The Alzheimer A\(\beta\) Peptide Develops Protease Resistance in Association with Its Polymerization into Fibrils*  

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An intriguing property of the polypeptide constituents of amyloid is that they apparently can escape the proteolytic mechanisms that normally catalyze turnover and prevent abnormal tissue accumulation of polypeptides. Here, we demonstrate that the A\(\beta\) peptide, the principal component of cerebrovascular amyloid deposits in Alzheimer's disease, becomes resistant to an array of proteases as a result of structural changes associated with its polymerization into amyloid fibrils. It is further demonstrated that fibril formation per se does not lead to protease resistance but probably structural changes associated with polymerization. The results suggest that higher order structural changes, regulated by the primary structure, enable amyloidogenic polypeptides to escape proteolytic degradation and accumulate in tissues.

Amyloid is a term used to describe proteins with specific ultrastructural and tinctorial properties accumulating in the body in the course of certain diseases (1, 2). The amyloid deposits contain polymerized protein with a characteristic fibrillar substructure. In contrast to most proteins, amyloid proteins are apparently not degraded by the proteolytic defense mechanisms that prevent tissue deposition of excessive or aged proteins. It has therefore been suggested that amyloidosis represents a pathway for final tissue deposition of proteins (3).

Alzheimer's disease is associated with parenchymal and cerebrovascular amyloidosis (4). The principal component of the amyloid, the A\(\beta\) peptide (5–7), is generated through proteolytic processing of the Alzheimer A\(\beta\) amyloid precursor protein (APP), a large multidomain protein composed of a short cytoplasmic domain, a single transmembrane domain, and a long intraluminal or extracellular domain. The A\(\beta\) peptide corresponds to parts of the transmembrane and intraluminal or extracellular domains (8–14). APP can be degraded by at least two separate pathways. One leads to cleavage of the APP molecule within the A\(\beta\) domain (15) and precludes amyloidogenesis, whereas the other generates intact A\(\beta\) (16–19). The principal structural variants of the A\(\beta\) peptide contain 39–43 amino acid residues (5–7, 20, 21). Incubation of synthetic A\(\beta\) peptide in physiological solution leads to formation of fibril-like structures similar to those seen in amyloid formed in vivo. In addition to polymerization, fibril formation involves conformational changes such as adoption of \(\beta\)-sheet structures (22–24). Polymerization and fibril formation involve a multitude of structural changes. We will therefore refer to all structural alterations associated with polymerization and fibril formation as "higher order structural changes."

In vitro, the A\(\beta\) peptide has been found to be sensitive to proteolytic enzymes (7, 20, 21). How it is able to escape the proteolytic mechanisms that catalyze degradation and turnover of proteins in the body and form deposits in the brain is still largely unknown. Here, we have studied if higher order structures may be important in regulating the susceptibility of A\(\beta\) to proteolysis.

**EXPERIMENTAL PROCEDURES**

Materials—Synthetic A\(\beta\)40 was obtained from Dr. David Teplow, the Biopolymer Laboratory at Harvard University. All other A\(\beta\)-derived peptides were obtained from Bachem (Bubendorf, Switzerland). Trypsin was obtained from Worthington. All other enzymes were from Boehringer Mannheim (Bromma, Sweden).

Polymerization, Enzymatic Digestion, and Chromatographic Separation of A\(\beta\) Peptide—Synthetic A\(\beta\)40 was dissolved in 1.1,1,3,3,3-hexafluoro-2-propanol and aliquoted in test tubes. The solvent was then removed by lyophilization. After addition of Tris-buffered saline (TBS), pH 7.4, containing 0.02% NaN\(_3\), the peptides were allowed to polymerize by incubation in a shaking water bath at 37 °C for 48 h. The final concentration of all polypeptides used was 200 \(\mu\)M. Following polymerization, proteases (50 \(\mu\)g/ml, unless otherwise indicated) were added and the peptides digested for 5 h at 37 °C in a total volume of 100 \(\mu\)l. In separate controls, trypsin was added directly, i.e. without prior polymerization. As an additional control, polymerized A\(\beta\) was depolymerized using 90% formic acid for 30 min. The acid was then removed by lyophilization before addition of trypsin. The enzymatic reaction was stopped by freezing. After lyophilization, the samples were dissolved in 90% formic acid, diluted 10 times with 0.1% trifluoroacetic acid in water, and separated on a Vydac C-4 RPLC column (0.21×15 cm) using a solvent system containing 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid, 100% acetonitrile (buffer B). Elution was monitored by measuring absorbance at 214 nm. The percentage of intact peptide remaining after cleavage was calculated according to the following equation: 100 × (amount of peptide after cleavage/amount of peptide before cleavage).

