(M + H)^+$ = 2315 Da), Aβ(4−19) (2000 Da), and Aβ(20−34) (1492 Da). Following cross-linking of Aβ(1−42) with TGase, pepsin digestion produced two additional species at 3474 and 3983 Da, corresponding to Aβ(4−19) linked to Aβ(20−34) and Aβ(4−19) linked to Aβ(4−19), respectively.[12]

ESI PIM-MS Analysis of Synthetic Aβ(1−40) Monomer Cross-Linked with TGase. We initially confirmed by ESI-MS the previously reported pepsin cleavage sites, this time with Aβ(1−40). A MS scan of pepsin-digested Aβ(1−40) monomer from m/z 500−2000 clearly showed multiply charged species of the expected fragments (Figure 2 and Table 1): $A\beta(1-19)^{+4,+3,+2}$, $A\beta(4-19)^{+4,+3,+2}$, $A\beta(20-34)^{+2}$, and $A\beta(35-40)^{+1}$. The charged species were assigned based on ultrazoom scans and MS/MS analyses. All intact monomers were digested, as no charge species of Aβ(1−40) was observed. Higher signal intensities for Aβ(1−19) relative to Aβ(4−19) species suggested that pepsin cleaved between F19 and F20 more rapidly than between E3 and F4 (Figure 2), a conclusion also reached previously.[39]

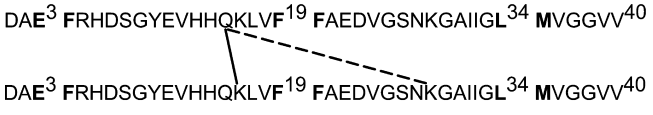

Figure 1. Amino acid sequence of the Aβ(1−40) peptide. Pepsin cleaves specifically between the pairs of residues highlighted in bold to generate Aβ(1−19), Aβ(4−19), and Aβ(20−34) fragments.[12] TGase catalyzes formation of a covalent bond between a γ carboxamide moiety of glutamine and a free ε amino group of lysine in peptides.[34] Therefore, TGase can generate a cross-link between Q15 and K16 (solid line) or Q15 and K28 (dashed line).

On the basis of the previous reports,[12,39] one would predict five possible cross-linked fragments from TGase cross-linked and pepsin-digested Aβ(1−40): Aβ(1−19)--(1−19), Aβ(1−19)--(4−19), Aβ(4−19)--(4−19), Aβ(1−19)--(20−34), and Aβ(4−19)--(20−34). Their respective masses are 4612, 4298, 3982, 3789, and 3474 Da. Our ESI-MS analyses were able to detect the last three of these cross-linked fragments (Figure 3 and Table 1): Aβ(4−19)--(4−19), [m/z 797^{+5}]; Aβ(1−19)--(20−34), [m/z 758^{+5}, 948^{+4}]; and Aβ(4−19)--(20−34), [m/z 695^{+5}, 869^{+4}, 1158^{+3}]. The latter two cross-links are unlikely to have resulted from intrapeptidyl covalent linkage because such a cross-link would result in a loss of 17 Da from the mass of Aβ(1−40) prior to pepsin digestion and no species with this mass was observed.[12] Additional peaks in Figure 3 correspond to residual noncross-linked peptic fragments observed in Figure 2 and to one additional peak $A\beta(1-15)^{+3}$ (X in Figure 3), possibly due to an additional less-specific pepsin cleavage.

