Amyloid β (Aβ) is a 39–43-residue protein that originates from proteolysis of the β-protein precursor (βPP) and accumulates in senile plaques in brains of Alzheimer’s disease (AD) patients. Mutant βPP, which incorporates an AD-causing double mutation at positions 671–688, has been shown to enhance Aβ production in transfected cells. In this work we investigate the susceptibility of the mutant βPP sequence to proteolytic cleavage by proteases from human brain. Internally quenched fluorogenic substrates were used that encompass the NH₂-terminal sequence of Aβ from wild-type βPP, the double mutant, and the two single substitutions. Protease activity in brain extract cleaved the mutant substrate 100-fold faster than the wild-type substrate and the partial mutants 25-fold faster. The major cleavage site in all substrates was at the amyloidogenic Ap1 site. The brain activity appeared to be cathepsin D (CD), as indicated by similarities to purified CD in 1) the rate and site of substrates cleavage, 2) the pH optima, and 3) the sensitivity to pepstatin A. The increased activity against the mutant substrate was not shared by cathepsins B and C, pepsin, HIV protease, and Candida albicans Asp-protease. Furthermore, CD cleaved a substrate that incorporates the COOH terminus of Aβ at positions equivalent to Thr³⁹ and Ala⁴², at ratios of 68% and 32%, respectively. CD degraded Aβ 1-40 into six fragments but Aβ 1-42 was completely resistant to digestion, probably because of its aggregation characteristics. These results indicate that CD is capable of producing the cleavage resulting in Aβ production and that it may prove to be a suitable therapeutic target.

The formation of extracellular amyloid deposits in brains of Alzheimer’s disease (AD) patients is one of the hallmarks of the disease. The major component of the amyloid is the 39–43-residue peptide, known as amyloid-β (Aβ) (see Ref. 1 for review), which is derived by proteolytic processing of a 110–135-kDa integral membrane protein termed amyloid-β precursor protein (βPP) (1). Considerable evidence implicates Aβ in the etiology of AD, the strongest of which was obtained from a number of mutations in or immediately flanking the Aβ region of βPP that are linked to an early onset form of the disease (see Refs. 2 and 3 for reviews). Therefore, understanding of the processing pathways of βPP in the brain is central to understanding the etiology of AD.

Two mutually exclusive pathways have been described for βPP. A secretory pathway generates a large secreted NH₂-terminal region, which was observed in cerebrospinal fluid (4) and in the media of cell cultures transfected with βPP cDNAs. In various cell cultures (5) and in brains (6), the secretory cleavage occurs inside the Aβ region, which precludes the evolution of the Aβ peptide.

A second pathway that involves the endosomal-lysosomal system has been shown to produce COOH-terminal fragments of 8–22 kDa that include potentially amyloidogenic forms with the 4-kDa Aβ region (7–9). Specifically, aspartyl proteinases seem to be involved in generating the COOH-terminal fragments, while cysteinyl proteinases are involved in degradation of the fragments (9). Whether these forms actually give rise to Aβ is still equivocal. A few observations suggest that βPP processing in lysosomes may be involved in the disease process and, therefore, in Aβ production. These include: lysosomal hydrolases accumulate around the amyloid plaques (10), acidic pH between 3.5 and 6.0 promotes the aggregation of Aβ (11), and potentially amyloidogenic fragments, similar to those generated in lysosomes, are neurotoxic (12, 13). Conversely, more direct evidence suggests that Aβ production occurs in other acidic compartments such as endosomes and late Golgi or secretory vesicles. This suggestion is supported by experiments showing that lysosomotropic inhibitors failed to inhibit Aβ production (14) and by the observation of Aβ production in I-cells, in which some lysosomal functions are deficient due to a genetic defect in mannose phosphorylation (15). In addition, leupeptin, a serine and cysteine protease inhibitor that inhibits 70% of lysosomal protease activities (16), failed to inhibit Aβ secretion (14, 17). This last observation, however, does not address the possibility that Aβ is produced by an aspartyl protease. In one study, alkalinizing agents completely inhibited the generation of COOH-terminal fragments of βPP, but did not inhibit Aβ production, suggesting that Aβ is not generated in an acidic compartment (17). This observation, however, was not reproduced by others (18, 19).

One of the βPP mutations that is associated with early onset AD in two related Swedish families consists of a double replacement of Lys⁶⁷⁰ → Asp⁶⁷⁰ and Met⁶⁷¹ → Leu⁶⁷¹ (the “Swedish mutation”) (19). When Swedish mutant βPP was transfected into neuroblastoma or kidney cells, 6–8-fold more Aβ was secreted to the medium and 5–fold more potentially amyloidogenic COOH-terminal fragments were produced (20–22). The Swedish mutation is located just upstream of the NH₂-terminal amyloidogenic cleavage and could affect substrate binding and catalytic efficiency of the protease producing the cleavage. Therefore, we compared the susceptibility of the Swedish and wild-type sequences of βPP to proteolytic cleavages by brain

The formation of extracellular amyloid deposits in brains of Alzheimer’s disease (AD) patients is one of the hallmarks of the disease. The major component of the amyloid is the 39–43-residue peptide, known as amyloid-β (Aβ) (see Ref. 1 for review), which is derived by proteolytic processing of a 110–135-kDa integral membrane protein termed amyloid-β precursor protein (βPP) (1). Considerable evidence implicates Aβ in the etiology of AD, the strongest of which was obtained from a number of mutations in or immediately flanking the Aβ region of βPP that are linked to an early onset form of the disease (see Refs. 2 and 3 for reviews). Therefore, understanding of the processing pathways of βPP in the brain is central to understanding the etiology of AD.

