Identification of a Metalloprotease from Alzheimer's Disease Brain Able To Degrade the β-Amyloid Precursor Protein and Generate Amyloidogenic Fragments†

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ABSTRACT: A 4.2-kDa polypeptide termed β protein (Aβ) accumulates in senile plaques and blood vessels in Alzheimer's disease and Down's syndrome. It is widely believed that Aβ is the product of the posttranslational processing of a larger precursor protein, the β amyloid precursor protein (APP). The proteolytic processes involved in the generation of the Aβ are virtually unknown. Here the purification and characterization of a protease from Alzheimer's disease brain capable of cleaving a 10 amino acid synthetic substrate flanking the N terminus of Aβ at the Met–Asp bond are described. Most importantly, the purified protease degrades human recombinant APP and generates a 15-kDa amyloidogenic fragment. The protease requires the presence of a reducing agent for its activity. Its pH optimum is around physiological pH, while the enzyme is inactive at acidic pH (below pH 5.0) and basic pH (over pH 7.6). The enzyme is inhibited by *N*-ethylmaleimide, (hydroxymercuri)benzoate, 1,10-phenanthroline, EDTA, and EGTA. Phenylmethanesulfonyl fluoride has no effect on its activity. This protease is devoid of caseinolytic or gelatinase activities, as well as activities against cathepsin B and cathepsin L substrates. Sequence analysis reveals high homology to the rat metallopeptidase EC 3.4.24.15, a protease involved in neuropeptide processing.

Alzheimer's disease (AD) and Down's syndrome neuropathology is characterized by extracellular proteinaceous deposits, termed amyloid, in senile plaques and in blood vessel walls. The amyloid deposits in AD consist mostly of a 39–42 amino acid fragment, called the β protein (Aβ). Aβ is derived from the β amyloid precursor protein (APP) of 110–135 kDa (Selkoe et al., 1988). Three major isoforms of APP are present in brain: APP695, APP751, and APP770 [for review see Muller-Hill and Beyreuther (1989) and Selkoe (1991)]. The accumulation of the Aβ may have neurotoxic effects and may lead to neurodegeneration and dementia in AD (Yankner et al., 1989, 1990; Roher et al., 1991; Koh et al., 1991; Kowall et al., 1991).

Little is known about the proteolytic processes involved in the normal secretion and turnover of the APP or about the proteases involved in the APP processing leading to the generation of Aβ. Esch et al. (1990) reported that soluble fragments of the APP695 and APP751 secreted by cells in culture contained only the first 15 amino acids from the NH_2[1] terminus of the Aβ, suggesting a proteolytic cleavage before or after the 16th amino acid residue Lys of the Aβ. The protease responsible for this cleavage, termed secretase, releases a large amino-terminal fragment into the culture medium, leaving a membrane-bound carboxyl terminus. Sisodia and co-workers described a domain around amino acids Val18 and Asp20 of the Aβ important for recognition and cleavage by secretase (Sisodia et al., 1990), as well as a cleavage occurring after Lys16 of Aβ in Chinese hamster ovary cells transfected with APP770 (Wang et al., 1991). It appears that during constitutive secretion of APP there is an initial proteolytic cleavage at the central portion of the Aβ, which would not allow the generation, accumulation, and deposition of the Aβ in senile plaques. Hence, an alternative proteolytic processing pathway is necessary to generate amyloidogenic APP fragments. The cellular site of this process is still a matter of controversy. Although the endosomal–lysosomal system has been shown to play a role in the degradation of APP and the generation of potentially amyloidogenic fragments (Golde et al., 1992; Haass et al., 1992a), there is no evidence that Aβ is generated in lysosomes.

The biochemical processes involved in the generation of the Aβ remain elusive. Several investigators proposed that this proteolytic cleavage could be a result of a chymotrypsin-like enzyme, especially in light of the strong association of α1-antichymotrypsin (ACT) with the Aβ in neuritic plaques (Abraham et al., 1988, 1991; Nelson & Siman, 1990). Razzaboni and colleagues recently reported a calcium-dependent serine protease partially purified from normal adult monkey brain and able to degrade APP (Razzaboni et al., 1992). This protease is strongly inhibited by ACT, aprotinin, and PMSF, and is similar in character to a calcium-activated serine protease partially purified from AD brain (Abraham et al., 1991). Recently, Haass et al. (1992b) reported that

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[1] Abbreviations: COOH, carboxyl; CPP, *N*-[1(*R*,*S*)-carboxy-3-phenylpropyl]; Davis–PAGE, Davis polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; E-64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; HMB, (hydroxymercuri)benzoate; IPTG, isopropyl β-D-thiogalactopyranoside; MeO, methoxy; NEM, *N*-ethylmaleimide; NH_2, amino; pAB, *p*-aminobenzoate; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; pNA, *p*-nitroanilide; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Suc, succinyl; TIMP-2, tissue inhibitor of metalloproteases; TLC, thin-layer chromatography; Z, benzyloxycarbonyl; ZINCOV, *N*-[2-[(hydroxyamino)carbonyl]-4-methyl-1-oxopentyl]-L-alanylglycinamide].

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Aβ is produced normally in a soluble form in cell cultures of untransfected and APP-transfected cells. Their findings agree with findings of other groups who purified and sequenced Aβ and a novel Aβ fragment from human cerebrospinal fluid and conditioned medium of human mixed-brain cells grown in vitro (Seubert et al., 1992; Shoji et al., 1992).

Additional information on the chemical processes that might lead to the aggregation of Aβ in AD was offered by Dyrks et al. (1992). They reported that metal-catalyzed oxidation systems are able to transform Aβ and the APP COOH-terminal 100 residue fragments into aggregating molecules in vitro. These researchers also pointed out that oxidation of enzymes might lead to their inactivation and the inhibition of intracellular protein turnover.

The formation of Aβ by one or more proteases can be enhanced by the recently discovered mutations in the APP molecule. One mutation is found in the APP gene in patients with hereditary cerebral hemorrhage with amyloidosis of Dutch origin and is localized inside the Aβ (Levy et al., 1990), while a second mutation is located just outside the carboxyl terminus of the Aβ and is detected in DNA from patients with familial AD (Goate et al., 1991; Murrell et al., 1991; Chartier-Harlin et al., 1991). A third double mutation alters the lysine and methionine next to the amino terminus of Aβ to asparagine and leucine (Mullan et al., 1992). These mutations may predispose the APP to abnormal proteolytic cleavage and the generation of the Aβ. Citron et al. (1992) reported increased levels of secreted Aβ from human kidney 293 cells transfected with APP containing the Swedish FAD double mutation when compared to cells expressing native APP. Similarly, Cai et al. (1993) reported elevated amounts of Aβ from human neuroblastoma (M17) cells transfected with the APP carrying the Swedish mutation.