Because the sites of TGase cross-linking and pepsin cleavage of Aβ(1−40) are known, the new signals corresponding to cross-linked Aβ peptic fragments in Figure 3 were easily assigned by ultrazoom scan and MS/MS analyses. With biological samples of Aβ aggregates, TGase cross-linking may not be the dominant means of covalent modification. As a result, a MS method that is not limited to recognition of Aβ fragments specific to one type of structural modification would be highly desirable in order to survey a spectrum of possible post-translationally modified, endogenous Aβ species. A MS/MS methodology that can systematically identify all precursor ions that give rise to a known Aβ product ion would be very effective in elucidating the structures of covalently modified endogenous Aβ oligomers because known MS/MS product ions can be generated both from predicted peptic fragment precursor ions derived from unmodified Aβ species and from unknown peptic fragment precursor ions produced from covalently modified Aβ species. PIM is precisely such a methodology. In a PIM experiment, a MS/MS product ion is selected and a range of m/z is scanned to determine all of its precursor ions. The identity of individual unpredicted precursor ions then can be determined by further ultrazoom and tandem MS analyses.

To demonstrate this PIM methodology, we returned to the pepsin-digested TGase-cross-linked synthetic Aβ(1−40) sample analyzed in Figure 3. Now we assumed that the covalent cross-linking modifications in our sample are not initially known and that we cannot predict the masses of the modified Aβ peptic fragments that are of interest. Unlike previous studies that utilized PIM to identify post-translational modifications by

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Table 1. m/z Assignments of Pepsin-Digested Noncross-Linked and TGase Cross-Linked Aβ(1−40) Monomer Fragments[a]

noncross-linked	symbol	m/z	cross-linked	symbol	m/z
Aβ(1−19)$^{+4}$	a^{+4}	579.8	Aβ(4−19)--(4−19)$^{+5}$	e^{+5}	797.1
Aβ(1−19)$^{+3}$	a^{+3}	772.8	Aβ(1−19)--(20−34)$^{+5}$	f^{+5}	758.1
Aβ(1−19)$^{+2}$	a^{+2}	1158.0	Aβ(1−19)--(20−34)$^{+4}$	f^{+4}	948.0
Aβ(4−19)$^{+4}$	b^{+4}	500.9	Aβ(4−19)--(20−34)$^{+5}$	g^{+5}	695.1
Aβ(4−19)$^{+3}$	b^{+3}	667.5	Aβ(4−19)--(20−34)$^{+4}$	g^{+4}	869.2
Aβ(4−19)$^{+2}$	b^{+2}	1000.3	Aβ(4−19)--(20−34)$^{+3}$	g^{+3}	1158.1
Aβ(20−34)$^{+2}$	c^{+2}	746.4			
Aβ(35−40)$^{+1}$	d^{+1}	561.5			

[a]The fragments and corresponding charges have been confirmed by MS/MS and ultrazoom scan analyses.

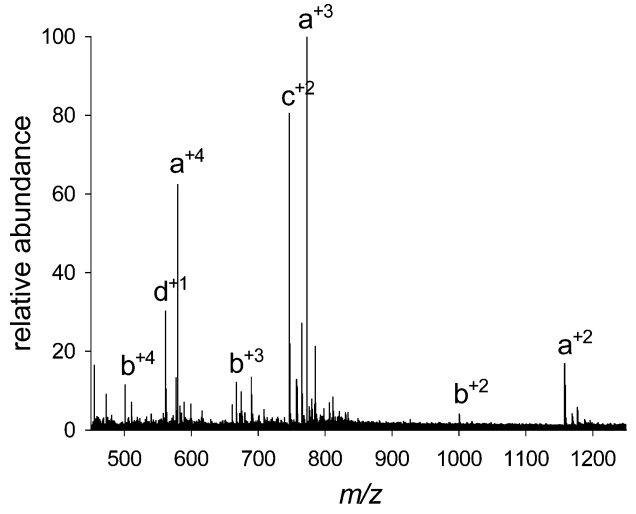

Figure 2. ESI-MS analysis of pepsin-digested Aβ(1−40) monomer. The symbols a−d represent the peptic fragments: Aβ(1−19), Aβ(4−19), Aβ(20−34), and Aβ(35−40), respectively. Refer to Table 1 for complete m/z values and assignments.

