The amyloid β-protein (Aβ) that is progressively deposed in Alzheimer's disease (AD) arises from proteolysis of the integral membrane protein, β-amyloid precursor protein (βAPP). Although Aβ formation appears to play a seminal role in AD, only a few studies have examined the chemical structure of Aβ purified from brain, and there are discrepancies among the findings. We describe a new method for the rapid extraction and purification of Aβ that minimizes artefactual proteolysis. Aβ purified by two-dimensional reverse-phase HPLC was analyzed by combined amino acid sequencing and mass spectrometry after digestion with a lysylendopeptidase. The major Aβ peptide in the cerebral cortex of all five AD brains examined was aspartic acid 1 to valine 40. A minor species beginning at glutamic acid 3 but blocked by conversion to pyroglutamyl was also found in all cases. A species ending at threonine 43 was detected, varying from 5 to 25% of total Aβ COOH-terminal fragments. Peptides ending with valine 38, isoleucine 41, or alanine 42 were not detected, except for one brain with a minor peptide ending at valine 39. Our findings suggest that Aβ1-40 is the major species of β-protein in AD cerebral cortex. Aβ1-40 and Aβ1-43 peptides could arise independently from βAPP, or Aβ1-43 could be the initial excised fragment, followed by digestion to yield Aβ1-40. These analyses of native Aβ in AD brain recommend the use of synthetic Aβ1-40 peptide to model amyloid fibrillogenesis and toxicity in vitro.

Alzheimer's disease (AD) is the most common cause of progressive intellectual failure in aged humans. The filamentous lesions that define AD occur within neurons (neurofibrillary tangles), in extracellular cerebral deposits (amyloid plaques), and in meningeal and cerebral blood vessels (amyloid angiopathy) (1). A peptide with a molecular weight of ~4,000, designated the amyloid β (Aβ) protein, is the subunit of vascular and plaque amyloid filaments in individuals with AD (2-9), trisomy 21 (Down's syndrome) (10) hereditary cerebral hemorrhage with amyloidosis (HCHWA)-Dutch type (11, 12), and normal brain aging (13). The cloning of cDNAs encoding the Aβ protein demonstrated that it was a fragment of a large integral membrane polypeptide, the β-amyloid precursor protein (βAPP), the gene for which is located on the long arm of human chromosome 21 (14-17). Elucidation of the exon-intron structure of the βAPP gene revealed that the Aβ protein is derived from portions of two exons and must therefore arise by proteolysis of the precursor polypeptide (18).

Several lines of evidence indicate that progressive cerebral deposition of Aβ plays a seminal role in the pathogenesis of Alzheimer's disease and can precede cognitive symptoms by years or decades (reviewed in Ref. 1). Importantly, missense mutations at residue 717 of the 770-amino acid isoform of βAPP have been found in affected members of at least nine families with autosomal dominant Alzheimer's disease (19-23). In addition, a mutation at residue 693 has been identified as the cause of HCHWA-Dutch (24). There is great interest in understanding the proteolytic processing of βAPP and identifying the proteases responsible for cleaving at the NH2 and COOH termini of the Aβ region. However, only a few studies have examined the chemical structure of the native Aβ protein purified from human brain, and there are discrepancies among the reported findings. Sequencing of Aβ isolated from meningeal vascular amyloid has consistently yielded an aspartic acid (position 672 of βAPP) as the amino terminus (2, 7-10, 12). However, there has been disagreement about the sequence of compacted amyloid plaque cores. One laboratory reported ragged amino termini from cores isolated using a protocol that includes overnight digestion with pepsin, with the major species beginning with the phenylalanine at position 4 of Aβ (3). Another laboratory could not obtain NH2-terminal sequence from cores purified in a sodium decyl sulfate-containing buffer without protease treatment and postulated a blocked amino terminus in compacted plaque amyloid (4). Similarly, two other laboratories obtained no interpretable NH2-terminal sequences from plaque cores isolated by other methods (6, 9). The precise carboxyl terminus of Aβ has likewise been in dispute. Two groups reported the valine at Aβ position 40 as the COOH terminus of meningeal vascular amyloid in Alzheimer's disease (7, 9), and another laboratory identified valine 39 as the last residue of meningeal vascular amyloid in AD (8) and in HCHWA-Dutch (12). The laboratory that reported the NH2-terminal sequencing of amyloid plaque cores (3) identified alanine 42 or threonine 43 as the COOH terminus (15).

