Substrate and Inhibitor Profile of BACE (β-Secretase) and Comparison with Other Mammalian Aspartic Proteases*

The full-length and ectodomain forms of β-site APP cleavage enzyme (BACE) have been cloned, expressed in Sf9 cells, and purified to homogeneity. This aspartic protease cleaves the amyloid precursor protein at the β-secretase site, a critical step in the Alzheimer's disease pathogenesis. Comparison of BACE to other aspartic proteases such as cathepsin D and E, napsin A, pepsin, and renin revealed little similarity with respect to the substrate preference and inhibitor profile. On the other hand, these parameters are all very similar for the homologous enzyme BACE2. Based on a collection of decameric substrates, it was found that BACE has a loose substrate specificity and that the substrate recognition site in BACE extends over several amino acids. In common with the aspartic proteases mentioned above, BACE prefers a leucine residue at position P1. Unlike cathepsin D etc., BACE accepts polar or acidic residues at positions P2 and P1 but prefers bulky hydrophobic residues at position P2. BACE displays poor kinetic constants toward its known substrates (wild-type sub-

Alzheimer's disease is characterized by the extracellular deposition of insoluble amyloid plaques. The main component of amyloid plaques is the 39–43-amino acid β-amyloid peptide (Aβ),1 which derives from a larger protein precursor (amyloid precursor protein, APP). Aβ is excised from APP by the sequential action of two proteases known, respectively, as β-secretase, which cuts amino-terminal to Aβ, and γ-secretase, which cleaves at the carboxyl terminus. Several reports appeared recently describing the cloning and characterization of β-secretase (1–5). This protein, designated Asp-2, BACE, or memapsin 2, according to the laboratory in which it was discovered, is a novel transmembrane aspartic protease that cleaves APP at the β-secretase site. BACE possesses all the characteristics expected for β-secretase in terms of substrate preference, pH optimum for activity, tissue distribution, and subcellular localization. In addition, two recent reports indicate that Aβ levels

The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor protein; DMA, N,N-dimethylacetamide; Fmoc, fluorenylmethoxycarbonyl; HATU, N-(dimethylamino)-1H-1,2,3-triazole[4,5-b] pyridine-1-yl-methylmethanaminium hexafluorophosphate N-oxide; HPLC, high performance liquid chromatography; FRET, fluorescence resonance energy transfer; MES, 4-morpholinolinosulfonic acid; BACE, β-site APP cleavage enzyme.

* 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. Fax: 41 61 688 19 29; E-mail: heinz.doebeli@roche.com.

1 The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor protein; DMA, N,N-dimethylacetamide; Fmoc, fluorenylmethoxycarbonyl; HATU, N-(dimethylamino)-1H-1,2,3-triazole[4,5-b] pyridine-1-yl-methylmethanaminium hexafluorophosphate N-oxide; HPLC, high performance liquid chromatography; FRET, fluorescence resonance energy transfer; MES, 4-morpholinolinosulfonic acid; BACE, β-site APP cleavage enzyme.

In the brains of BACE knockout mice are reduced by more than 90% compared with control mice (6, 7). In addition to cleaving APP at the β-secretase site, BACE cuts APP further downstream within the amyloid region (between Tyr-10 and Glu-11 of Aβ), generating a truncated form of Aβ that is probably still amyloidogenic (3, 8). Parallel to the discovery of BACE, a second, homologous transmembrane aspartic protease termed Asp-1, BACE2, memapsin 1, or down region aspartic protease was reported (4, 5, 9, 10). Preliminary analysis of BACE2 indicated that it can also function as a β-secretase in vitro (8, 9).