Two mutually exclusive pathways have been described for βPP. A secretory pathway generates a large secreted NH₂-terminal region, which was observed in cerebrospinal fluid (4) and in the media of cell cultures transfected with βPP cDNAs. In various cell cultures (5) and in brains (6), the secretory cleavage occurs inside the Aβ region, which precludes the evolution of the Aβ peptide.

A second pathway that involves the endosomal-lysosomal system has been shown to produce COOH-terminal fragments of 8–22 kDa that include potentially amyloidogenic forms with the 4-kDa Aβ region (7–9). Specifically, aspartyl proteinases seem to be involved in generating the COOH-terminal fragments, while cysteinyl proteinases are involved in degradation of the fragments (9). Whether these forms actually give rise to Aβ is still equivocal. A few observations suggest that βPP processing in lysosomes may be involved in the disease process and, therefore, in Aβ production. These include: lysosomal hydrolases accumulate around the amyloid plaques (10), acidic pH between 3.5 and 6.0 promotes the aggregation of Aβ (11), and potentially amyloidogenic fragments, similar to those generated in lysosomes, are neurotoxic (12, 13). Conversely, more direct evidence suggests that Aβ production occurs in other acidic compartments such as endosomes and late Golgi or secretory vesicles. This suggestion is supported by experiments showing that lysosomotropic inhibitors failed to inhibit Aβ production (14) and by the observation of Aβ production in I-cells, in which some lysosomal functions are deficient due to a genetic defect in mannose phosphorylation (15). In addition, leupeptin, a serine and cysteine protease inhibitor that inhibits 70% of lysosomal protease activities (16), failed to inhibit Aβ secretion (14, 17). This last observation, however, does not address the possibility that Aβ is produced by an aspartyl protease. In one study, alkalinizing agents completely inhibited the generation of COOH-terminal fragments of βPP, but did not inhibit Aβ production, suggesting that Aβ is not generated in an acidic compartment (17). This observation, however, was not reproduced by others (18, 19).

One of the βPP mutations that is associated with early onset AD in two related Swedish families consists of a double replacement of Lys⁶⁷⁰ → Asp⁶⁷⁰ and Met⁶⁷¹ → Leu⁶⁷¹ (the “Swedish mutation”) (19). When Swedish mutant βPP was transfected into neuroblastoma or kidney cells, 6–8-fold more Aβ was secreted to the medium and 5–fold more potentially amyloidogenic COOH-terminal fragments were produced (20–22). The Swedish mutation is located just upstream of the NH₂-terminal amyloidogenic cleavage and could affect substrate binding and catalytic efficiency of the protease producing the cleavage. Therefore, we compared the susceptibility of the Swedish and wild-type sequences of βPP to proteolytic cleavages by brain

* This work was funded in part by National Institutes of Health, National Institute on Aging Grant AG10481. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. 46Y, AP-10, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064. Tel.: 708-937-2104; Fax: 708-938-2258.

‡ The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid-β; βPP, amyloid-β precursor protein; CD, cathepsin D; DAB CYL, 4-(4-dimethylaminophenylazo)benzoic acid; EDANS, 5-(2-aminoethyl)-aminolnaphthalene-1-sulfonic acid; RP, reverse-phase; HPLC, high performance liquid chromatography; MOPS, 4-morpholinopropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; HIV, human immunodeficiency virus.
proteinases. A proteinase that cleaves the Swedish sequence at least 8-fold faster than the wild type may be the amyloidogenic proteinase and account for the observations in transfected cells. Indeed, we found that cathepsin D (CD) (EC 3.4.23.5) cleaved the Swedish NH2-terminal sequence (termed Swedish substrate) about 100-fold faster than the wild-type sequence (native substrate). Furthermore, CD produced the COOH-terminal amyloidogenic cleavages at positions equivalent to Ala32 and Thr39 of Aβ. Finally, CD degraded Aβ 1-40 into small peptides but not Aβ 1-42, suggesting that CD is capable of producing Aβ from its precursor protein.

MATERIALS AND METHODS

Biological Materials—Brain tissue samples (frontal cortex) were obtained from a donor with no evidence of neurological disorder. Samples were homogenized in a glass/Teflon homogenizer for 1 min in 25 mM MOPS/Tris, pH 7.5, 0.5 M sucrose, and 2.5 mM EDTA, at a 1:5 g/mL tissue buffer ratio. The homogenized samples were centrifuged twice for 15 min at 5000 × g at 4 °C.