N-terminal Sequencing—The N-terminal sequence was determined by adiabatic biphasic column technology using an HP G1005A protein sequenator (Hewlett-Packard protein chemistry system, Palo Alto, CA).

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Resistance against Tryptic Degradation—The principal
to addition resistance against tryptic degradation. After
treatment with trypsin and subsequently separated by RPLC. The chro-
monic peptide, contains 40 amino acid residues and can be sepa-
and then adding TBS; periods treated with trypsin and subsequently separated by RPLC. The chroma-
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monic peptide, contains 40 amino acid residues and can be sepa-
remaining after tryptic cleavage was determined by RPLC.

RESULTS

Fig. 1. Incubation of Ap1-40 in TBS for 48 h leads to increased resistance against tryptic degradation. Synthetic Ap1-40 was treated with trypsin and subsequently separated by RPLC. The chromatograms show peptides eluting between -20 and 60% acetonitrile. The peak corresponding to Ap1-40 is indicated in each chromatogram. The peak eluting after -24.5 min (B-D) corresponds to Ap7-28, and the peak eluting after ~28 min corresponds to Ap28-40 (B-D). A, Ap1-40 not exposed to trypsin; B, Ap1-40 exposed to trypsin without incubation in TBS; C, Ap1-40 incubated in TBS for 48 h prior to addition of trypsin; D, Ap1-40 incubated in TBS for 48 h and then treated with formic acid prior to addition of trypsin; E, Ap1-40 was incubated in TBS for the indicated periods of time prior to addition of trypsin. Percentage of intact Ap1-40 remaining after tryptic cleavage was determined by RPLC.

Electron Microscopy—Synthetic polypeptides were prepared for electron microscopy by placing 5 μl of the polymerized and non-polymerized peptide solutions on grids covered by a carbon-stabilized Formvar film and then adding 5 μl of freshly prepared 1.5% uranyl acetate in water. After 2–3 min, excess fluid was removed with a filter paper and the grids were air-dried. The negatively stained specimens prepared in this way were examined and photographed in a JEOL EM 100CX at 60 kV.

Prolonged Incubation of Ap1-40 in TBS Leads to Increased Resistance against Tryptic Degradation—The principal Ap variant, Ap1-40, contains 40 amino acid residues and can be separated by RPLC (Fig. 1A). Treatment of Ap1-40 with trypsin (50 μg/ml) for 5 h led to degradation of more than 90% of the peptide (Fig. 1B). In contrast, Ap1-40 that had been incubated for 48 h in TBS prior to addition of trypsin displayed a high degree of resistance against the enzyme, and the bulk of the molecules remained intact (Fig. 1C). Formic acid treatment of Ap1-40 that had been incubated in TBS for 48 h forced the peptide back to a trypsin-sensitive state (Fig. 1D). Interestingly, previous findings indicated that formic acid is capable of dissociating Ap fibrils from Alzheimer brain (6). Two tryptic fragments eluting after ~24.5 and ~28 min are visible in Fig. 1, B-D. These were identified as Ap17-28 and Ap29-34 by N-terminal sequencing. The sequence data show that Ap1-40 was cleaved after Lys16 and Lys28, which is in agreement with the predicted substrate specificity of trypsin (see legend to Fig. 4). Fig. 1E shows a representative experiment demonstrating that the trypsin resistance of Ap developed in a time-dependent manner, increasing rapidly during the first 48 h of incubation and then marginally between 48 and 96 h. The findings that the trypsin-resistant state was (i) induced spontaneously during incubation without addition of enzymes or co-factors and (ii) reversed by treatment with formic acid indicate that it did not involve covalent modifications but reversible higher order structural changes of the peptide.