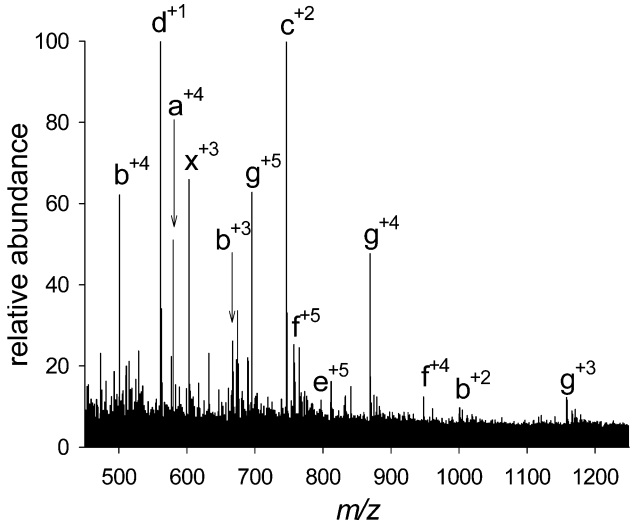

Figure 3. ESI-MS analysis of pepsin-digested TGase cross-linked Aβ(1−40) monomer. The symbols a−d represent the noncross-linked peptic fragments: Aβ(1−19), Aβ(4−19), Aβ(20−34), and Aβ(35−40), respectively, and e−g represent the TGase cross-linked fragments: Aβ(4−19)--(4−19), Aβ(1−19)--(20−34), and Aβ(4−19)--(20−34), respectively. Refer to Table 1 for complete m/z values and assignments.

relying on CID reporter ion(s) specific to the type of modification(s), such as chemical cross-linkers,[40] oxonium,[31] and immonium ions [30] for detection of peptide cross-linking, glycosylation, and phosphorylation, respectively, our PIM analyses focused on unmodified CID product ions distal from the site of modification in the precursor ion. To identify these candidate MS/MS product ions for PIM analysis, we analyzed the MS/MS spectra from each peptic fragment observed for unmodified Aβ(1−40) in Figure 2. MS/MS fragment ions m/z 992.5, 1091.5, and 1228.7, corresponding to the singly charged Aβ(4−11), Aβ(4−12), and Aβ(4−13) fragments, respectively, were relatively intense in the MS/MS spectrum of Aβ(4−19)$^{+2}$ (m/z 1000) (data not shown). The +1 charge state on these fragments was confirmed by ultrazoom scan analysis.

To conduct PIM analysis of the TGase cross-linked and pepsin digested Aβ(1−40) sample from Figure 3, we then selected the intensity of the Aβ(4−13) m/z 1228.7 product ion as a reporter for monitoring during a PIM scan over the range of m/z 450−2000. Several precursor ions were identified (Figure 4, Table 2). These included several m/z already observed in Figure 2 that corresponded to known peptic fragments from unmodified Aβ(1−40). However, additional peptic fragment precursor ions were detected that were not found in Figure 2, including m/z 869, 996, and 1075. Zoom scans indicated that these m/z corresponded to +4 charge states, and additional MS/MS analyses allowed them to be assigned as [Aβ(4−19)--(4−19)]$^{+4}$, [Aβ(4−19)--(20−34)]$^{+4}$, and [Aβ(1−19)--(4−19)]$^{+4}$, respectively (Table 2). Moreover, the quality of the PIM scan in Figure 4 was excellent. Virtually all of the major peaks in Figure 4 are assigned in Table 2, and no additional peptic fragments from Figure 3 were detected that could not produce Aβ(4−13) from a single MS/MS bond cleavage (e.g., Aβ(1−19)--(20−34), Aβ(35−40), and Aβ(1−15)). Similar results were obtained from PIM scans that monitored product ions Aβ(4−11) and Aβ(4−12) as reporters (see Supporting Information Figure S1). Preliminary analysis in our laboratory indicates that, for example, Aβ(20−24) derived from the unmodified Aβ(20−34) peptic fragment is an additional potential MS/MS reporter ion candidate (see Supporting Information Figure S2). Because the PIM methodology focuses only on a specific group of ions (e.g., only the precursor ions of Aβ(4−13)$^{+1}$), background noise from the solvent is mostly eliminated and the observed PIM spectrum has better signal-to-noise ratios than those of the original MS spectrum in Figure 3. As a result, some ions such as Aβ(4−19)--(4−19)$^{+3}$ [m/z 1328] and Aβ(1−19)--(4−19)$^{+4}$ [m/z 1075] that were suppressed or buried under noise in the original spectrum can be identified unambiguously in Figure 4.

