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Isolation, Chemical Characterization, and Quantitation of Aβ 3-Pyroglutamyl Peptide from Neuritic Plaques and Vascular Amyloid Deposits

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From the neuritic plaques and vascular walls of the brains of patients with Alzheimer disease, we have purified and quantified an Aβ peptide which starts at residue 3Glu in the form of pyroglutamyl (Aβ3pE). The N-terminally truncated Aβ3pE comprised 51% of the Aβ in the neuritic plaques. This was followed by 30% starting at position 1Asp which included 20% in the isomerized form (IsoAsp). In contrast, the vascular amyloid only contained an average of 11% in the form of Aβ3pE with the major component starting at residue 1Asp (69%), which included only 6% in the form of IsoAsp. The presence of Aβ3pE has important structural consequences since it is more hydrophobic than other forms of Aβ, thus increasing the insolubility of Aβ. In addition, Aβ3pE, with its blocked N-terminus to the action of common aminopeptidases, may result in the profuse accumulation of Aβ in the neuritic plaques of Alzheimer disease.

The conditions leading to the formation of cytotoxic soluble and aggregated beta-amyloid (Aβ) peptide in Alzheimer disease (AD) still remain a mystery (1). However, it is widely accepted that the obnoxious effects of Aβ are due to a higher content of an antiparallel β-pleated sheet. In addition, post-translational modifications in the amino acid sequence of Aβ such as isomerizations, racemizations, oxidations and N-terminal degradations, have been reported (2-5) which may play a role in the stability of Aβ. As a continuation of our studies dealing with the molecular structure of Aβ, in this communication we give an account of the purification, chemical characterization and quantitation of Aβ with a residue of pyroglutamyl at position 3 (Aβ3pE), isolated from both the amyloid of the neuritic plaques and the leptomeningeal vascular deposits.

MATERIALS AND METHODS

Human tissue. In this study we utilized a total of nine brains from demented individuals who died of AD. The brains were removed in the immediate post-mortem (delay 1-3 h). In all cases the neuropathology reports indicated a large number of neurofibrillary tangles and neuritic plaques to meet the histopathological criteria for the diagnosis of AD as stipulated by the NIH Neuropathology Panel for AD (6) and by the Consortium to Establish a Registry for AD (7).

Methodology. Neuritic plaque cores were purified in three independent occasions. In each preparation, three cerebral hemispheres were utilized. The amyloid cores were isolated following procedures previously outlined (2) with some minor modifications. In brief, cerebral hemispheres were coronally sliced (1 cm thick), and the leptomeninges carefully removed and set aside for the preparation of vascular amyloid. The cerebral cortex was dissociated from the underlying white matter, finely minced and incubated in 10 volumes of disaggregation buffer (DAB-S buffer) containing 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2mM EDTA, 200 μg/ml phenylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7μg/ml pepstatin, 50 μg/ml gentamicin sulfate and 0.25 μg/ml amphotericin B. After filtration through a series of stainless steel meshes the suspension was submitted to 1.2 and 1.9 M sucrose separations (2). The pellets collected at the top of the tubes were washed three times with Tris-HCl buffer with 2mM $CaCl_2$ and submitted to collagenase and DNase digestion followed by SDS lysis under the conditions previously described (2). The SDS insoluble material, containing the cores of amyloid, was recovered by centrifugation and the pellet resuspended in 30 volume of 1.1M sucrose and centrifuged at 135,000 $\times$ g for 1 h. The amyloid cores were further purified by sucrose density gradient centrifugation (2), followed by their solubilization in 80% formic acid and fractionation by size exclusion FPLC on a Superose 12 column. The fractions carrying the Aβ peptides were concentrated by vacuum centrifugation and dialyzed against water and then ammonium bicarbonate (100 mM). The Aβ was digested by trypsin, at an approximate enzyme substrate ratio of 1:50, for 15 h at 37°C and freeze dried. The resulting tryptic peptides were separated by C18 RP-HPLC. Peptides were submitted to automatic amino acid analyses and mass spectrometry. Two synthetic peptides, Aβ 3-5 and Aβ 3-42, both containing pyroglutamic acid at position 3 were used as references. These peptides, as well as Aβ 1-40, Aβ 1-42, Aβ 1-42 (1IsoAsp, 7IsoAsp) and Aβ 17-42, were custom made by California Peptide Research Inc. (Napa, CA).