Substrate Preparation—Fluorogenic substrates were prepared that span the amino acid sequence near the NH2-terminal cleavage site of Aβ (see Fig. 1), from Glu414 to Phe525 of wild-type βPP770 (native substrate), and of mutant βPP from a Swedish family with early onset AD where the Lys-Met at positions 670-671 are replaced by Asn-Leu (Swedish substrate) (19). Two additional substrates were made, each incorporating one of the amino acid replacements: Lys to Asn (Asn substrate) and Met to Leu (Leu substrate). A substrate that spans the sequence near the COOH-terminal cleavage site of Aβ (substrate C) from Val714 to Ile716 was also made. Polyether linkers were incorporated into the substrates to distance the fluorescent and absorbent moieties from the amino acid sequence and to enhance solubility.

Protease Assays—Protease assays were performed at 37 °C, in a 500-μl reaction volume consisting of 40 μM buffer, 10 μM substrate, from 1 mM stock in dimethyl sulfoxide, and 5–10 μl of brain extract. Buffers used were malate, pH 3.0; formate, pH 3.5–4.0; acetate, pH 4.5–5.0; MES, pH 5.5–6.0; MOPS, pH 7.0; and Tris, pH 7.5. The principle of operation of the fluorogenic assays employing fluorescence quenching have been described for the secretory and amyloidogenic site of βPP (23). Fluorescence measurements were made on a Shimadzu RF5000U spectrofluorimeter with excitation and emission wavelengths set at 340 and 490 nm, respectively.

Identification of Cleavage Sites—Substrates were incubated with brain extracts or purified enzymes under the conditions specified for protease assays with a substrate concentration of 20 μM at 23 °C. Extract was diluted 50–100-fold into the reaction mixture. After incubation of 40 or 240 min, samples were analyzed by RP-HPLC using an Applied Biosystems C18 column (220 × 4.2 mm). Substrate fragments were eluted with a linear H2O/acetonitrile gradient at 3% acetonitrile/min, containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Sequential absorbance and fluorescence measurements of the column eluant were performed. Absorbance was measured simultaneously at the substrates to distance the fluorescent and absorbent moieties from the amino acid sequence and to enhance solubility.

Hydrodynamic Measurements—Sedimentation velocity measurements were performed on a Beckman XL-A analytical ultracentrifuge and analyzed as described by Holzman and Snyder (24). The experiments were carried out under the reaction condition specified for protease assays with Aβ concentration of 200 μM at 23 °C.

RESULTS

Cleavage of NH2-terminal Substrates of Aβ by Human Brain Extract—Four NH2-terminal substrates were used to assay human brain proteinase activities: a "native substrate" (wild type), a Swedish substrate that incorporates the double mutation of Lys-Met to Asn-Leu, the "Asn substrate" that incorporates the Met to Leu replacement (Fig. 1). With all substrates, maximum activity was observed at pH 3.0–3.5 (Fig. 2), suggesting that proteinases from an acidic compartment are active against these substrates. Activity against the Swedish substrate was 100–130-fold higher than the rate against the native substrate at pH 3.0–3.5 and the difference diminished as the pH increased. Both the Asn and Leu substrates resulted in the activities at high pH and against the Swedish substrate at pH 3.0–5.0.

FIG. 1. Five substrates used in this study. Native substrate, that encompasses the NH2-terminal amyloidogenic cleavage site from Glu414 to Phe525 of wild-type βPP770; Swedish substrate, that encompasses the Swedish sequence and to enhance solubility. The cleavage sites in all substrates were determined by separation on a Finishing Mat ESI mass spectrometer or a Bruker Reflex laser desorption mass spectrometer.

FIG. 2. Effect of pH on protease activities in extracts from normal human brain against four substrates that encompass the NH2-terminal amyloidogenic cleavage site of Aβ from wild-type βPP, Swedish mutant βPP and partial mutations of Lys-Asn and Met-Leu, as described in Fig. 1. Inset, the same activity on a scale 90-fold larger to show the activities at high pH and against the native substrate. Five μl of extract (pH 3.0–5.0) or 10 μl of extract (pH 5.5–7.5) were added to a total volume of 300 μl consisting of 40 mM buffer and 10 μM substrate.

substrate was 100–130-fold higher than the rate against the native substrate at pH 3.0–3.5 and the difference diminished as the pH increased. Both the Asn and Leu substrates resulted in the activities at high pH and against the Swedish substrate at pH 3.0–5.0.

The cleavage sites in all substrates were determined by sepa-
Cleavage of NH₂ and COOH Termini of Aβ by Cathepsin D

Fig. 4. Effect of pH on human liver CD activity against the four substrates that encompass the NH₂-terminal amyloidogenic cleavage sites, described in Fig. 1. Conditions were as in Fig. 2, except that 2 µl of enzyme preparation (10 units/ml) were used in the reaction. Inset, as in Fig. 2.

were ~25-fold higher.