Isolation and Purification of Aβ from 7PA2 CHO Cell Conditioned Media. The most challenging aspect in

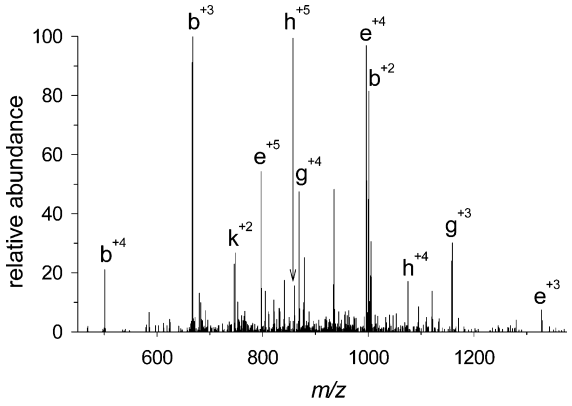

Figure 4. PIM analysis monitoring the Aβ(4−13)$^{+1}$ product ion m/z 1228.7 from pepsin-digested TGase cross-linked Aβ(1−40) monomer. The symbols b and k represent the noncross-linked peptic fragments: Aβ(4−19) and Aβ(4−15), respectively, and e, g, and h represent the TGase cross-linked fragments: Aβ(4−19)--(4−19), Aβ(4−19)--(20−34), and Aβ(1−19)--(4−19), respectively. Refer to Table 2 for complete m/z values and assignments.

Table 2. m/z Assignments of PIM Analysis of Pepsin-Digested TGase Cross-Linked Aβ(1−40) Monomer Fragments in Figure 4

peptic fragments	symbol	m/z
Aβ(4−19)$^{+4}$	b^{+4}	501.1
Aβ(4−19)$^{+3}$	b^{+3}	668.0
Aβ(4−19)$^{+2}$	b^{+2}	1001.1
Aβ(4−19)--(4−19)$^{+5}$	e^{+5}	797.0
Aβ(4−19)--(4−19)$^{+4}$	e^{+4}	996.1
Aβ(4−19)--(4−19)$^{+3}$	e^{+3}	1328.0
Aβ(4−19)--(20−34)$^{+4}$	g^{+4}	869.0
Aβ(4−19)--(20−34)$^{+3}$	g^{+3}	1159.0
Aβ(1−19)--(4−19)$^{+5}$	h^{+5}	860.0
Aβ(1−19)--(4−19)$^{+4}$	h^{+4}	1075.0
Aβ(4−15)$^{+2}$	k^{+2}	748.0

characterizing the structures of endogenous Aβ oligomers is obtaining sufficient amounts for most biophysical techniques. The Chinese hamster ovary (CHO) cell line denoted 7PA2, which expresses a mutant form of the amyloid precursor protein (APP) found in familial AD, was one of the first sources from which endogenous Aβ oligomers were isolated.[19] Aβ oligomers isolated from media conditioned by these cells have been reported to inhibit hippocampal long-term potentiation.[10] Importantly, the stability of these oligomers after boiling in 1−2% SDS corresponded to that of Aβ oligomers isolated from AD brain tissue; thus, analyses of these secreted oligomers provides a convenient bridge in progressing from structural studies of synthetic Aβ oligomers to those of brain oligomers.