In view of the potential therapeutic importance of inhibiting the proteolytic cleavages that liberate the NH2 and COOH termini of Aβ in Alzheimer's disease, the precise chemical
nature of the native peptide must be established. We report a new method for the rapid isolation and purification of intact Aβ protein that minimizes the likelihood of artifactual proteolysis during the preparation. The purified Aβ was analyzed by both amino acid sequencing and mass spectrometry, the latter method allowing precise determination of the lengths of the cerebral Aβ proteins. Our results explain and resolve several previous discrepancies about the nature of Aβ protein in Alzheimer’s disease.

MATERIALS AND METHODS

Purification of Aβ Protein from Cerebral Cortex—Aβ protein was prepared from frozen (−80 °C) cerebral cortex of 15 histopathologically confirmed cases of AD. Mini-scale preparations (30–50 mg wet weight starting cortex) followed by immunoblotting with an antibody to Aβ1–42 (25) revealed that 5 of the 15 brains had high contents of Aβ, and these were used for the purification and quantitative analysis described here. Frozen cortex (1–5 g) was homogenized in a buffer containing 10% SDS, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.6, and the protease inhibitors leupeptin (1 μg/ml), pepstatin (0.1 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), DFP (0.1 mM), and 1-chloro-2,4-dinitrobenzene (1 μg/ml). The homogenate was centrifuged at 100,000 × g for 40 min (20 °C). The pellet was rehomogenized in the same buffer and sedimented again. This pellet was washed with 2% SDS, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.6, and recentrifuged. The washed pellet was extracted in 100% formic acid (3) for 40 min (20 °C). After centrifugation at 100,000 × g for 20 min, the Aβ protein was obtained in the formic acid-soluble fraction. This supernatant was chromatographed on a C4 reverse-phase HPLC column (Baker, Inc.) according to our previous method for purifying fragments of ubiquitin from Alzheimer paired helical filament preparations (20). The Aβ-immunoreactive peak was then chromatographed on the same column but using a different elution gradient (see “Results”). The final purified Aβ was confirmed as such by SDS-polyacrylamide gel electrophoresis (Coomassie Blue staining) and by immunoblotting with a rabbit polyclonal antibody raised to synthetic Aβ1–42.

Immunocytochemical staining with anti-Aβ1–42 was conducted on slices of cortex taken at autopsy directly from the fresh brain specimen before freezing the latter for the biochemical experiments. These slices, which were thus immediately adjacent to the tissue used to purify Aβ, were fixed briefly (3 h) in neutral buffered formalin, sectioned on a Vibratome, and immunostained conventionally (23).

Fragmentation of Aβ Protein with a Lysylendopeptidase—Purified Aβ protein (100 μg) was digested with the lysylendopeptidase Achromobacter protease I (API), 2 μg in 500 μl of 2 M urea, 50 mM Tris-HCl, pH 9.0, for 15 h (37 °C). The reaction was stopped by adding guanidine-HCl to 6 M, trifluoroacetic acid to 0.1%, and 2-mercaptoethanol to 0.1%. After filtration through Millex GV (Millipore), the sample was injected on a 4.6 × 250-mm butyl (C4) HPLC column (Baker) and eluted with a linear gradient of 0–80% isopropanol/acetoneitrile (7:3) in 0.1% trifluoroacetic acid.