HPLC analyses of the cleavage sites produced by CD revealed that all the substrates were cleaved at the amyloidogenic Met⁴-Asp⁵ or Leu⁵-Asp⁶ bonds (Fig. 5), as was observed with brain extract. Very low levels of cleavage at other sites were detectable when pure CD was used, suggesting that the minor cleavages observed with brain extract, may be produced by other proteinases that are present at low levels.

Activities by Other Proteinases—The activities of the Cys proteinases cathepsin B and C, and Asp proteinases pepsin, HIV proteinase, and Candida albicans proteinase were assayed to evaluate whether the susceptibility of the Swedish sequence to proteolysis is unique to CD or common to other enzymes (Table I). Relative to all other proteinases, the activity of CD was extremely high against both substrates and especially against the Swedish substrate. Of the additional proteinases tested, only pepsin showed a 10-fold preference for the Swedish substrate relative to the native. However, at pH 3.5 its activity was highest against the Asn substrate and not the Swedish (not shown). Thus, the results indicate that the strong preference for the Swedish substrate is unique to CD.

Effects of Inhibitors—The effects of pepstatin A on brain extract activity and on CD were compared in order to obtain additional evidence about the identity of brain activities against the native and Swedish substrates. Pepstatin A (at 9.6 nm) inhibited both CD and the brain activity against the Swedish substrate by 98% with IC₅₀ values of 1.4 and 2.1 nm, respectively (Fig. 6A). These IC₅₀ values are reasonably close, suggesting that the brain extract activity is indeed CD. Similar results were obtained with a novel aspartyl proteinase inhibitor (data not shown) with considerable specificity for CD.² These results provide additional evidence that the brain activity against the Swedish substrate is that of CD.

Pepstatin A at 14.4 nm, inhibited the brain activity against the native substrate only by 50% and E64, a cysteiny1 proteinase inhibitor, together with pepstatin inhibited the activity by 90% (Fig. 6B). However, CD activity against the native substrate was inhibited 95% with IC₅₀ of 2 nm. Thus, cysteiny1 proteinases also hydrolyze the native substrate.

Cleavage of the native substrate by brain extract was further analyzed in the presence of pepstatin A and E64 (Fig. 7). Pepstatin A inhibited the cleavage at Met⁶ by 98% and the cleavage by Purified Human Cathepsin D—The brain activities against Asn and Leu substrates with normal brain extract. Substrates (20 µM) were incubated for 40 min in 50 µl of reaction volume at pH 3.5, with 1 µl (native substrate) or 0.5 µl (other substrates) of human brain extract, at room temperature. Inset, chromatogram of the reaction with the native substrate on a scale 12-fold larger to show details. The substrate, eluting at 17.4 min, appeared relatively small in these chromatograms because its fluorescence is quenched about 40-fold when the DABCYL group is still attached. HPLC conditions were described under "Materials and Methods." Italicized letters show the amyloidogenic cleavage site.

rating the cleavage products by RP-HPLC and subjecting them to mass spectroscopy for molecular weight (Mₕ) determination. Four NH₂-terminal peptides were produced from the native substrate, eluting at 11.5, 12.8, 13.0, and 13.4 min (inset in Fig. 3). The peak of 11.5 min had a Mₕ of 996, corresponding to cleavage at the amyloidogenic Met⁶-Asp⁷ or Leu⁵-Asp⁶ bonds. The small peaks eluting at 13.4, 12.6, and 11.2 min, resulted from Met⁶-Asp⁷ cleavage. The largest peak at 13.4 min reflects the amyloidogenic cleavage site.

Cleavage of the Swedish, Asn, and Leu substrates, each produced a major peptide eluting at 13.4, 12.6, or 14.2 min, respectively (Fig. 3). These peptides had Mₕs of 1095, 1114, or 1110, respectively, corresponding to cleavages at the amyloidogenic Met⁴-Asp⁵ or Leu⁵-Asp⁶ bonds. The small peaks eluting at 10.6 or 11.5 min had Mₕs of 982 or 996, corresponding to cleavages at the COOH-side of Asn⁵ or Lys⁵, respectively.

Cleavage by Purified Human Cathepsin D—The brain activity against these substrates peaked at pH 3.0 suggesting that a proteinase from an acidic compartment, such as CD, may be involved. Indeed, the activity of purified human liver CD at pH 3.0–4.0 appeared quantitatively similar to that of human brain extract (Fig. 4). As in brain extract, activity against the Swedish substrate was 100-fold higher than activity against the native substrate, and activities against Asn and Leu substrates

were ~25-fold higher.

HPLC analyses of the cleavage sites produced by CD revealed that all the substrates were cleaved at the amyloidogenic Met⁴-Asp⁵ or Leu⁵-Asp⁶ bonds (Fig. 5), as was observed with brain extract. Very low levels of cleavage at other sites were detectable when pure CD was used, suggesting that the minor cleavages observed with brain extract, may be produced by other proteinases that are present at low levels.