The Selkoe group reported that from 75 mL of 7PA2 cell medium conditioned overnight, about 10 pmol of Aβ monomer through tetramer was obtained.[41] We increased the scale to obtain 300 mL of conditioned serum-free medium from twelve 225 cm^2 culture flasks per week and concentrated this medium to 10 mL with Amicon Ultracel 3 kDa centrifugal filters. Aβ species in this concentrated conditioned medium were immunoaffinity purified as described in the Experimental Procedures, and 0.5 mL fractions were eluted with 100 mM ammonium hydroxide. Western blot analysis of these fractions showed only Aβ monomers (data not shown). It is possible that the minute amount of Aβ oligomers in the concentrated sample either failed to bind efficiently to the resin or bound too tightly to be eluted with ammonium hydroxide. Further optimization of our procedures for isolation of endogenous Aβ oligomers is necessary to acquire sufficient amounts for MS analyses.

ESI-MS Analysis of Endogenous Aβ Monomer. We took the opportunity to analyze this immunoaffinity purified endogenous Aβ monomer to illustrate the application of ESI PIM-MS technique to an endogenous Aβ species. On the basis of ELISA, the peak eluted fraction contained about 20 pmol of Aβ. MALDI-TOF MS analysis of an aliquot of this fraction showed a range of C-terminally truncated N-terminal Aβ fragments (Figure 5). The initial ESI-MS scan of another

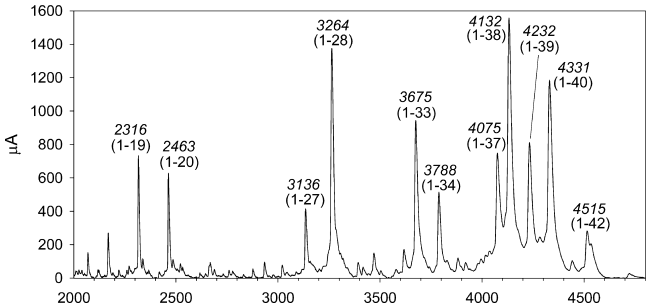

Figure 5. MALDI-TOF MS of immunoaffinity-purified endogenous Aβ monomer. Dried 50 μL aliquots were resuspended in 5 μL of matrix solution containing sinapinic acid, and 1 μL (containing 400 fmol of A$\beta(x-40)$ by ELISA) was dried on the gold sample strip. Observed masses (italics) and corresponding Aβ sequences (in parentheses) are indicated.

aliquot of this fraction was dominated by low m/z peaks <600, perhaps mAb fragments that eluted from the immunoaffinity resin, and resulted in very weak signals for Aβ peptides. A +5 charge species of Aβ(1−40) at m/z 867.1 gave one of the strongest Aβ signals but was barely visible among the noise (Figure 6A). MS/MS analysis of this Aβ(1−40)$^{+5}$ peak indicated an Aβ(1−34)$^{+4}$ product ion (m/z 942.7), which was selected for further PIM analysis. This particular fragment could be derived from all A$\beta(1-x)$ peptides, where $x \geq 35$. The resulting PIM spectrum showed excellent signal-to-noise (Figure 6B) and was in good agreement with the MALDI-TOF spectrum in Figure 5: a number of precursors from Aβ(1−35) up to Aβ(1−42) were obtained (Figure 6B and Table 3). Furthermore, m/z values corresponding to oxidized forms of several of these peptides were detected, presumably derived from oxidation of M35 to a methionine sulfoxide. Small incompletely resolved peaks corresponding to some of these oxidized peptides were also observed in the MALDI-TOF spectrum in Figure 5, but the assignments were more robust in Figure 6B.