Pyroglutamate Aminopeptidase Reaction—After mass spectrometry analysis (see “Results”) of fraction B from the above C4 column, the nitrocellulose target was extracted 5 times with 50% isopropanol. The resultant extract was treated with pyroglutamatepeptidase (1-pyroglutamyl peptide hydrolase, EC 3.4.19.3) (0.4 milliunits, Nakarai Tesque Co.) in 10 mM sodium phosphate buffer, pH 7.8, 1 mM EDTA and 10 mM dithiothreitol for 5 h (37 °C). The reaction was stopped by addition of formic acid, and the fraction was applied to a C4 reverse-phase HPLC column. A new fraction that eluted with a shorter retention time than the original fraction B was collected and subjected to amino-terminal sequencing.

Protein Sequencing—HPLC-purified Aβ protein and its API-generated fragments were sequenced on an Applied Biosystems 477A/120A protein sequenator. Some fragments were sequenced after analysis by mass spectrometry (see “Results”).

Mass Spectrometry—The purified Aβ protein or its API-generated fragments were applied to nitrocellulose targets, dried by flushing with nitrogen gas, and rinsed with 0.1% trifluoroacetic acid. Plasma desorption mass spectrometry was performed on a Bio Ion 20 Biopolymer Mass Analyzer (Applied Biosystems). Atomic masses were determined after calibration with hydrogen (H) and nitrous oxide (NO) in each spectrum. The theoretical molecular weights of the peptide fragments were calculated as their singly protonated molecular ions. An acceleration voltage of 15 kV was used. The channel resolution was 0.66 and 1.5 atomic mass units at m/z 1,000 and 5,000, respectively.

RESULTS

Mass Spectrometry of Purified Aβ Protein—Aβ protein was purified from AD cerebral cortex by two sequential reverse-phase HPLC runs (Fig. 1). The first run used a linear gradient of 0–80% isopropanol/acetonitrile (7:3) in 0.1% trifluoroacetic acid. The fraction containing Aβ protein (Fig. 1A, arrow), as judged by Western blotting with anti-Aβ1–42, was then rechromatographed using a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid (Fig. 1B). The purified Aβ protein from the second HPLC run was then analyzed by mass spectrometry as described under “Materials and Methods” (Fig. 2). An unusually broad peak was observed that distributed asymmetrically around a mass of 4356.7, suggesting that Aβ protein was not homogeneous. Protein sequencing of this fraction from the second HPLC run yielded Asp-Ala-Glu-Phe-Arg-His as the first 6 amino acids. Taken together, these data suggest that the major molecular species of Aβ protein was a 40-residue peptide beginning with Asp-1 and ending with Val-40. We next attempted to obtain proteolytic fragments of Aβ protein in order to determine formally the precise chemical structure of its amino and carboxyl termini.

NH2-terminal Sequencing of Lysylendopeptidase-generated Fragments of Aβ Protein—Purified Aβ protein was digested...
Mass Spectrometry of Purified Amyloid β Protein in AD

FIG. 2. Mass spectrometry of purified Aβ protein. Aβ protein was isolated and purified by HPLC as shown in Fig. 1 and as described under "Materials and Methods." The sample was applied to the nitrocellulose target and analyzed with a Bio-Ion 20 mass analyzer as described under "Materials and Methods."

as previously reported (26, 27) with the protease API, which specifically cleaves peptide bonds between lysine and the adjacent COOH-terminal amino acid. Since Aβ has 2 lysines in its sequence (positions 16 and 28), at least three fragments were expected: β1-16, β17-28, and β29-42 (or β29-42/3 if one assumes the chemical structure of Aβ is Asp-1 to Ala-42 or Thr-43; Ref. 15). The resultant fragments were separated by reverse-phase HPLC, as described under "Materials and Methods."