Activities by Other Proteinases—The activities of the Cys proteinases cathepsin B and C, and Asp proteinases pepsin, HIV proteinase, and Candida albicans proteinase were assayed to evaluate whether the susceptibility of the Swedish sequence to proteolysis is unique to CD or common to other enzymes (Table I). Relative to all other proteinases, the activity of CD was extremely high against both substrates and especially against the Swedish substrate. Of the additional proteinases tested, only pepsin showed a 10-fold preference for the Swedish substrate relative to the native. However, at pH 3.5 its activity was highest against the Asn substrate and not the Swedish (not shown). Thus, the results indicate that the strong preference for the Swedish substrate is unique to CD.

Effects of Inhibitors—The effects of pepstatin A on brain extract activity and on CD were compared in order to obtain additional evidence about the identity of brain activities against the native and Swedish substrates. Pepstatin A (at 9.6 nm) inhibited both CD and the brain activity against the Swedish substrate by 98% with IC₅₀ values of 1.4 and 2.1 nm, respectively (Fig. 6A). These IC₅₀ values are reasonably close, suggesting that the brain extract activity is indeed CD. Similar results were obtained with a novel aspartyl proteinase inhibitor (data not shown) with considerable specificity for CD.² These results provide additional evidence that the brain activity against the Swedish substrate is that of CD.

Pepstatin A at 14.4 nm, inhibited the brain activity against the native substrate only by 50% and E64, a cysteiny1 proteinase inhibitor, together with pepstatin inhibited the activity by 90% (Fig. 6B). However, CD activity against the native substrate was inhibited 95% with IC₅₀ of 2 nm. Thus, cysteiny1 proteinases also hydrolyze the native substrate.

Cleavage of the native substrate by brain extract was further analyzed in the presence of pepstatin A and E64 (Fig. 7). Pepstatin A inhibited the cleavage at Met⁶ by 98% and the cleavage

² J. Erickson, personal communication.
the aspartyl proteinase. Thus, an aspartyl proteinase, not a cysteinyl proteinase, cleaves the native substrate at the amyloidogenic site and therefore, is likely to be the same enzyme responsible for the resulting amyloid into small fragments. To test whether CD is capable of digesting and degrades AP further, peak Thr4 declined while peaks Ile4 and Ala5 increased, suggesting that Gly1-Ile4 evolved by exopeptidase activity (not shown).

Purified human CD produced two amino terminal peptides, Gly1-Thr2 and cleavage at the Thr2-Ala5 bond. This is equivalent to a cleavage at Thr43 of AP, which would yield an AP of 43 amino acids. The peak of 13.0 min had a Mf of 923, which corresponds to peptide Gly1-Ala5 (cleavage at Ala6); and the peak of 13.5 min, with Mf of 852, corresponded to peptide Gly1-Ile4 (cleavage at Ile5). HPLC analysis every 40 min showed that initially the peak of 12.6 min, Thr2, accounted for 69% of the cleavage products, and peak Ala5 accounted for 91%. However, at longer time points, as the substrate concentration diminished, peak Thr2 declined while peaks Ile4 and Ala5 increased, suggesting that Gly1-Ile4 evolved by exopeptidase activity (not shown).

Cleavage of Carboxyl-terminal Substrate—The presence of proteinase(s) capable of producing carboxyl-terminal cleavage of AB was studied using a substrate encompassing bPP sequence from position 711 to 716 (substitue C). Brain extract activity against substrate C peaked at pH 3.5 (Fig. 8A), suggesting the involvement of acidic proteinases. Cleavage site analysis revealed 3 NH2-terminal peptides. The peak eluting at 12.6 min had a Mf of 1024 (Fig. 8B), corresponding to peptide Gly1-Thr2 and cleavage at the Thr2-Ala5 bond. This is equivalent to a cleavage at Thr43 of AP, which would yield an AP of 43 amino acids. The peak of 13.0 min had a Mf of 923, which corresponds to peptide Gly1-Ala5 (cleavage at Ala6); and the peak of 13.5 min, with Mf of 852, corresponded to peptide Gly1-Ile4 (cleavage at Ile5). HPLC analysis every 40 min showed that initially the peak of 12.6 min, Thr2, accounted for 69% of the cleavage products, and peak Ala5 accounted for 91%. However, at longer time points, as the substrate concentration diminished, peak Thr2 declined while peaks Ile4 and Ala5 increased, suggesting that Gly1-Ile4 evolved by exopeptidase activity (not shown).

Purified human CD produced two amino terminal peptides, Gly1-Thr2, which accounted for 69% of the cleavage products, and Gly1-Ala5, accounting for 32% (Fig. 8B). Even long incubation periods did not alter these ratios significantly, indicating that cleavage at Thr43 precluded the cleavage at position Ala6. These results suggest that the major activity observed in human brain extract is CD, and that CD is capable of producing amyloidogenic cleavages at Thr43 or Ala6, but not at Ile5.