In seeking to develop a disease-modifying therapy for Alzheimer's Disease, BACE presents itself as an ideal drug target. It belongs to a well understood class of protease where inhibitors have previously been developed for therapeutic use (renin, human immunodeficiency virus protease). Two different peptidomimetic inhibitors of BACE with nanomolar activity have already been described (3, 11). In addition, an x-ray crystal structure has been published (12), which should facilitate rational design of new inhibitors. However, because the treatment of Alzheimer’s disease will be a long term therapy, a β-secretase inhibitor has to be very selective. Aspartic proteases are widely distributed in the body. BACE2, for instance, is found at low levels in most peripheral organs (13). Here we describe the enzymatic properties of BACE in comparison with its homologue, BACE2, and other relevant mammalian aspartic proteases, namely pepsin, cathepsin D, cathepsin E, napsin A and renin.

EXPERIMENTAL PROCEDURES

Cloning and Expression of BACE and BACE2
cDNAs encoding the aspartyl proteases BACE and BACE2 were modified by PCR in the 5’ non-coding region to optimize ribosomal recognition and at the 3’ end by adding sequences encoding 6xHis residues to enable rapid purification of the recombinant proteins. Expression in Sf9 insect cells via recombinant baculovirus resulted in higher yields than expression in Escherichia coli, Schizosaccharomyces pombe, or HEK293 cells. Thus the cDNAs were cloned into the pFAST-BAC1 vector (Invitrogen) as BamHI × XbaI fragments for expression in insect cells and the PCR products were confirmed by sequencing. After recombination into the baculovirus genome, the purified viral DNA was transformed into the insect cells. Sf9 cells were cultured at 27 °C in TC100 medium (BioWhittaker) with 5% (v/v) fetal calf serum. Virus stocks were generated with a titer of 1.5 × 106 plaque-forming units/ml. For large scale production of BACE and BACE2, 24-liter fermenters of Sf9 cells were infected with a multiplicity of infection of 1.

Preparation of Enzymes

BACE Ectodomain—The first step in purification of BACE ectodomain is immunonaffinity chromatography using a monoclonal antibody (BSC-1) generated in-house by Dr. M. Brockhaus. The antibody was generated by standard methods from a mouse immunized with BACE purified from E. coli. The specificity of BSC-1 (IgG1) was established by enzyme-linked immunosorobent assay and Western blot assays. Thus 5 liters of cell-free Sf9 fermentation broth was concentrated 10-fold by ultrafiltration and loaded directly onto a 1.6 × 4-cm BSC-1-Sepharose immunoaffinity column that had been equilibrated in 50 mM Tris-HCl,

This paper is available on line at http://www.jbc.org

Received for publication, September 25, 2001, and in revised form, November 15, 2001
Published, JBC Papers in Press, December 7, 2001, DOI 10.1074/jbc.M109266200

Fiona Grüninger-Leitch, Daniel Schlatter, Erich Küng, Peter Nerböck, and Heinz Döbeli‡
From Hoffmann-La Roche Ltd, Grenzacherstrasse 124, CNS Research, CH-4070 Basel, Switzerland

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 7, Issue of February 15, pp. 4687–4693, 2002
Printed in U.S.A.

The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor; DMA, N,N-dimethylacetamide; Fmoc, fluorenylmethoxycarbonyl; HATU, N-(dimethylamino)-1H-1,2,3-triazole[4,5-b] pyridine-1-yl-methylmethanaminium hexafluorophosphate N-oxide; HPLC, high performance liquid chromatography; FRET, fluorescence resonance energy transfer; MES, 4-morpholinolinosulfonic acid; BACE, β-site APP cleavage enzyme.
The extract was then loaded on a 2.6 M NaCl, 0.1% Triton X-100. The column was then eluted with 50 mM Tris-HCl, pH 7.4. This material was passed over a Mono S HR 5/5 column (Amersham Biosciences, Inc.) that had been previously equilibrated in 50 mM Tris-HCl, pH 7.4. BACE, which is obtained in the final concentration of 10 mM. The extract was then loaded on a 2.6 M NaCl, 0.1% Triton X-100.