FIG. 3. HPLC profile of lysylendopeptidase-derived Aβ peptide fragments. Purified Aβ protein was digested with lysylendopeptidase at 37 °C for 15 h as described under "Materials and Methods." The digest was chromatographed on a C4 reverse-phase column with a linear gradient of 0–80% isopropanol/acetonitrile (7:3) in 0.1% trifluoroacetic acid for 40 min. Each fraction (A–D) was analyzed by amino acid sequencing and by mass spectrometry. The partial amino acid sequences for fractions A, C, and D were D-A-E-F-R, L-V-F-F-A-E-D, and G-A-I-I-G, respectively. Fraction B failed to yield any sequence (see text). All peaks besides A-D contained no protein and were also present in runs conducted without loaded protein; they presumably represent solvent-derived signals.

Mass Spectrometry of the Lysylendopeptidase-generated Fragments of Aβ Protein—Mass spectra of the lysylendopeptidase-derived Aβ fragments (fractions A–D) are shown in Fig. 4. A mass of 1956.4 was observed for fraction A (Fig. 4A). This value agreed well with the theoretical molecular weight of 1956 for the peptide Asp-1 to Lys-16. Taken together with the protein sequencing data for this fraction, fraction A was concluded to be the amino-terminal fragment (β1-16) of the Aβ protein.

A mass of 1752.5 was observed for fraction B (Fig. 4B). This value did not correspond to any of the masses predicted for lysylendopeptidase-generated fragments of the Aβ protein. A computer search suggested that the peptide Glu-3 to Lys-16 might constitute fraction B, because its theoretical molecular weight, 1770, was within approximately 18 atomic mass units of the experimentally observed mass of 1752.5. We speculated that this mass difference might be the result of dehydration of glutamic acid to form pyroglutamate. Such a modification could also explain the failure of the NH₂-terminal sequencing of this fragment. In an attempt to confirm the presence of pyroglutamate as the first amino acid of the peptide in fraction B, the fraction was treated with pyroglutamylpeptidase followed by protein sequencing. As expected, Phe-Arg-His-Asp-Ser were identified as the second through sixth amino acids of this peptide. Hence, we conclude that fraction B was another amino-terminal fragment (β1-16) of Aβ protein that was blocked at position 3, and that this fragment represented a minor species (15–20%) of the total Aβ NH₂-
terminal fragments, as judged from the HPLC chromatogram (Fig. 3). Fraction C produced the spectrum shown in Fig. 4C. The mass of 1326.3 corresponds well with that predicted for the peptide Leu-17 to Lys-28, 1326.5. The predominant peak at 1347.5 is consistent with the sodium-cationized molecular ion of this peptide. Taken together with the protein sequence data, fraction C was concluded to contain the middle portion (β^{29-43}) of Aβ protein without any detectable modification.

The mass spectrometric analysis of fraction D showed two families of peaks separated by ~285 atomic mass units (Fig. 4D); both had the same amino-terminal sequence (see above). The ions at masses 1085.9 and 1370.1 correspond closely to the theoretical masses for β^{29-40} (1086.4) and β^{29-43} (1371.7), respectively. The theoretical mass difference between these, 285.3, agrees well with the difference observed experimentally (284.2). The signals of mass 1104.8 and 1123.7 were thought to represent essentially the same peptide as that of mass 1085.9, with the former likely to represent the sodium-cationized molecular ion of β^{29-40}. The mass of signal 1390.7 is also consistent with the sodium-cationized form of the peptide of mass 1370.1. Fig. 4D shows the spectrum of the one case among the five Alzheimer cases we examined that had the highest peak for the Gly-29 to Thr-43 peptide. In all five brains, the latter peptide was consistently present, although the content of this peak varied from 5 to 25% of the total COOH-terminal fragments. Taken together with the protein sequence data for this fraction (above), the two molecules were concluded to be the Aβ carboxy-terminal fragments, β^{29-40} and β^{29-43}, respectively.