An important question was to investigate if the conditions used (e.g. substrate:enzyme ratio and incubation time) allowed the cleavage reaction to proceed to completion. Near maximal degradation of both incubated and control Ap1-40 was obtained with 0.5 μg/ml trypsin (substrate:enzyme ratio, ~9600), an enzyme concentration 100 times lower than that used in the earlier experiments (Fig. 2). Incubation of the substrate with trypsin for 16 h yielded essentially identical results as when incubated for 5 h (data not shown). A reasonable conclusion from these experiments is that the conditions used indeed allowed the reaction to proceed to completion. Moreover, since trypsin concentrations above 0.5 μg/ml and prolonged incubation time did not add to the degradation, only a limited number of Ap1-40 were probably accessible to the enzyme. These non-resistant molecules may correspond to a population of Ap1-40, which had failed to adopt a trypsin-resistant structure.

Ultrastructure of Ap1-40 and Ap-derived Peptides Incubated in TBS—Synthetic Ap1-40 incubated in a buffer of physiological pH and ionic strength polymerizes into fibrillar structures with a morphology closely resembling that of amyloid fibrils. Electron microscopic examination revealed that the fibrils formed by Ap1-40 during incubation in TBS for 48 h were typically a few hundred nm in length with a tendency to adhere both side to
AP peptide were incubated in TBS for 48 h and then processed for electron microscopy. The percentage of the indicated number of experiments. The ratios between the percentage of intact AP remaining after cleavage of incubated and not incubated peptide (I/N) are indicated to the right in the figure. The substrate specificities of the proteases are as follows: trypsin, -P,-P', -(P = Lys or Arg); collagenase, -P,-P', -(P = uncharged, non-aromatic; P' = nonspecific residue); Lys-C, -Lys,-P', -(P' = nonspecific residue); pepsin, -P,-P', -(P = nonspecific residue; P' cannot be Val, Ala, or Gly); carboxypeptidase Y, -P,-P', -(both residues are nonspecific); chymotrypsin, -P,-P', -(P = aromatic; P' = nonspecific residues); proteinase K, -P,-P', -(both residues are nonspecific).

FIG. 4. APβ-40 not incubated in TBS or incubated in TBS for 48 h was subjected to proteolysis with the indicated proteases for 5 h. Data are expressed as mean values of the indicated number of experiments. A longer peptide, APβ-35, did not form typical fibrils but still developed protease resistance. Another short peptide, APβ-2, yielded large fibrils. The APβ-1 peptide, which contains amino acid residues from the transmembrane region of APP, was in fact quite resistant even without prior incubation in TBS.

The polymers formed displayed different morphologies. Trypsin Sensitivity of Polymerized and Non-polymerized APβ-derived peptides—The same peptides that had been allowed to polymerize in TBS for 48 h and then examined by electron microscopy had at least one tryptic cleavage site and were subjected to tryptic cleavage. In contrast to APβ-40, APβ-2, that had polymerized into fibrils was only slightly more resistant to trypsin than the corresponding non-polymerized peptide (Fig. 3E). This indicates that fibril formation per se is insufficient to induce resistance. In contrast to APβ-40 and APβ-2, the two shorter peptides, APβ-12-28 and APβ25-35, did not form typical fibrils when incubated under the conditions used here. Nevertheless, the two latter peptides both developed trypsin resistance similar to that of APβ-40. The APβ25-35 molecule, which contains 7 amino acid residues from the transmembrane domain of the APP molecule, suggesting that hydrophobic interactions possibly are important in protecting the single tryptic cleavage site present in this region of the APP molecule. The fact that APβ-12-28 and APβ25-35 did not form typical fibrils but still developed protease resistance is further evidence that fibril formation per se is not the actual cause of protease resistance. Instead, it is possible that conformational alterations closely associated with polymerization lead to protease resistance.