In this paper, we describe the detailed purification and characterization of a metalloprotease from AD brain which cleaves the Met–Asp bond in a 10 amino acid synthetic peptide flanking the amino terminus of the Aβ. Sequence analysis of the purified protease reveals high homology to the rat metalloendopeptidase 24.15. This activity was partially characterized in Papastoitsis and Abraham (1991). Most importantly, the protease cleaves native APP generating, among other fragments, a fragment that comigrates with a recombinant APP polypeptide (C-100) corresponding to the APP 596–695 amino acids of APP695. Since its cleavage site specificity in the 10 amino acid synthetic peptide coincides with the amino terminus of the Aβ, this enzyme becomes a reasonable candidate in the generation of Aβ.

EXPERIMENTAL PROCEDURES

Homogenization of the Brain Tissue. Brain tissue from AD patients (a gift from D. J. Selkoe, Harvard University) was homogenized in ice-cold 5× (v/w) Tris-acetate buffer, pH 7.4, containing 1% (v/v) Triton X-100 and 1 mM dithiothreitol (DTT) in a Waring blender. After homogenization, the solution was stirred for 30 min, on ice, and then centrifuged at 100000*g* for 60 min. The supernatant was subjected to 0–40 and 40–80% ammonium sulfate fractionation. After centrifugation in a Sorvall RC-58 refrigerated centrifuge at 10000*g* for 30 min, the two precipitates were redissolved in Tris-acetate buffer with 1 mM DTT, pH 7.4, and dialyzed extensively against the same buffer before further purification. All purification steps were carried out at 4 °C.

Assay for Synthetic Substrate Degrading Activity. The different protease fractions were monitored for proteolytic activity against the peptide HSEVKMDAEF (P1) which was iodinated on the His residue as described elsewhere ([^{125}I]-P1) (Abraham et al., 1990). Incubations were carried out at 37 °C in 50 mM Tris-acetate, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM DTT. The proteolytic products were separated by TLC on cellulose microcrystalline plates (J. T. Baker), followed by autoradiography. The TLC solvent contains 1-butanol, pyridine, acetic acid, and water (15:10:3:12 v/v) (Tempst & Van Beeumen, 1983). After TLC and autoradiography, radioactive products from the conversion of P1 to HSEVKM were quantified with a Molecular Dynamics ImageQuant v3.0 scanning densitometer or a Bioscan System 200 imaging scanner. Activity was reported as the percentage of P1 present at time zero converted to HSEVKM (Met cut) after the appropriate incubation time. The relationship is linear from 0 to approximately 60% conversion. One unit of Met cut protease activity under these conditions was defined as converting 1 nmol of P1 to HSEVKM in 1 min.

DEAE-Trisacryl M Ion-Exchange Chromatography. The 40–80% ammonium sulfate fraction, which contained the majority of the activity, was applied on a DEAE-trisacryl M ion-exchange column (1.5 × 20 cm). The column was previously equilibrated with 50 mM Tris-acetate plus 1 mM DTT, pH 7.4. Fractionation was carried out by eluting with a linear gradient of 0–0.5 M NaCl at a flow rate of 20 mL/h. Three-milliliter fractions were collected. Active fractions were pooled and dialyzed overnight against 50 mM Tris-acetate, 0.5 M $(NH_4)_2SO_4$, and 1 mM DTT, pH 7.4.

Phenyl-Sepharose Hydrophobic Interaction Chromatography. The dialyzed pool was loaded on a phenyl-Sepharose CL-4B column (1.6 × 2.6 cm). Proteins were eluted with a linear gradient (300 mL total) from 0.5 to 0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 20 mL/h, and 3.7-mL fractions were collected.

Sephacryl S-200 Gel Filtration Chromatography. The active fractions from phenyl-Sepharose chromatography were pooled and dialyzed against 50 mM Tris-acetate plus 1 mM DTT, pH 7.4, and concentrated to 3.8 mL by ultrafiltration under nitrogen pressure in an Amicon filter (YM-10, 10-kDa cutoff). The concentrated pool was then loaded on a Sephacryl S-200 gel filtration column (2.5 × 63 cm) equilibrated and eluted with 50 mM Tris-acetate plus 1 mM DTT, pH 7.4. The flow rate was adjusted to 10 mL/h, and 2.5-mL fractions were collected.

HA-Ultrogel Hydroxyapatite Chromatography. In the last step the pool containing protease activity from the Sephacryl S-200 column was chromatographed on an HA-Ultrogel column (1.6 × 6.5 cm). The pool and the column were equilibrated with 10 mM KH_2PO_4 plus 1 mM DTT, pH 7.4. The column was eluted with a linear gradient (100 mL total) of 10 mM–0.3 M KH_2PO_4 plus 1 mM DTT, pH 7.4, at 5 mL/h. Fractions of 1.5 mL were collected.

Molecular Weight Determination of the Protease. The apparent molecular weight of the protease was determined by SDS–PAGE according to the method of Laemmli (1970). The molecular weight standards used were myosin (H-chain, 228 000), phosphorylase *b* (109 600), bovine serum albumin (70 000), ovalbumin (44 000), carbonic anhydrase (27 900), and β-lactoglobulin (19 000). Gels were silver-stained using a Bio-Rad silver stain kit according to the manufacturer's instructions (Bio-Rad, Richmond, CA). The bands of the final protease sample were quantified with a Molecular Dynamics ImageQuant v3.0 scanning densitometer.

Mechanistic Examination of the Purified Protease. To examine the effects of various additives on the activity degrading the synthetic substrate (P1), the sample was

incubated with the appropriate amount of inhibitor for 60 min at 4 °C and assayed for remaining activity, as described above. Control reactions with no reagent, or containing only the solvent of the reagent, were always included. Stock solutions of PMSF, E-64, 1,10-phenanthroline, calpain inhibitor, and Z-Phe-Phe-CH_2F (Enzyme Systems Products, Livermore, CA) were dissolved in ethanol. Bestatin, EGTA, EDTA, HMB, iodoacetamide, leupeptin, NEM, TIMP-2 (a gift from W. Stetler-Stevenson, National Cancer Institute), and CPP-Ala-Ala-Phe-pAB (a gift from M. Orlowski, Mount Sinai School of Medicine) were dissolved in water. ZINCOV (Calbiochem) and phosphoramidon (Sigma) were also dissolved in water. Pepstatin A was dissolved in methanol. The susceptibility of the protease activity to various divalent cations was determined by including metals at a final concentration of 2 mM in the assay reaction mixture.

pH Optimum of the Purified Protease. The protease was incubated as described above except that the buffers were a series of citrate/phosphate buffers formulated according to the procedure of McIlvaine (McIlvaine, 1921) with pHs ranging from 4.0 to 7.6.