We failed to detect peptides with Ile-41 or Ala-42 as the last amino acids of Aβ protein, although care was taken to search for ions of 1199.5 or 1270.6 that would correspond to peptides with the sequence Gly-29 to Ile-41 or Gly-29 to Ala-42, respectively.

Taking all the data into account, Aβ protein was principally composed of the 40-residue sequence Asp-1 to Val-40, associated with a minor species of the peptide, pyroglutamate-3 (pGlu-3) to Val-40. The longest Aβ protein species detected extended to Thr-43 at its carboxyl terminus.

**DISCUSSION**

The new method for purifying Aβ protein from human brain reported here provides a faster, more efficient means of isolating intact Aβ protein because it does not require an incubation time of 1–2 days for digestion with proteases like pepsin (3) or collagenase (2, 7, 10), nor the time for gel filtration prior to HPLC purification (2, 10). Homogenization of frozen tissue directly into a high concentration of SDS in the presence of multiple protease inhibitors was designed to minimize possible postmortem proteolysis by an exopeptidase. On the other hand, it is possible that some degree of NH₂-terminal heterogeneity may occur in vivo in amyloid deposits of varying age and location among AD subjects. There is evidence of allelic heterogeneity among the autosomal dominant AD families genetically linked to chromosome 21 that have been examined to date (19, 22, 23, 32). In addition, many cases have no identifiable genetic basis, making it probable that environmental factors (for example, head trauma) (Ref. 33) might play a role in initiating some cases of widespread cerebral β-amyloidosis. Although deposition of Aβ in plaque-like deposits is an invariant feature of AD, biochemical microheterogeneity in the composition of these deposits, including the presence of longer βAPP fragments that contain the Aβ region, cannot
be ruled out and might be expected.

Regarding the carboxy terminus of Aβ, we found the major species of cortical Aβ to end at residue Val-40 in all five cases examined. However, a minor species ending at Thr-43 was detected in each case, varying in amount from ~5 to 25% of the total COOH-terminal fragments. We failed to detect peptides ending with Val-39, Ile-41, or Ala-42 in these five cases, except for one case that had a small amount of Aβ29-39 fragment after lysylendopeptidase digestion (data not shown).

Our current data are consistent with at least two possibilities: 1) that Aβ29-43 is the initial amyloid fragment excised from βAPP, and that many of these molecules are digested by carboxypeptidase(s) in the tissue to yield a major species of Aβ31-40, or 2) that Aβ31-40 and Aβ39-43 arise independently from precursor molecules of different cellular origin (e.g. vascular versus neuronal) or in different anatomical sites (e.g. neurons in one cortical layer versus another). Available data indicate that vascular deposits can contain Aβ31-40 (7, 9), but these are unlikely to be the sole source of the abundant Aβ31-40 molecules in our extracts, since microvascular deposits were less abundant than diffuse and compacted plaques in the cases we studied.

The mechanisms by which the known mutations in human βAPP (βAPP695 Glu → Gln; βAPP717 Val → Ile, Val → Gly or Val → Phe) lead to enhanced cerebral and vascular deposition of Aβ are currently unclear. One report has suggested that synthetic Aβ peptides containing the βAPP695 Gln substitution form fibrils more rapidly in vitro than the wild-type peptide (34). The βAPP717 mutations occur carboxy-terminal to the Aβ peptides that we have detected to date in AD cerebral cortex. However, it may be that future studies will identify minor species of extracellular Aβ peptides that extend beyond Thr-43, toward the carboxy terminus of βAPP. This possibility raises the question of whether several distinct proteases may be involved in the progressive processing of amyloidogenic βAPP fragments intracellularly and extracellularly prior to the occurrence of the apparently stable Aβ31-40 or Aβ31-42 peptides. The peptide composition of Aβ deposits in Alzheimer brain tissue must be understood in detail if drugs designed to prevent the formation of Aβ peptides are to be truly effective.

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