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Mass spectrometry of Aβ peptides were obtained by matrix-assisted laser desoption ionization on a Kratos Kompak III spectrometer. The specimens (0.3 μl) were loaded on the sample slide followed by the addition of 0.3 μl of a saturated solution of α-cyano-4-hydroxycinnamiic acid in 50% ethanol. The spectra of the Aβ peptides, 50 average shots, was obtained at 20kV extraction voltage.

The vascular amyloid was isolated from the leptomeningeal membranes from nine cerebral hemispheres of individuals with histopathologically confirmed AD utilizing the technique previously described by our laboratory (8). Solubilization of amyloid was achieved by the addition of 12 ml of 80% glass distilled formic acid and after standing at room temperature for 15 min the specimen was centrifuged at 500,000 $\times$ g at 10°C for 20 min. The formic acid volume was reduced by vacuum centrifugation to approximately 3 ml and dialyzed (dialysis tubing 1000 Da cutoff) against 2 changes, 1 liter each, of 5 M guanidine-HCl, 100 mM Tris-HCl, pH 8.5 (2). Small amounts of solid Tris were added to the second buffer change to obtain a final pH of 7.5. The white fluffy precipitate mainly formed by Aβ 1-42 and other insoluble material was recovered by centrifugation. The more soluble Aβ 1-40 remained as a soluble fraction in the supernatant. Both specimens were submitted to FPLC chromatography on a Superose 12 column (2). The fractions containing the Aβ peptides were subsequently studied and characterized as described above for the neuritic plaque Aβ.

RESULTS

The neuritic plaque and vascular amyloids were chromatographed on a Superose 12 column to separate the Aβ from other contaminants. The Aβ peptides were digested by trypsin and separated by RP-HPLC (Figure 1). The amino acid composition of these peptides was established by automatic amino acid analyses and by mass spectrometry (Table 1). A number of these peptides accounted for a heterogeneous Aβ starting at position 1Asp, 2Ala, 3Glu, 8Ser and 9Gly. As can be appreciated in Figure 1 and Table 1, the tryptic peptide residues 1-5 was duplicated, due to the partial modification of Asp to IsoAsp (2). The shift of the peptide bond from the Asp α-carboxyl to the Asp β-carboxyl group, confers to the IsoAsp peptide a shorter chromatographic retention time, under the employed experimental conditions (2). Amino acid analysis of the tryptic peptide number 4 yielded the Aβ sequence of residues 3-5 (Glu-Phe-Arg). However, the molecular mass of this peptide indicated the loss of 18 mass units, which resulted from the cyclization of the N-terminal Glu to yield a pyroglutamyl derivative (3pE). As shown in Figure 1, the chromatographic retention time of the synthetic peptide 3pE-5 was exactly the same as that of the Aβ derived 3pE-5 peptide (18.7 min). Figure 1 and Table 1 also show the chromatographic profile and mass spectrometry of the six tryptic peptides resulting from the N-terminal domain, residues 1-16, of the leptomeningeal vascular Aβ. In this case, the N-terminus appears to be less degraded and only minimally modified with most of the Aβ peptide starting at residue 1Asp. In three independent Aβ preparations (see Table 2), the percentage of Aβ3pE yielded an average ratio of 51% for a total of nine AD brains investigated. This was followed by 30% starting at residue 1Asp, which included the isomerized form (20%). In contrast, the vascular Aβ (Table 2) only contained an average of 11% in the form of 3pE, with the major component starting at position 1Asp (69%), which included both the Asp and IsoAsp (63% and 6%, respectively). The remaining 20% corresponded to the Aβ starting at position 2Ala.