Substrate Gel Electrophoresis. To examine the substrate specificity of the purified protease pool, aliquots of the enzyme were electrophoresed on SDS substrate gels, containing 1 mg/mL casein or gelatin. The protease pool was mixed in a 1:1 ratio with 2x Laemmli (Laemmli, 1970) sample buffer without mercaptoethanol and loaded on a 12% SDS–polyacrylamide gel. Electrophoresis was carried out at 4 °C at 20 mA. After electrophoresis, the SDS was removed by washing the gel twice in 2.5% Triton X-100 for 30 min each at 25 °C. The Triton X-100 was then removed by washing the gel twice in double-distilled water for 15 min each. The gel was then incubated in 50 mM Tris-acetate, 1 mM $MgCl_2$, 0.02% (w/v) NaN_3, and 1 mM DTT, pH 7.4, for 2 days at 37 °C while shaking. The gel was stained in 0.1% Coomassie brilliant blue and destained. Alternatively, purified protease was mixed (1:1) with 0.1 M Tris-acetate plus 10% (v/v) glycerol, pH 7.0. The samples were then subjected to native PAGE in the Davis system (Davis, 1964) using 7.5% acrylamide, containing 1 mg/mL casein or gelatin. Electrophoresis was carried out at 25 mA at 4 °C. Following electrophoresis the gel was incubated in 50 mM Tris-acetate, 1 mM $MgCl_2$, and 1 mM DTT, pH 7.4, overnight at 37 °C while shaking. The gel was stained in 0.1% Coomassie brilliant blue and destained. Z-Val-Lys-Arg-AFC (a cathepsin B substrate) and Z-Phe-Arg-AFC (a cathepsin L substrate) enzyme overlay membranes were purchased from Enzyme Systems Products. The purified protease was mixed (1:1) with 0.1 M Tris-acetate plus 10% glycerol, pH 7.0, sample buffer and subjected to native PAGE as above. Following electrophoresis the gels were incubated in 50 mM Tris-acetate, 2 mM $MgCl_2$, and 1 mM DTT, pH 7.4, for 15 min at 25 °C and then placed on a moist filter paper inside a humidity chamber. At the same time the enzyme overlay membranes were dipped quickly in the same buffer and layered on top of the gels. The gels and the attached membranes were incubated at 37 °C for 30 min. Activity was monitored with an ultraviolet (UV) lamp.

Other Enzymatic Assays. Enzyme preparations were also assayed against a number of chromogenic substrates and full-length human recombinant APP751 produced by the baculovirus system in SF21 insect cells [Savage, M. J., Iqbal, M., Loh, T., Scott, R., & Siman, R. Cathepsin G: Localization in human temporal cortex and degradation of β-amyloid precursor protein (submitted for publication)]. The protease was incubated with 2 mM substrate solutions of MeO-Suc-Glu-Val-Lys-pNA, MeO-Suc-Glu-Val-Lys-Met-pNA (a gift from M. Iqbal, Cephalon, Inc.), Leu-pNA, Lys-pNA, Ala-pNA, and Met-pNA (Sigma) in 50 mM Tris-acetate, 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol, pH 7.4. Changes in absorbance were followed at 410 nm in a Titertek Multiskan ELISA reader. Control reactions contained no enzyme or no substrate. One unit of activity was defined as the hydrolysis of 1 μmol of substrate/min. The activity of the protease was tested against full-length human recombinant APP751 (1.6 μg). To avoid nonspecific degradation, APP was preincubated with 4 mM PMSF and 0.3 mM E-64 or 10 mM DFP and 0.3 mM E-64 followed by incubation in citrate/phosphate, pH 7.0, buffer (McIlvaine, 1921) with the purified protease. The reactions were incubated for 15 min at 37 °C. The products were separated on SDS–PAGE according to the discontinuous procedure of Schagger and Von Jagow (1987). The separated polypeptides were transferred to PDVF membranes (Millipore) according to the method of Towbin et al. (1979). The blots were immunostained with rabbit antibodies, 61C, targeting the last 30 amino acids of the COOH terminus of APP (Yamaguchi et al., 1990), and APP fragments were detected by using the ECL Western blotting detection system (Amersham). To prove specificity, the antibody was preabsorbed with its antigen (5 μg of peptide/μL antibody) overnight at 4 °C. Alternatively, reactions were incubated for 15 min and the products were separated and transferred to PVDF membranes as described above. The blots were immunostained with rabbit antibodies, C7, targeting the last 20 amino acids of APP (Podlisny et al., 1991), and APP fragments were detected using the ECL method. The molecular weight standards used were ovalbumin (44 000), carbonic anhydrase (27 900), β-lactoglobulin (19 000), lysozyme (14 300), bovine trypsin inhibitor (6200), and insulin (α and β chains, 3000 Da).

Synthesis of APP C-100. A DNA fragment encoding the COOH-terminal 99 amino acids of APP was generated by PCR using APP751 cDNA as template and two specific primers, carrying recognition sites for the restriction enzymes *Nde*I and *Bam*HI, respectively. PCR products were purified by electroelution after agarose gel electrophoresis, digested with *Nde*I and *Bam*HI, and ligated into an accordingly prepared expression vector. Bacterial transformants were grown to an A_{600} of 0.8, and expression was induced by addition of 1 mM IPTG. Bacteria were harvested 3 h after induction, and production of the recombinant protein was verified by Western blot analysis using an APP C-terminal-specific antibody. The APP C-100 was purified by ion-exchange chromatography and HPLC, and its identity was confirmed by amino acid analysis.

Digestion, HPLC, and Peptide Sequence Analysis. Protein sequence analysis was performed by William S. Lane from the Harvard University Microchemistry Facility according to the method of Aebersold et al. (1987) with minor modifications. The proteins were separated on a 10% SDS–PAGE gel and electroblotted onto nitrocellulose. After digestion with lys C endopeptidase, the resultant peptides were separated by narrow-bore reversed-phase HPLC. Optimal fractions for Edman microsequencing were screened for length and homogeneity by mass analysis on a mass spectrometer (Lane et al., 1991). Samples were subjected to automated Edman degradation on an ABI Model 477A protein sequencer using the manufacturer's recommendations for faster cycle time.