DISCUSSION

The pathology associated with AD is thought to involve misfolding and aggregation of Aβ peptides. More than two decades of research on synthetic Aβ variants have provided some insight into their aggregated structures. However, these synthetic Aβ aggregates do not reproduce important features of neurotoxic endogenous oligomers that may contribute their higher structural stability such as potential post-translational covalent modification between peptides. Furthermore, the low levels of endogenous oligomers make their structural character-

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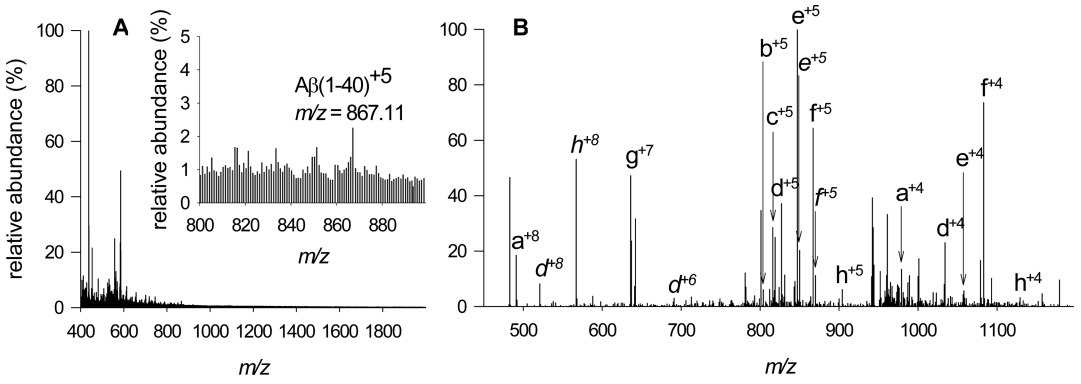

Figure 6. ESI-MS and PIM analysis of immunoaffinity-purified endogenous Aβ monomer. A dried 50 μL aliquot (containing 2 pmol of A$\beta(x-40)$ by ELISA) was resuspended in 20 μL of a mixture of 50% methanol:50% water:0.5% formic acid and injected into the LTQ Velos mass spectrometer. (A) The ESI-MS scan indicates a weak A$\beta(1-40)^{+5}$ signal (m/z 867.1). (B) PIM analysis on the A$\beta(1-34)^{+4}$ product ion (m/z 942.7) reveals precursor ions m/z and assignments indicated in Table 3. Italics indicate methionine-oxidized fragments.

Table 3. m/z Assignments of PIM Analysis on A$\beta(1-34)^{+4}$ Product Ion m/z 942.7 in Figure 6B[a]

Aβ fragment	symbol	m/z	Aβ fragment	symbol	m/z
A$\beta(1-35)^{+8}$	a^{+8}	491.0	*A$\beta(1-39)^{+5}$*	e^{+5}	850.0
A$\beta(1-35)^{+4}$	a^{+4}	979.0	A$\beta(1-39)^{+4}$	e^{+4}	1058.0
A$\beta(1-36)^{+5}$	b^{+5}	805.0	A$\beta(1-40)^{+5}$	f^{+5}	867.0
A$\beta(1-37)^{+5}$	c^{+5}	816.0	*A$\beta(1-40)^{+5}$*	f^{+5}	870.0
A$\beta(1-38)^{+8}$	d^{+8}	521.0	A$\beta(1-40)^{+4}$	f^{+4}	1083.0
A$\beta(1-38)^{+6}$	d^{+6}	691.0	A$\beta(1-41)^{+7}$	g^{+7}	636.0
A$\beta(1-38)^{+5}$	d^{+5}	827.0	*A$\beta(1-42)^{+8}$*	h^{+8}	567.0
A$\beta(1-38)^{+5}$	d^{+5}	831.0	A$\beta(1-42)^{+5}$	h^{+5}	904.0
A$\beta(1-38)^{+4}$	d^{+4}	1034.0	A$\beta(1-42)^{+4}$	h^{+4}	1129.0
A$\beta(1-39)^{+5}$	e^{+5}	847.0			

[a]The fragments and corresponding charges have been confirmed by MS/MS and ultrazoom scan analyses. Italics indicate methionine-oxidized fragments.

ization one of the most difficult albeit essential challenges in AD.