**Digestion of Amyloid-β 1-40 and Aβ 1-42 by Human Cathepsin D**—We show above that CD is capable of cleaving both the amino and carboxyl ends of Aβ. However, CD would be an unlikely amyloidogenic proteinase if it is capable of digesting the resulting amyloid into small fragments. To test whether CD degrades Aβ further, Aβ 1-40 and Aβ 1-42 were incubated with CD. Aβ 1-40 was quickly digested into 6 distinct fragments at pH 3.5 (Fig. 9A). At pH 5.0 Aβ was digested into the same fragments at pH 3.5 (Fig. 9B).
fragments but at a much slower rate (not shown). Conversely, Aβ 1-42 was not digested even after 16 h (Fig. 9B). This result was further confirmed by mass spectrometry analysis of the reaction mixture, which revealed the presence of Aβ with M, of 4511 and no other small peptides.

The resistance of Aβ 1-42 to digestion by CD may result from the aggregation of Aβ 1-42, which could prevent accessibility of the enzyme to the cleavage sites (11). Therefore, the aggregation states of Aβ 1-40 and 1-42 were determined by analytical ultracentrifugation under the reaction conditions used. This analysis showed that the sedimentation coefficient of Aβ 1-40 was ~11 S, which is consistent with a protein of a few hundred kilodaltons in mass (Fig. 10). The sedimentation coefficient of Aβ 1-42, however, was about 140 S, which is consistent with aggregate mass of tens of thousands of kilodaltons. Thus, the large aggregates of Aβ 1-42, or their different structure probably prevented access of the enzyme to the cleavage sites.

**DISCUSSION**

A mutation that was reported to cause early onset AD in a Swedish family, involves the substitution of the Lys-Met at position 670-671 of βPP with Asn-Leu (19). The mutant βPP, when transfected into cells, produced 6–8-fold more Aβ than wild-type βPP (20–22). The mutation is localized just upstream of the amyloidogenic cleavage site and could affect the susceptibility of the Met-Asp bond to proteolytic cleavage. To test this hypothesis, we used fluorogenic substrates that encompass the N- and C-terminal amyloidogenic sequence. Panel A shows the effect of pH on protease activity from brain extract. Conditions were as in Fig. 2; Panel B, chromatograms of EDANS-containing peptides from a 40-min reaction with brain extract (upper) or from an 8-h reaction with CD (lower). Conditions were as in Fig. 3.

Consistent with our previous conclusion, we observed in this work that an acidic protease activity is present in human brains that cleaved the Swedish substrate at a rate about 100-fold faster than the native substrates and that both substrates were cleaved at the amyloidogenic sites. A similar activity was observed in extracts from AD brains and from bovine brains (not shown). The brain activity is likely to be CD as indicated by the following observations: 1) the brain activity produced similar cleavage rates with all four substrates and peaked at pH 3.0–3.5, as did CD activity; 2) the brain activity produced a cleavage pattern in three of the substrates that was similar to that of CD; and 3) pepstatin A inhibited the brain activity against the Swedish substrate with IC50 similar to that of CD. The brain activity against the native substrate consisted of both cysteiny1 and aspartyl proteinases, however, it appears that only the aspartyl proteinase produced the cleavage at the amyloidogenic site.

The brain extracts also had activity that was capable of producing the COOH-terminal amyloidogenic cleavage, which also appears to be CD. Both the brain activity, and purified CD cleaved substrate C at two distinct sites, equivalent to positions Thr43 and Ala42 of Aβ. The major activity was at Thr43 and accounted for ~68% of the total cleavage while the activity at Ala42 accounted for 32%. These ratios did not change even after a long incubation of purified CD with the substrate, indicating
that the cleavage at Thr43 precluded the cleavage at Ala42. However, an additional activity was present in brain extract that degraded the major product further to produce a cleavage at position Ile41. Thus, our observations can account for the ragged COOH terminus of Aβ. Unfortunately, our data did not provide any information concerning cleavages at positions Val4′ or Val5′ of Aβ. A cleavage at the COOH-side of Val (Val6′ of Aβ) was not observed, but the absence of a peptide bond on the NH2-terminal side of this residue may have prevented such a cleavage. Longer COOH-terminal substrates that incorporate more upstream residues were synthesized, to allow analysis of cleavages at positions Val4′ or Val5′ or Val6′.