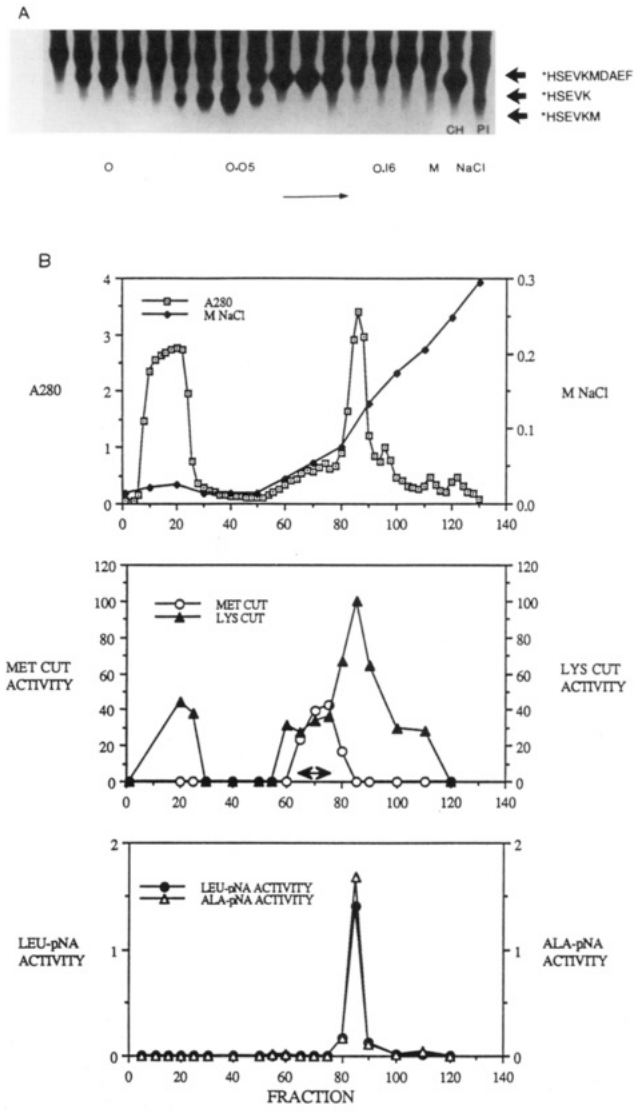

FIGURE 1: (A) Examination of Met and Lys cut activities of DEAE-trisacryl M fractions. Fractions were assayed with [^{125}I]P1 and proteolytic products separated by TLC and visualized by autoradiography. The positions of the uncleaved peptide and its proteolytic products are indicated by arrows. CH, sample loaded on the column; P1, uncleaved peptide. Activity profiles of DEAE-trisacryl M ion-exchange chromatography of a 40–80% ammonium sulfate fraction of AD brain homogenate. For details of the chromatographic conditions see Experimental Procedures. The fractions containing Met cut activity were pooled as indicated (middle box).

RESULTS

Purification of the Protease. Crude extract of human brain, as well as the 40–80% ammonium sulfate fraction, contained a number of proteolytic activities able to degrade P1. An activity cleaving between Lys and Met in P1 was termed Lys cut activity and between Met and Asp, Met cut activity. These included serine proteases, metalloproteases, and thiol proteases. Application of the 40–80% ammonium sulfate fraction to DEAE-trisacryl M resulted in the retention of many of these activities on the column. However, the Met cut protease eluted from the column at the beginning of the salt gradient. Activities that hydrolyzed Leu-pNA and Ala-pNA (Mantle et al., 1989) and two activities that cleave between the Lys and the Met amino acids of the synthetic substrate were also separated from the Met cut protease (Figure 1). The Met cut protease pool from the DEAE-trisacryl M was chromatographed on a phenyl-Sepharose column at pH 7.4. The Met cut protease eluted as a broad peak between 0.15 and 0 M

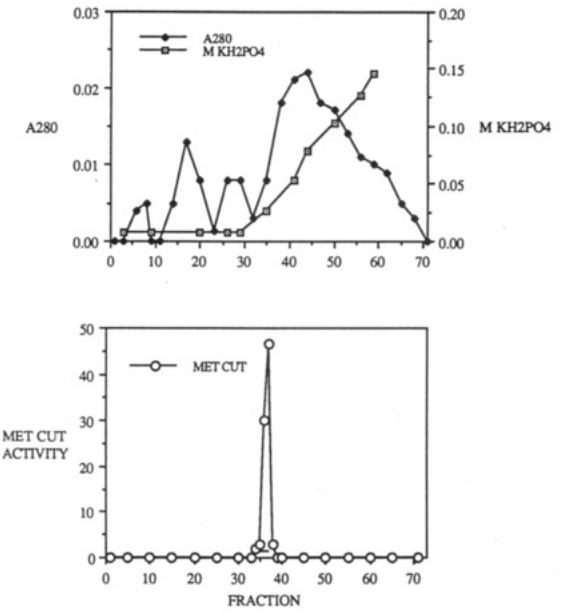

FIGURE 2: Hydroxyapatite chromatography on an HA-Ultrogel column. The Met cut activity containing fractions from the Sephacryl S-200 were chromatographed on an HA-Ultrogel column as described under Experimental Procedures. The active fractions of this column represent the final protease preparation.

$(NH_4)_2SO_4$. The fractions containing the peak of the Met cut protease were pooled, dialyzed, concentrated, and subjected to gel filtration on a Sephacryl S-200 at pH 7.4. The Met cut protease separated from the bulk of other proteins. Finally, the activity was pooled and chromatographed on an HA-Ultrogel column at pH 7.4 (Figure 2). The Met cut activity eluted at the beginning of the gradient in a sharp peak. This peak was pooled, concentrated, and used as the final protease preparation. The final preparation contained the Met (major) and Lys (minor) cuts. Despite the use of various chromatographic techniques in earlier trials (data not shown) and as shown here, we have been unable to separate the two activities from each other. In addition, both cuts were equally susceptible to all protease inhibitors used for the mechanistic examination of the Met cut protease, including the specific inhibitor CPP-Ala-Ala-Phe-pAB (see below). Thus, we concluded that the Met cut protease is also able to cleave between the Lys and the Met amino acids of the synthetic substrate but to a lesser extent (~30% of the total activity). Chromatography of the 40–80% ammonium sulfate fraction on a DEAE-trisacryl M, phenyl-Sepharose, Sephacryl S-200, and HA-ultrogel provided an approximately 114-fold purification of the Met cut protease with an overall yield of 16% (Table 1). A problem that we faced in quantifying the recovery of the protease during purification was that the Met cut activity was effectively masked by other proteases prior to the first chromatographic step (DEAE-trisacryl M). These activities cleaved the P1 between Lys and Met amino acids (Lys cut proteases). In fact, it is only after the first step that we were able to detect the Met cut activity (Figure 1).