We describe here a well-known MS technique called *precursor ion mapping* (PIM) that may be applicable to structural studies of the endogenous Aβ oligomers. It is an effective tool that has been routinely used to characterize or identify various structural modifications in peptides and proteins, both in vitro and in vivo. On the basis of our review of the literature, however, our PIM approach has not yet been applied to detect post-translational modifications of endogenous Aβ oligomers or, for that matter, any other endogenous or synthetic Aβ species. Furthermore, no other analytical technique has provided structural information about endogenous Aβ oligomers despite the fact that these agents are widely regarded to be among the most important etiologic agents in Alzheimer's disease. While efforts to obtain sufficient amounts of endogenous Aβ oligomers for PIM analysis remain ongoing, it is important to demonstrate the power of this method with Aβ samples that are currently available. First, we characterized peptic fragments of a model system, TGase cross-linked synthetic A$\beta(1-40)$ monomer. We successfully identified and assigned three cross-linked fragments out of a possible five peptic fragment species from a $4-13^{+1}$. Then, we analyzed an endogenous Aβ monomer sample, which had been immunoaffinity purified from conditioned media of 7PA2 CHO cells, by PIM in order to show its applicability to biological samples. Multiple C-terminally truncated Aβ precursor ions (i.e., 1−35 to 1−42) were identified. Furthermore, the MS/MS product ion A$\beta(1-34)^{+4}$ m/z 942.7 selected for this analysis allowed a survey of all endogenous Aβ modifications that span residues 35−42. The only modification that has been reported for this region is M35 oxidation,[42−44] and the PIM analysis in Figure 6B readily detected this modification in several of the Aβ peptides. More MS studies are required before it can be concluded that this M35 oxidation is not an artifact because some MS solvents induce M35 oxidation.[45] However, given that the concentration of this endogenous monomer sample is only 1/10 that of the TGase cross-linked A$\beta(1-40)$ monomer sample in Figure 3 and that the +5 charge A$\beta(1-40)$ species of the former (Figure 6A) is barely visible (i.e., ∼2% of the total intensity) on the full MS spectrum, the relatively clean result in Figure 6B clearly showed that PIM would be a very powerful tool for studying the structures of all endogenous Aβ species including oligomers.

Success with this PIM methodology does require that the MS/MS fragment selected for monitoring not include the site(s) that is covalently modified in the endogenous Aβ oligomer of interest. Because this site is unknown, it may be necessary to investigate a number of potential product ions that can arise from Aβ peptic fragments. In the present studies, we have mainly selected the MS/MS *b* ions or N-terminal product ion fragments of Aβ peptide for PIM analysis, simply for a proof of concept. In fact, PIM analysis on the MS/MS *y* ions or C-terminal fragments of Aβ peptide may give other unexpected N-terminally truncated or modified fragments, which otherwise would not have been detected by analyzing only the *b* ions. This would be especially important for analyzing the extract from AD brain, where a wide spectrum of structural modifications to Aβ is expected.

In conclusion, the variety of prospective product ions that can be selected for PIM analysis allows this technique to be applied not only to TGase cross-linked Aβ peptides but also to other Aβ species that have been modified in vivo by endogenous cross-linking agents or post-translational modifications. From a broader perspective, knowing the structure of endogenous Aβ oligomers may greatly assist in the development both of novel diagnostic tools for preventative screening and of therapeutic treatments for slowing disease progression in AD.

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■ ASSOCIATED CONTENT

Supporting Information

PIM analysis monitoring. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Aβ, amyloid-β; AD, Alzheimer's disease; SDS/LDS, sodium(/lithium) dodecylsulfate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MS, mass spectrometry; PIM, precursor ion mapping; TGase, transglutaminase; APP, amyloid precursor protein; SEC, size-exclusion chromatography; MW, molecular weight; CHO, Chinese ovary cell; MS/MS, tandem MS; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; ESI, electrospray ionization; Da, dalton; *m*/*z*, mass-to-charge ratio; mAb, monoclonal antibody

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