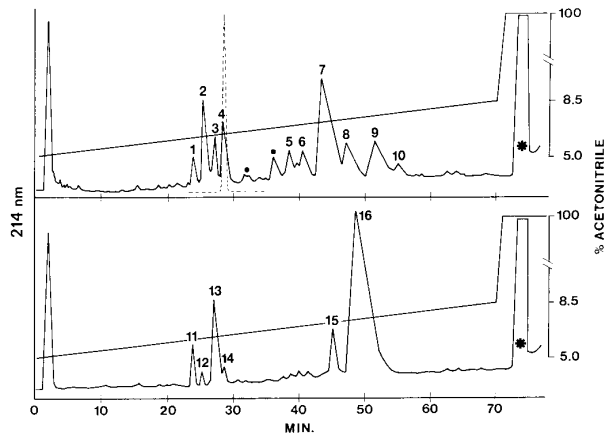

FIG. 1. Chromatographic profiles of the tryptic peptides derived from neuritic plaque core Aβ (top panel) and vascular amyloid (bottom panel). Both HPLC were performed on an LKB-RP-ODS C18 column (125 mm $\times$ 4 mm, 3 μm bead size) using a water/ 0.05% TFA as solvent A and acetonitrile/ 0.05% TFA as solvent B. A linear gradient was developed from 5% to 8.5% of solvent B in 70 min at a flow rate of 0.7 ml/min, at room temperature and the signal recorded at 214 nm. In the chromatograms the tryptic peptides derived from the neuritic plaque Aβ are numbered from 1 to 10, and those derived from the vascular Aβ are number from 11 to 16. Their respective amino acid sequences and molecular masses are presented in Table 1. The relative quantities of the peptides containing N-termini Aβ sequences were calculated from their underlying areas using the SpectraSystem Software PC1000 from Thermo Separation Products. Since the chromatograms were recorded at 214 nm, the number of the peptide bonds was considered in the final estimation. The relative yields of these peptides is shown in Table 2. The peptides numbered as 2 and 12 corresponded to residues 1-5 with 1Asp as IsoAsp, as previously described (2). The peptides numbered as 4 and 14 have their N-termini Glu as pyroglutamyl. The chromatographic retention time of these peptides (18.7 min) perfectly overlapped with the synthetic peptide: pyroglutamyl-phenylalanyl-arginine (hatched peak). Peptides numbered as 7 to 10 (neuritic plaque Aβ) corresponded to Aβ sequences of residues 6-16 containing at position 7: L-IsoAsp, D-IsoAsp, L-Asp, D-Asp. Peptides 15 and 16 (vascular Aβ) contained L-IsoAsp and L-Asp, respectively. The two dots on the top chromatogram indicate peptides derived from trypsin autolysis. The asterisks indicate fractions containing the tryptic peptides residues 17-28 and 29-40. The insoluble tryptic peptide residues 29-42 was further cleaved by CNBr. These peptides were separated by extending the acetonitrile gradient to 60% in 30 min (data not shown) as previously described (2, 8).

DISCUSSION

Several laboratories (4, 10-12) including our own (2, 9), have reported the presence of Aβ with multiple N-termini which suggests a variable degree of *in situ* degradation by amino-peptidases. The presence of 3pE was

TABLE 1

Mass Spectrometry of Aβ Tryptic Peptides Derived from Neuritic Plaque Core and Vascular Amyloid

Sequence	MALDI −MS	M_r (Calculated)	Aβ residues
Neuritic plaque Aβ peptides			
1 AEFR	522.9	522.6	2-5
2 DAEFR	637.7	637.7	1IsoD-5
3 DAEFR	637.5	637.7	1-5
4 pEFR	433.6	433.5	3pE-5
5 GYEVHHQK	998.1	998.1	9-16
6 SGYEVHHQK	1085.7	1085.2	8-16
7 HDSGYEVHHQK	1338.0	1337.4	6-16
8 HDSGYEVHHQK	1337.4	1337.4	6-16
9 HDSGYEVHHQK	1338.4	1337.4	6-16
10 HDSGYEVHHQK	1337.4	1337.4	6-16
Vascular Aβ peptides			
11 AEFR	522.2	522.6	2-5
12 DAEFR	635.5	637.7	1IsoD-5
13 DAEFR	637.3	637.7	1-5
14 pEFR	433.5	433.5	3pE-5
15 HDSGYEVHHQK	1335.9	1337.4	6-16
16 HDSGYEVHHQK	1336.7	1337.4	6-16

initially detected by mass spectrometry in complex mixtures of Aβ peptides (4,11), but because of its low yield it was considered a minor component. However, immunochemical studies suggested that Aβ3pE was one of the major peptides deposited in the AD and Down's syndrome brains (13,14). The presence of Aβ3pE has important structural consequences since it blocks the N-terminal region to degradation by common amino-peptidases. In addition, the isomerization of residues 1Asp and 7Asp, the latter representing up to 75% of the neuritic plaque amyloid (2) also inhibit enzymatic degradation of Aβ (15).