Purity and Molecular Weight Determination of the Purified Protease. Electrophoresis of the final protease preparation in the Laemmli system (1970) followed by silver staining revealed only two bands (Figure 3). The lower molecular weight species accounted for approximately 67% in the preparation and corresponded to an apparent molecular weight of 43 000. The higher molecular weight species with an apparent molecular weight of 85 000 accounted for 33% of the staining.

Table 1: Purification Scheme of the Brain Met Cut Protease

step	vol (mL)	total protein (mg of protein)	total protease activity[a] (units)	spec activity (units/mg of protein)	purifn factor	yield (%)
crude extract	250	3600	nd[b]	nd		
$(NH_4)_2SO_4$ fraction (40–80%)	50	59	nd	nd		
DEAE-Trisacryl M	60	70.7	518	7.3	1	100
phenyl–Sepharose	5.8	8.7	39.5	4.5	0.6	7.6
Sephacryl S-200	11	2.8	47.2	16.9	2.4	9.1
HA-Ultrogel	1	0.1	83	830.0	113.6	16.0

[a] One unit of Met cut activity was defined as the conversion of 1 nmol of P1 to HSEVKM/min. The units represent the average value of three different assays for each purification step. [b] nd, not determined.

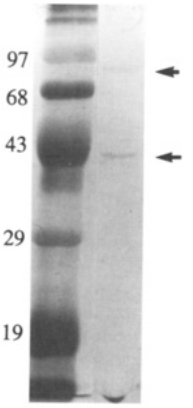

FIGURE 3: Protein composition and molecular weight determination of the final Met cut protease preparation. The purified sample was electrophoresed on a 12% SDS–polyacrylamide gel followed by silver staining. The position of two protein species is indicated by arrows. MW, molecular weight standards; lane 1, purified protease sample.

pH Optimum and Mechanistic Class of the Protease. The purified Met cut protease exhibited a pH optimum around pH 6.0. The enzyme was inactive below pH 5.0 or above pH 7.6 (data not shown). Strong inhibition of the Met cut enzyme was observed with NEM and HMB, less with E-64 (Table 2). The inhibition of the HMB was readily reversed at stoichiometrically increasing concentrations of the reducing agent (DTT). Iodoacetamide and leupeptin had a partial inhibitory effect on the protease (34 and 36.3%, respectively). A reducing agent, i.e., dithiothreitol, was required for the activity of the Met cut protease at low concentration. Strong inhibition was obtained by 1,10-phenanthroline, ZINCOV [a chelator of Zn(II)], EDTA, and EGTA, suggesting the requirement for a metal. It was only after including a relatively high concentration of EGTA (7.5 mM) that we achieved inhibition of the Met cut protease with this reagent. EGTA is known to be less potent in chelating zinc and magnesium when compared to calcium. CPP-Ala-Ala-Phe-pAB, a specific inhibitor of endopeptidase 24.15 (Orlowski et al., 1988), strongly inhibited the Met cut protease. To further assess the metal requirements of the protease, the enzyme activity was assayed in the presence of a number of divalent ions at 2 mM conentration. The protease was slightly activated in the presence of Mg(II) (30.4%), less by Mn(II), while Ca(II) had no significant effect on the Met cut activity. Strong inhibition occurred in the presence of Zn(II), Cu(II), and Hg(II) (Table 2).

Substrate Specificity. The purified protease was inactive toward several *p*-nitroanilide substrates, including MeO-Suc-Glu-Val-Lys-pNA, MeO-Suc-Glu-Val-Lys-Met-pNA, Leu-pNA, Lys-pNA, Ala-pNA, and Met-pNA as well as azocasein. The purified protease was also electrophoresed on SDS or Davis substrate gels containing casein or gelatin and incubated at 37 °C. No activity was observed with either of the two substrates. Also, the purified Met cut protease was electrophoresed on Davis gels and overlayed with either Z-Val-Lys-Lys-Arg-AFC (a cathepsin B substrate) or Z-Phe-Arg-AFC (a cathepsin L substrate) substrate overlay membranes followed by incubation at 37 °C for 30 min. There was no activity against either of these substrates.

Table 2

reagent	reagent concn[a] (mM)	% activity remaining
control		100
N-ethylmaleimide	5.0	7.0
(hydroxymercuri)benzoate	1.6	0
E-64	0.14	57.5[b]
E-64	0.14	27.3[c]
iodoacetamide	8.0	66
calpain inhibitor	1.0	92.9
Z-Phe-Phe-CH_2F	0.2	82.7
leupeptin	0.2	63.7
PMSF	7.0	100
aprotinin	0.03	100
soybean trypsin inhibitor	0.01	100
1,10–phenanthroline	4.0	0
ZINCOV	1.0	42.4
phosphoramidon	1.0	89.5
CPP-Ala-Ala-Phe-pAB	0.15	28.0
CPP-Ala-Ala-Phe-pAB	0.015	47.5
EDTA	3.0	33.2
EGTA	7.5	0
TIMP-2	0.03	100
bestatin	0.002	60
pepstatin A	0.15	100
dithiothreitol	0.0	0
dithiothreitol	1.0	100
dithiothreitol	2.5	70.8
dithiothreitol	5.0	25.5
Mg(II)	2.0	130.4
Mn(II)	2.0	119.1
Ca(II)	2.0	104.0
Cu(II)	2.0	0
Zn(II)	2.0	0
Hg(II)	2.0	0

[a] The purified enzyme preparation and reagent (at the indicated concentration) were incubated at 4 °C for 45 min except for E-64, which was incubated for 15 or 30 min ([b] and [c], respectively). Iodoacetamide was incubated at 37 °C for 30 min. The reaction mixtures were then assayed for remaining Met cut activity using the standard assay described under Experimental Procedures. The percent activity remaining is expressed relative to controls which were incubated without any reagent but in the presence of the same solvent used for the reagents.