The COOH-terminal cleavage site of Aβ is embedded in the membrane spanning region of βPP which should limit the accessibility of water or the active site of proteinases to the cleavage site. Thus, the cleavage must be facilitated by membrane damage or by dislodging the βPP from the membrane. The possibility that membrane damage occurs prior to Aβ production is unlikely in some systems, as Aβ is produced as a part of the normal βPP processing in cultured or transfected cells, and is present in CSF of normal individuals (17, 18, 25, 26). If Aβ is produced by the same mechanism in transfected cells, in normal individuals and in AD patients, then βPP is dislodged from an undamaged membrane before the COOH-terminal cleavage can occur. How βPP may be dislodged from the membrane is not known; membrane anchorage and secretory processing of βPP have been shown to be facilitated by the triplet lysine residues at positions 724 to 726, just down stream of the membrane spanning region (27). Thus, a cleavage at this site may release the βPP molecule into the endosomal milieu after internalization through a clathrin coated pit (7). Alternatively, βPP may be directed into lysosomes with a fragment of a membrane targeted for degradation. In this case, lysosomal phospholipases and lipases may degrade the membrane (28, 29), making the βPP accessible to proteolysis. This route does not necessarily involve prior presence of βPP on the plasma membrane, however, it is inconsistent with some data that suggest that Aβ is generated in an acidic but non-lysosomal compartment (14, 15, 18).

CD is present in clathrin coated vesicles (30), endosomes (31), lysosomes (32), trans-Golgi vesicles (32, 33), and transport vesicles (34). Thus, this enzyme would be accessible to βPP in various cell compartments, most likely early endosomes or trans-Golgi vesicles. The role of CD in the secretion of the membrane protein Galβ1-4GlcNAc α2-6-sialyltransferase from the trans-Golgi during acute-phase response (33) suggests a similar role in the secretion of βPP. This suggestion is supported by recent observations: 1) potentially amyloidogenic fragments were produced in βPP-transfected cells by an aspartic proteinase (9); 2) secreted βPP from cells transfected with the Swedish mutant, was cleaved at the amyloidogenic site (or β-secretory site (22)) that we find is very susceptible to cleavage by CD; and 3) potentially amyloidogenic fragments and Aβ are likely to be produced in an acidic compartment (see above). Thus, we propose that the NH2-terminal amyloidogenic cleavage by CD occurs in both wild type and mutant cases, during secretion in the trans-Golgi and gives rise to potentially amyloidogenic fragments. However, in mutant cases, the rate of cleavage at the amyloidogenic site may be higher, resulting the accumulation of Aβ. In other AD cases, the accumulation of Aβ may be mediated by different mechanisms such as binding to apolipoprotein-E, which may prevent the clearance of Aβ from the brain. Since potentially amyloidogenic fragments accumulate in cells transfected with mutant βPP, the COOH-terminal...
Cleavage of NH₂ and COOH Termini of Aβ by Cathepsin D

cleavage is likely to occur later, or at a substantially lower rate.

Other proteolytic enzymes present in these compartments may also come in contact with βPP. The susceptibility of the NH₂-terminal substrates to proteolysis was tested with a few other lysosomal or aspartyl proteinases (Table 1). However, only cathepsin E showed activity against the Swedish substrate that was greater than ~100-fold the rate against the native substrate. Nonetheless, cathepsin E is present primarily in the stomach and is absent from brain tissue, making it unlikely to be the amyloidogenic proteinase (35).

Our results suggest that CD is capable of producing both the NH₂- and COOH-terminal cleavages in peptide substrates. If so, it may further digest Aβ into smaller fragments. Indeed, CD digested Aβ 1-40 into six distinct fragments, but Aβ 1-42 was completely resistant to digestion. Aβ 1-42 formed aggregates that were about a 100-fold larger than the aggregates of Aβ 1-40. Thus, the large aggregates of Aβ 1-42, or their different structure, may prevent the accessibility of the enzyme to cleavage sites within Aβ.

Our suggestion that CD may be the amyloidogenic proteinase should be tested with additional experiments with "physiological βPP" isolated from human brain membranes, in order to establish the role of CD in the formation of Aβ. If such a role for CD is indeed confirmed, this proteinase may prove to be a viable therapeutic target. A recent report suggesting that there is a balance between formation and clearance of Aβ (36), supports the notion that inhibition of amyloidogenic proteinases may clear some of the amyloid from patients brains.

Acknowledgments—We gratefully acknowledge the support and encouragement of Drs. Thomas Perun and Michael Williams in Pharmaceutical Discovery Research at Abbott Laboratories. We thank Dr. David Bennett of Rush Alzheimer's Disease Center in Chicago, Illinois, for providing the brain samples.

REFERENCES
1. Selkoe D. J. (1991) Neuron 6, 487-498
2. Goate A. M., and Crawford F. (1992) BioEssays 14, 727-734
3. Selkoe D. J. (1993) Trends Neurosci. 16, 403-409
4. Palmert M. R., Siedlik S. L., Podlisny M. B., Greenberg B., Shelton E. R., Chan M. W., Usik M., Selkoe D. J., Perry G., and Younkin S. G. (1989) Biochem. Biophys. Res. Commun. 165, 182-188
5. Esch F. S., Keim P. S., Beattie E. C., Blacher R. W., Cutler A. R., Ellendorf T., Mckinley D., and Ward F. J. (1980) Science 210, 1125-1124
6. Pasternack J. M., Palmert M. R., Usik M., Wang R., Zurcher-Neeley H., Goelz-DeWhitt P. A., Fairbanks M. B., Cheung T., Blades D., Heintz A. L.,