A pyrrolidonecarboxyl peptidase capable of removing N-terminal pyroglutamyl exist in normal and AD brains at comparable levels (16,17). Failure to hydrolyze 3pE from the AD brain by this enzyme could result from conformational steric hindrance. The removal of Aβ residues 1Asp and 2Ala and the cyclyzation of 3Glu results in the loss of one positive and two negative charges, and a loss in polar groups. These losses render Aβ3pE-42 more hydrophobic than Aβ1-40 and Aβ1-42, adding to its molecular insolubility. We explored the differences in polarity of various synthetic Aβ peptides on a reverse-phase C3 chromatographic column. The results indicate that the most polar peptide is Aβ1-40, followed by the cluster of Aβ1-42(1IsoAsp, 7IsoAsp), Aβ1-42 and Aβ3pE-42, and finally by the most hydrophobic of them all, Aβ17-42. By this criterion the differences between Aβ1-42 and Aβ3pE-42, appear to be minimal. However, we have observed that, at equimolar quantities, Aβ3pE-42 aggregates more readily than Aβ1-42. We have also found that isomerization of Asp in Aβ1-42 also leads to a faster rate of aggregation. Thus, the described post-translational modifications may promote the accumulation of Aβ peptides in the form of insoluble cores in the neuritic plaques.

The origins of N-terminally modified Aβ3pE-42 remains obscure. *In vitro* and *in vivo* experiments have not clarified whether the post-translational modifications occur intra-cellularly and/or extracellularly. A mass spectrometry study of secreted Aβ produced by cultured cells described over 40 N- and C-terminally truncated peptides in addition to Aβ1-40 and Aβ1-42, but no detectable Aβ3pE (18). Several laboratories have reported degraded Aβ by a variety of proteases (19-21), but have yet to determine if these activities are responsible for the truncated peptides found in the neuritic plaques. Recent studies have suggested that microglia may terminally truncate Aβ1-42 associated with plaques (22), yielding Aβ1-40. In Down's syndrome, Aβ3pE-42 appears in plaques before Aβ1-42 (14). No obvious protease or microglial activity correlates with the appearance of Aβ3pE-42 (22). However, its early appear-ance suggest Aβ3pE-42 as a nidus for the development of the neuritic plaque.

Although fibrillar Aβ kills neurons in culture (23), another cell type, smooth muscle cells are killed by soluble Aβ1-42 (24). In addition, soluble Aβ1-42 suppresses acetylcholine synthesis in primary cultures of rat basal forebrain cholinergic neurons (25). Enhancing the aggregation of Aβ, even marginally by amino-terminal deletions (26), may favor deposition reducing the diffusion of soluble Aβ. We and others have also ob-

TABLE 2

Percentage of Aβ N-terminal Tryptic Peptides from Neuritic Plaque Core and Vascular Amyloid

Sequence	Aβ residues	A[a]	B[a]	C[a]	Average
Neuritic plaque Aβ peptides					
1 AEFR	2-5	9	7	9	8
2 DAEFR	1IsoD-5	24	13	22	20
3 DAEFR	1-5	11	7	11	10
4 pEFR	3pE-5	46	62	45	51
5 GYEVHHQK	9-16	5	4	6	5
6 SGYEVHHQK	8-16	5	7	7	6
Vascular Aβ peptides					
11 AEFR	2-5	23	19	20	20
12 DAEFR	IlsoD-5	5	7	5	6
13 DAEFR	1-5	60	64	65	63
14 pEFR	3pE-5	12	10	10	11

[a] A, B, C represent three independent neuritic plaque amyloid preparations; D, E, F represent three independent vascular amyloid preparations.

served that N-terminal truncation also renders Aβ less able to activate complement (27). Thus, N-terminal truncation may confer a degree of protection to brains in which Aβ1-42 is made. The absence of inflammatory reactions around early plaques in Down's syndrome (28), which reportedly are composed of Aβ3pE-42 (14), may suggest that N-terminal truncation of Aβ1-42 represents a means of defense in the brain parenchyma.

The presence of pyroglutamyl and aspartyl isomerizations, which are the predominant modifications observed in the cores of amyloid of neuritic plaques, apparently increase the insolubility and resistance of Aβ peptides to proteolytic degradation. If these modifications occur soon after Aβ cleavage from βAPP, they may promote amyloidogenesis. Alternatively, these modifications could occur after fibrillogenesis as a factor of time. In both scenarios they would increase the proteolytic resistance and stability of Aβ leading to amyloid accumulation in the Alzheimer disease brain.

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