Polymerization of APβ-40 into Fibrils Leads to Increased Resistance against a Wide Array of Proteases—It was also investigated whether polymerized APβ becomes insensitive to proteolytic enzymes other than trypsin. A number of mammalian and non-mammalian exo- and endopeptidases with varying substrate specificities (25) indicated in the legend to Fig. 4 were tested for their ability to degrade polymerized and control APβ-40. Incubation in TBS and polymerization were associated with increased resistance to all enzymes tested (Fig. 4). Since a large number of potential cleavage sites is present within the peptide, structural changes throughout its entire length may be implicated in the development of protease resistance. The finding that the bacterial enzyme proteinase K was able to degrade the bulk of polymerized APβ-40 strongly argues against side and end to end, giving rise to loosely arranged aggregates of moderate size (Fig. 3A). A shorter peptide, APβ-28, corresponding to only the hydrophilic, intraluminal, or extracellular part of APβ, polymerized and produced fibrils of a similar character, aggregating into thin bundles, often of considerable length (Fig. 3B). An even shorter peptide, APβ28-35, yielded large polymers of a different character. Even if occasional thin fibrils could be detected in this preparation, plateletlike structures (about 50–200 nm in width and up to 1 μm or more in length) with a diffuse fibrillar substructure predominated (Fig. 3C). Another short peptide, APβ25-35, covering a hydrophobic transmembrane part of APβ and a few amino acids in the intraluminal or extracellular part, likewise polymerized in TBS forming plateletlike structures of defined width and length (Fig. 3D). In controls in which APβ1-40 and the other peptides described above were prepared for electron microscopy immediately after being dissolved in TBS, no or only occasional short (<100 nm) fibrillar structures could be detected (not shown). In conclusion, all polypeptides studied had the capacity to polymerize, although...
the polymers formed AP peptide has been demonstrated here. The AP peptide can too dense for the proteolytic enzymes to penetrate. That resistance was caused by formation of peptide aggregates in neuronal and vascular tissue forming amyloid deposits. If the secreted peptide fails to develop protease resistance, a postulated prerequisite for the phenomenon, perhaps by allowing excessive intracellular accumulation of non-digestible AP peptide endocytosed from the culture medium.

Lansbury and co-workers (28) have shown that polymers of the AP peptide can serve as seeds that increase the rate by which the monomeric peptide polymerizes into fibrils. This process has been termed nucleation-dependent polymerization and may increase the probability that an individual Ap molecule secreted by a cell adopts protease resistance and thereby evades degradation. A schematic outline of the proposed fate of secreted AP peptide is given in Fig. 5. Notably, several other proteins polymerize into fibrils with a morphology similar to or indistinguishable from those formed by AP*-40 (29, 30). These proteins are also able to form amyloid in different tissues, apparently without being degraded. Hence, it is reasonable to assume that they become able to withstand proteolytic attacks in association with polymerization and fibril formation, perhaps through structural changes similar to those of Ap. We therefore propose that the general mechanism described here, polymerization-associated protease resistance, may be extended to other proteins capable of forming amyloid in vivo.

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FIG. 5. Schematic outline of the proposed fate of the Ap peptide in the body. Normal cells generate the Ap peptide by processing APP and release it extracellularly. If the secreted peptide fails to polymerize and develop protease resistance, it is degraded by proteolytic enzymes (to the left in the figure). If the peptide polymerizes and succeeds in developing protease resistance, it evades degradation (to the right in the figure). Polymerized peptides can serve as nucleation seeds driving newly formed monomeric Ap into the same resistant structure. Eventually, the protease-resistant Ap polymers accumulate in neuronal and vascular tissue forming amyloid deposits.

DISCUSSION
In summary, a novel and possibly pathogenic property of the Ap peptide has been demonstrated here. The Ap peptide can develop protease resistance, a postulated prerequisite for the final tissue deposition of amyloid, through higher order structural changes. Irrespective of the morphological appearances of the polymers formed (i.e. fibrils or plateletlike structures), it is likely that conformational changes closely associated with polymerization determine whether the peptide will acquire protease resistance or not. Apparently, these conformational changes make putative cleavage sites inaccessible to the proteases. The conclusion that polymerization is associated with resistance to proteases is in part supported by the findings of Bush et al. (26), who showed that incubation of the Ap peptide in the presence of ZnCl2 enhances the formation of Ap oligomers and polymers and increases resistance to tryptic cleavage at the ap-secretase site.

Reportedly, polymerization of Ap also affects its biological properties, making it toxic to neuronal cells (27). It is therefore possible that induced protease resistance is involved in this phenomenon, perhaps by allowing excessive intracellular accumulation of non-digestible Ap peptide endocytosed from the culture medium.