J. Kay, and E. W. Lees, personal communication.

Greenberg B. D., Cotter R. J., and Younkin S. G. (1992) Biochemistry 31, 10493-10494.
7. Haas C., Koo E. H., Mellon A., Hung A. Y., and Selkoe D. J. (1992) Nature 357, 500-503
8. Goate A. M., Estus S., Younkin L. H., Selkoe D. J., and Younkin S. G. (1992) Science 255, 728-730
9. Siman R., Mistretta S., Durkin J. T., Savage M. J., Levy T., and Scott R. W. (1993) J. Biol. Chem. 268, 16692-16699
10. Cataldo A. M., and Nixon R. A. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3861-3865
11. Burdick D., Soreghan B., Kwon M., Kosmoski J., Nauker M. F., Henschen A., Yates J., Cotman C., and Gibe C. G. (1990) J. Biol. Chem. 265, 544-554
12. Yankin B. A., Daou W. R., Fisher S., Villa-Komaroff L., Oster-Germain M. L., and Neve R. L. (1990) Science 245, 417-420
13. Pollock K., Supper B., Farquhar C. E., Smith A. C., Dang N. T., and Martin G. M. (1993) Neurosci. Lett. 154, 145-148
14. Haas C., Hung A. Y., Schlossmacher M. G., Teplow D. B., and Selkoe D. J. (1992) J. Biol. Chem. 268, 3021-3024
15. Podlisny, M., Haas, C., and Selkoe, D. J. (1993) Soc. Neurosci. Abstr. 19, 1276 (abstract)
16. Grinde B. and Seglen P. O. (1986) Biochem. Biophys. Acta 832, 73-86
17. Binciol G., Gubanov D. H., Matsuda M., and Yankin B. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2092-2096
18. Shoji M., Okada T. E., Hisojo J., Cheung T. T., Estus S., Shaffer L. M., Cai X. D., McKay D. M., Tintner E., Frangione B., et al. (1992) Science 254, 128-129
19. Mullin M. J., Crawford F., Axelrod K., Hoiden H., Lillins L., Whinlaid B., and Lennolet L. (1992) Nat. Genet. 1, 345-347
20. Citron M., Ellendorf T., Haas C., McConlogue L., Hung A. Y., Seubert P., Vigo-Felcay C., Lieberburg I., and Selkoe D. J. (1992) Nature 359, 672-674
21. Cai X. D., Golde T. E., and Younkin S. G. (1993) Science 259, 514-516
22. Felsenstein K., Hunihan L. W., and Roberts S. B. (1994) Nat. Genet. 6, 351-356
23. Ladró, U. S., Wang, G. T., Klein, W. L., Holzmann, T. F., and Krutf, G. A. (1994) J. Protein Chem., in press
24. Holzman, T. F. and Snyder, S. (1994) in Molecular Endocrinology: Advances in Modern Analytical Ultracentrifugation, Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems (Schuster, T. M. and Laue, T. M., eds) Springer-Verlag, Boston
25. Haas C., Schlossmacher M. G., Hung A. Y., Vigo-Felcay C., Mellon A., Otsasouwski B. L., Lieberburg I., Koo E. H., Schenk D., Teplow D. B., and Selkoe D. J. (1992) Nature 358, 322-325
26. Schubert Z., Vigo-Felcay C., Esch F. S., Lee M., Dey H. F., Davis D., Sinha S., Schlossmacher M. G., Whaley J., Swithelhurt C., McCormack H., Wollert R., Selkoe D. J., Lieberburg I., and Schenk D. (1992) Nature 359, 255-257
27. Usami M., Yamashigaya W., and Murray S. K. (1993) J. Neurochem. 61, 239-246
28. Minor L. K., Milklin F. H., Jerome W. C., Lewis J. D., Rothblatt G. H., and Glick J. M. (1991) Exp. Mol. Pathol. 54, 159-171
29. Schubert D., and Behl C. (1993) Brain Res. 629, 275-282
30. Sapirstein, V., Berg, M. L., Durrie, R., and Marks, N. (1993) Soc. Neurosci. Abstr. 19, 1276 (abstract)
31. Williams K. F., and Smith J. A. (1993) Arch. Biochem. Biophys. 305, 298-306
32. Ayasaka N., Goto T., Tsukuba T., Kido M. A., Nagata K., Kondo T., Yamamoto M., and Tanaka T. (1990) J. Biol. Chem. 265, 702-707
33. Lammers, G. and Janieses C. J. (1988) Biochem. J. 258, 623-631
34. Krieger T. J., and Hook V. Y. (1992) Biochemistry 31, 4223-4231
35. Muto N., Yamamoto M., Tanii S., and Yonemura S. (1993) J. Biochem. (Tokyo) 110, 629-632
36. Hyman B. T., Marzolf K., and Arriagada P. V. (1993) J. Neuropathol. Exp. Neurol. 52, 594-606