Purified human recombinant APP751 produced in the baculovirus system was incubated with the purified Met cut protease for 15 min at pH 7.0. Following incubation, the reactions were analyzed on a Tris-tricine SDS–PAGE, followed by Western blotting and staining with polyclonal antibodies targeting the last 30 amino acids of APP (61C). Antibody 61C revealed a fragment of approximately 15 000 (Figure 4A, lane 1). Judging from its molecular weight, this proteolytic fragment contains the Aβ segment. The staining of the fragment was completely abolished when the antibody

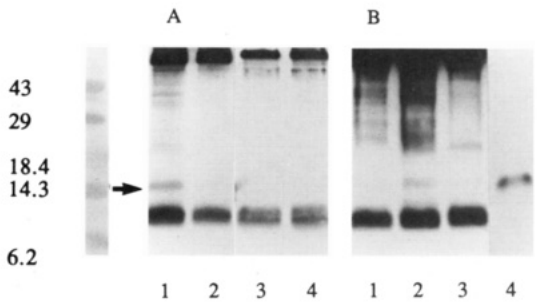

FIGURE 4: Degradation of human recombinant APP by the Met cut protease. APP (1.6 μg) was incubated with the purified Met cut protease at physiological pH. Incubations were carried out at 37 °C, and the reactions were separated on SDS–Tricine–PAGE and transferred to PVDF membranes. The blots were immunostained with rabbit antibodies targeting the last 30 or 20 amino acids of APP. (A) Lane 1, APP plus enzyme, 15 min; lane 2, APP alone, 15 min [the blot was immunostained with 61C antibody (Yamaguchi et al., 1990) targeting the last 30 amino acids of APP]; lanes 3 and 4, same as lanes 1 and 2, but the antibody was preabsorbed with its antigen. Position of amyloidogenic fragment is indicated by arrow. (B) Lane 1, APP alone, 15 min; lane 2, APP plus enzyme, 15 min; lane 3, APP plus enzyme and inhibitor; lane 4, C-100 [the blot was immunostained with antibody C7 (Podlisny et al., 1991) targeting the last 20 amino acids of APP]. Position of native APP is at the top of the blot. Note that a fragment comigrating with C-100 is seen in lane 2.

was preincubated with its antigen (Figure 4A, lane 3). Similarly, APP reactions were stained with polyclonal antibodies targeting the last 20 amino acids of APP (C7). These antibodies revealed a prominent band of approximately 15 000 (Figure 4B, lane 2), appearing to comigrate with the C-100 polypeptide (Figure 4B, lane 4) which represents the 596–695 amino acids of APP695. The production of this fragment was prevented when the protease was preincubated in the presence of CPP-Ala-Ala-Phe-pAB (Figure 4B, lane 3).

Amino Acid Sequence Analysis of the Two Protein Bands of the Final Preparation. Proteins were separated on SDS–PAGE, transferred onto nitrocellulose, digested, and sequenced as described under Experimental Procedures. Internal fragments of both the 85- and 43-kDa bands were obtained after digestion. Two peptides from the 85-kDa protein were sequenced and revealed a high degree of homology with sequences from rat metalloendopeptidase 24.15 (Table 3). We were unable to obtain sequence from a third fragment because its NH_2 terminus was blocked, suggesting that it is the NH_2 terminus of the protease. Similarly, sequences obtained from two fragments of the 43-kDa band revealed identity to sequences of rat aspartate aminotransferase (EC 2.6.1.1), a ubiquitous pyridoxal phosphate-dependent enzyme (Braunstein & Snell, 1985) (data not shown). Human aspartate aminotransferase was purchased from Sigma and assayed with the synthetic substrate P1. No proteolytic activity was observed under our incubation conditions.

DISCUSSION

In this paper we describe the purification and characterization of a metalloprotease from AD brain. The purification was monitored by using an assay based on the ability of the enzyme to cleave a synthetic peptide, HSEVKMDAEF (P1), between the Met and Asp amino acids, aspartic acid being the NH_2 terminus of Aβ. P1 was used as substrate because it provides a sensitive and highly selective assay for the protease. In addition to P1, the purified protease also digests human recombinant APP and generates potential amyloidogenic fragment(s). Internal peptide sequence analysis of the purified enzyme, termed Met cut protease, exhibits sequence highly homologous to the rat metallopeptidase EC 3.4.24.15.

A careful examination of the nature of the Met cut protease confirmed that it is a metalloprotease. Strong inhibition by 1,10-phenanthroline, ZINCOV, EDTA, and EGTA suggested that a metal is necessary for the activity of the protease. The purified Met cut protease was strongly inhibited by NEM and HMB and less by E-64, while dithiothreitol was required for its activity (Table 2). These results suggest that thiol groups are necessary for the activity of the protease. The homologous rat endoprotease 24.15 contains 18 Cys residues, including 1 located only 5 amino acid residues away from the active site of the protease (Pierotti et al., 1990). It appears that "bulky" inhibitors such as NEM and HMB might bind to a thiol group close to the active site of the protease and block access to the substrate molecule. CPP-Ala-Ala-Phe-pAB, a specific inhibitor of metallopeptidase 24.15, strongly inhibits the Met cut activity. Calpain inhibitor and TIMP-2, an inhibitor of matrix metalloproteases (W. Stetler-Stevenson, personal communication), did not inhibit the Met cut protease.

Because of the requirement that the protease has reduced thiol group(s), we also tested inhibitors of lysosomal cysteine proteases. Z-Phe-Phe-CH_2F (an inhibitor of cathepsin L and B) had no inhibitory effect on the protease activity, while leupeptin, an inhibitor of lysosomal cysteine and serine proteases, resulted in only limited inhibition (37.3%). These results, together with the inability of the Met cut enzyme to hydrolyze specific substrates of cathepsin B and L (Z-Val-Lys-Arg-AFC and Z-Phe-Arg-AFC), clearly distinguish it from these lysosomal cysteine proteases. Serine protease inhibitors such as PMSF, aprotinin, and soybean trypsin inhibitor had no effect on the Met cut activity. The Met cut protease is not an aspartyl protease since it was not inhibited by pepstatin A. Bestatin, an aminopeptidase inhibitor, had some inhibitory effect (40%) on the activity of the Met cut protease.

The inhibitor profile of the Met cut protease is similar to that of endopeptidase 24.15 purified from rat brain (Orlowski et al., 1983). Endopeptidase 24.15 is active against peptides such as bradykinin, neurotensin, and luteinizing hormone-releasing hormone. This protease is one of the two known neutral zinc metalloproteases found in brain. The other zinc metalloprotease, endopeptidase 24.11, seems to be identical to "enkephalinase" (Almenoff et al., 1981; Alstein et al., 1981; Orlowski & Wilk, 1981; Fulcher et al., 1982; Almenoff & Orlowski, 1983, 1984). Endopeptidase 24.15 was also purified from rat testes and shown to be closely related to the rat brain enzyme (Orlowski et al., 1989). While endopeptidase 24.11 is widely distributed in other tissues besides brain, endopep-

Table 3: Amino Acid Sequence (Residues) of Peptides Derived from Proteolytic Cleavage of the 85-kDa Band

	78
rat sequence:[a]	A-L-A-D-V-E-V-T-Y-T-V-Q
human sequence—peptide 1:	A-L-A-D-V-E-V-T-Y-T-V-Q
	200
rat sequence[a]	N-L-N-E-D-T-T-F-L-P-F-T-R-E-E-L-G-G-L-P
human sequence—peptide 2:	N-L-N-E-D-T-T-F-L-P-F-T-L-Q-E-L-G-X-L-P

[a] Pierotti et al. (1990).

tidase 24.15 is a much more tissue-specific protease, with the majority of its activity concentrated in the brain (Chu & Orlowski, 1985). Furthermore, substantial amounts (20–25%) of the protease activity were shown to be membrane-bound (Acker et al., 1987). Recently, Dahms and Mentlein (1992) described the purification of endopeptidase 24.15 from rat and pig brain. This group reported that approximately 20% of this endoprotease is associated with membranes. The latter characteristic becomes more significant in light of the latest evidence of some APP processing in intracellular compartments (Golde et al., 1992; Haass et al., 1992a).

Recently, McDermott et al. (1992) identified a proteolytic activity that cleaves acetyl-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-NH_2 at the Met–Asp bond. On the basis of the inhibitory profile of the protease they concluded that the purified protease is metallopeptidase 24.15. This group did not demonstrate activity against APP and did not obtain sequence information.

The final Met cut protease preparation, as well as less purified samples, exhibited no activity against gelatin or casein, azocasein, and a number of *p*-nitroanilide substrates such as MeO-Suc-Glu-Val-Lys-pNA, MeO-Suc-Glu-Val-Lys-Met-pNA, Leu-pNA, Lys-pNA, Ala-pNA, and Met-pNA. It appears that the above substrates do not meet the specificity criteria of the Met cut protease. Particularly, the inability of the Met cut protease to cleave smaller fragments of P1 (EVK-pNA and EVKM-pNA) suggests that at least the P1′, P2′, or P3′ position in the substrate's cleavage site is necessary for recognition, binding, and/or proteolysis. The purified Met cut protease degraded full-length APP and generated one fragment of approximetely 15 kDa comigrating with a recombinant APP segment (C-100), which is believed to be neurotoxic (Yankner et al., 1989). We cannot exactly pinpoint the cleavage site based on Western blot analysis, but as judged from the molecular weight of this fragment, its comigration with C-100 and its reactivity with two different antibodies against the COOH terminus of APP (Yamaguchi et al., 1990; Podlisny et al., 1991), the 15-kDa fragment should contain the Aβ segment of the protein. Even though the cleavage of the native APP by the protease appears to be limited in terms of amounts of proteolytic fragment(s) generated, there are several factors that could be responsible for these limitations. The in vitro proteolysis of the APP molecule by the Met cut protease might be hampered by conformational changes of the APP when it is not embedded in a cell membrane. Alternatively, the native APP might not be the primary target of the Met cut protease in vivo. Instead, the APP could be first cleaved by another protease and the fragments generated become then susceptible to proteolytic cleavage by the Met cut protease. In addition, Alzheimer's disease is a progressive disease and the accumulation of plaques in brain requires many years. It is resonable to suggest that the generation and accumulation of amyloidogenic fragments and Aβ are the result of moderate rather than robust proteolysis. Met cut protease is shown here to generate potentially amyloidogenic fragment(s). Further, the formation of the 15-kDa fragment from APP was prevented when the Met cut protease was preincubated with CPP-Ala-Ala-Phe-pAB, a specific inhibitor of metallopeptidase 24.15.

The primary structure data come in strong agreement with the characterization studies reported here confirming that the Met cut protease is a homolog of the rat metallopeptidase 24.15. Differences in the sequence of peptide 2 (see Table 3) could be either due to species variations or due to a different gene, i.e., another member of the same family.

Currently, it is not known which form of APP (membrane-bound or soluble) or which of its fragments are the precursors of Aβ. Seubert and colleagues reported a second secreted form of APP whose COOH-terminal amino acid appears to be methionine, precisely before the amino terminus of Aβ (Seubert et al., 1993). It is very likely that the above proteolytic cleavage of APP occurs at the trans-Golgi network or extracellularly.

The findings that Aβ and the APP COOH-terminal 100 amino acids accumulate in vitro after metal-catalyzed oxidations (Dyrks et al., 1992) add to the hypothesis that protein oxidation and enzyme dysfunction might be involved in aging and Alzheimer's disease (Smith et al., 1991; Stadtman, 1992). A decrease in activity of APP-processing enzymes due to oxidation and the aggregation of amyloidogenic fragments may compromise cellular integrity, leading to Alzheimer's disease. It is noteworthy that the Met cut protease could be particularly susceptible to oxidation due to the high number of Cys residues in its molecule (18), including 1 only 5 amino acids from the active site. Also, in our hands, the Met cut protease is inactive in the absense of a reducing agent, i.e., dithiothreitol.

The Met cut protease fulfills some of the essential criteria expected from an APP-processing enzyme. (A) It cleaves the 10 amino acid synthetic substrate flanking the NH_2 terminus of Aβ between Met and Asp amino acids, aspartic acid representing the NH_2 terminus of Aβ. (B) It cleaves the APP molecule at physiological pH, to generate potential amyloidogenic fragments. The tissue distribution and subcellular localization of the Met cut protease are currently being investigated, to establish the biological significance of this enzyme with respect to the formation of Aβ. Efforts are also under way to produce antibodies necessary for further studies of the protease.

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REFERENCES

Abraham, C. R., Selkoe, D. J., & Potter, H. (1988) *Cell 52*, 487–501.

Abraham, C. R., Ben-Meir, A., & Tempst, P. (1990) *Pept. Res. 3*, 211–215.

Abraham, C. R., Driscoll, J., Potter, H., Van Nostrand, W., & Tempst, P. (1991) *Biochem. Biophys. Res. Commun. 174*, 790–796.

Acker, G. R., Molineaux, C., & Orlowski, M. (1987) *J. Neurochem. 48*, 284–292.

Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E., & Kent, S. B. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6970–6974.

Almenoff, J., & Orlowski, M. (1983) *Biochemistry 22*, 590–599.

Almenoff, J., & Orlowski, M. (1984) *J. Neurochem. 42*, 151–157.

Almenoff, J., Wilk, S., & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun. 102*, 206–214.

Alstein, M., Mittman, S., & Vogel, Z. (1981) *Life Sci. 28*, 185–191.
Braunstein, A. E., & Snell, E. E. (1985) *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 2–35, Wiley, New York.
Cai, X. D., Golde, T. E., & Younkin, S. G. (1993) *Science 259*, 514–516.
Chartier-Harlin, M-C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J., & Mullan, M. (1991) *Nature 353*, 844–846.
Chu, T. G., & Orlowski, M. (1985) *Endocrinology 116*, 1418–1425.
Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., & Selkoe, J. (1992) *Nature 360*, 672–674.
Dahms, P., & Mentlein, R. (1992) *Eur. J. Biochem. 208*, 145–154.
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci. 121*, 404–427.
Dyrks, T., Dyrks, E., Hartmann, T., Masters, C., & Beyreuther, K. (1992) *J. Biol. Chem. 267*, 18210–18217.
Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., & Ward, P. J. (1990) *Science 248*, 1122–1124.
Fulcher, I. S., Matsas, R., Turner, A. J., & Kenny, A. J. (1982) *Biochem. J. 203*, 519–522.
Goate, A., Chartier-Harlin, M-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rosser, M., Owen, M., & Hardy, J. (1991) *Nature 349*, 704–706.
Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J., & Younkin, S. G. (1992) *Science 255*, 728–730.
Haass, C., Koo, E. H., Mellon, A., Hung, A. Y., & Selkoe, D. J. (1992a) *Nature 357*, 500–503.
Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., & Selkoe, D. J. (1992b) *Nature 359*, 322–325.
Hardy, J., & Allsop, D. (1991) *Trends Pharm. Sci. 12*, 283–288.
Koh, J.-Y., Yang, L. L., & Cotman, C. W. (1990) *Brain Res. 533*, 315–320.
Kowall, N. W., Beal, F., Busciglio, J., Duffy, L. K., & Yankner, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7247–7251.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lane, W. S., Galat, A., Harding, M. W., & Schreiber, S. L. (1991) *J. Protein Chem. 10*, 151–160.
Levy, E., Carman, M. D., Fernandez-Matrid, I. J., Power, M. D., Lieberburg, I., van Duinen, S., Bots, G. Th. A. M., Luyendijd, W., & Frangione, B. (1990) *Science 248*, 572–579.
Mantle, D., Lauffart, B., Perry, K. E., & Perry, H. R. (1989) *J. Neurol. Sci. 89*, 227–234.
McDermott, J. R., Biggins, J. A., & Gibson, A. M. (1992) *Biochem. Biophys. Res. Commun. 185*, 746–752.
McIlvaine, T. C. (1921) *J. Biol. Chem. 49*, 183–186.
Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., & Lannfelt, L. (1992) *Nature Genet. 1*, 1345–1347.
Muller-Hill, B., & Beyreuther, K. (1989) *Annu. Rev. Biochem. 58*, 287–307.
Murrell, J., Farlow, M., Ghetti, B., & Benson, M. D. (1991) *Science 254*, 97–99.
Nelson, R., & Siman, R. (1990) *J. Biol. Chem. 265*, 3836–3843.
Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942–4950.
Orlowski, M., Michaud, C., & Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81–88.
Orlowski, M., Michaud, C., & Molineaux, C. J. (1988) *Biochemistry 27*, 597–602.
Orlowski, M., Reznik, S., Ayala, J., & Pierotti, A. R. (1989) *Biochem. J. 261*, 951–958.
Papastoitsis, G., & Abraham, C. R. (1991) *J. Cell. Biochem. 15G*, 151.
Pave-Preux, M., Ferry, N., Bouguet, J., Hanoune, J., & Barouki, R. (1988) *J. Biol. Chem. 263*, 17459–17466.
Pierotti, A., Dong, K. W., Glucksman, M. J., Orlowski, M., & Roberts, J. L. (1990) *Biochemistry 29*, 10323–10329.
Podlisny, M. B., Tolan, D. R., & Selkoe, D. J. (1991) *Am. J. Pathol. 138*, 1423–1435.
Razzaboni, B. L., Papastoitsis, G., Koo, E. H., & Abraham, C. R. (1992) *Brain Res. 589*, 207–216.
Roher, A. E., Ball, M. J., Bhave, S. V., & Wakade, A. R. (1991) *Biochem. Biophys. Res. Commun. 174*, 572–579.
Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Selkoe, D. J. (1991) *Neuron 6*, 487–498.
Selkoe, D. J., Podlisny, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C., & Oltersdorf, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7341–7345.
Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I., & Schlenk, D. (1992) *Nature 359*, 325–327.
Seubert, P., Oltersdorf, T., Lee, M. G., Barbour, R., Blomquist, C., Davis, D. L., Bryant, K., Fritz, L. C., Galasko, D., Thai, L. J., Leiberburg, I., & Schenk, D. B. (1993) *Nature 361*, 260–262.
Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X. D., McKay, D. B., Tintner, R., Frangione, B., & Younkin, S. G. (1992) *Science 258*, 126–129.
Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., & Price, D. L. (1990) *Science 248*, 492–495.
Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A., & Markesbery, W. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10540–10543.
Stadtman, E. R. (1992) *Science 257*, 1220–1224.
Tempst, P., & Van Beeumen, J. (1983) *Eur. J. Biochem. 135*, 321–330.
Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wang, R., Meschia, J. F., Cotter, R. J., & Sisodia, S. S. (1991) *J. Biol. Chem. 266*, 16960–16964.
Yankner, B. A., Dawes, L. R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M., & Neve, R. L. (1989) *Science 243*, 417–420.
Yankner, B. A., Duffy, L. K., & Kirschner, D. A. (1990) *Science 250*, 279–282.
Yamaguchi, H., Ishiguro, K., Shoji, M., Yamazaki, T., Nakazato, Y., Ihara, Y., & Shansaku, H. (1990) *Brain Res. 537*, 318–322.