Full-length BACE—50 g (wet weight) of SF9 cells was suspended in 750 ml of phosphate-buffered saline, 2% Triton X-100 and homogenized with a hand-held glass homogenizer. The homogenate was stirred on ice for 30 min and then centrifuged at 100,000 g for 20 min. The supernatant was adjusted to pH 8.0, and imidazole was added to a final concentration of 10 mM. The extract was then loaded on a 2.6 M NaCl, 0.1% Triton X-100. The column was subsequently washed with this buffer and then with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1% Triton X-100. The column was then eluted with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 200 mM imidazole, 0.1% Triton X-100 (10 column volumes). Pooled fractions containing full-length BACE were pooled, dialyzed against 50 mM sodium phosphate, 10 mM Tris, 100 mM NaCl, 0.1% Triton X-100, pH 8.0. The column was subsequently washed with this buffer and then with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1% Triton X-100. The column was then eluted with 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton X-100 and re-loaded onto a second 5-ml HitTrap Q column that had been equilibrated in 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton X-100. The column was washed in this buffer and eluted with a gradient of 50 mM Tris-HCl, pH 7.4, 1 mM NaCl, 0.1% Triton X-100 (20 column volumes). Fractions containing BACE were pooled, dialyzed against 50 mM Tris-Cl, pH 8.0, 0.1% Triton X-100 and loaded on a Mono S HR 5/5 column (Amersham Biosciences, Inc.). The unbound material was collected. This material was then diluted 10-fold into 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton X-100 and re-loaded onto a second 5-ml HitTrap Q column that had been equilibrated in 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.1% Triton X-100. The column was washed in this buffer and eluted with a gradient of 50 mM Tris-HCl, pH 7.4, 1 mM NaCl, 0.1% Triton X-100 (20 column volumes). Fractions containing BACE were pooled, dialyzed against 50 mM Tris-Cl, pH 8.0, 0.1% Triton X-100 and loaded on a Mono S HR 5/5 column (Amersham Biosciences, Inc.).

Identification of the Cleavage Site of Soluble Substrates

The samples were dried in a Speed-Vac and then dissolved in 110 μl of 50% formic acid. 100 μl were subjected to reversed-phase HPLC using a AL12505 column (5 μm, 5 × 100 mm, YMC, gradient of 1% formic acid and acetonitrile). Peak fractions were collected and dried again in a Speed-Vac. The peptide fragments were analyzed by electrospray ionization-mass spectroscopy (using a PE SCIEX, model API-100LC).

Inhibitors

The BACE inhibitor P10-P4' StatVal (Lys-Thr-Glu-Ile-SerGlu-Val-Aan-Sta-Val-Ala-Glu-Phe, where Sta is a statin transition state mimetic), originally described by Sinha et al. (3), was purchased from Bachem (Bubendorf, Switzerland). The BACE inhibitor OM99-2 (Glu-Val-Asn-Mim-Ala-Glu-Phe, where Mim is a transition state mimetic), originally described by Sinha et al. (14). Recombinant human renin was purified according to legends. The progression of the fluorescence increase was measured at λemission = 520 nm with fluorescence excitation at λexcitation = 430 nm. Reaction kinetics were followed periodically for 30 min at various substrate concentrations. The detected signals were converted into moles of substrate hydrolyzed per second. Kinetic data were determined graphically from Lineweaver-Burk plots. It should be noted that data obtained by varying substrate concentrations had to be corrected for the effect of excess quenching capacity, which is typical of all the FRET substrates used here. Assays were performed at enzyme concentrations that warranted a linear progression of product formation.

Identification of Cleavege Sites Using Combinatorial Libraries

Substrate and Inhibitor Profile of BACE

The BACE inhibitor P10-P4' StatVal (Lys-Thr-Glu-Ile-SerGlu-Val-Aan-Sta-Val-Ala-Glu-Phe, where Sta is a statin transition state mimetic), originally described by Sinha et al. (3), was purchased from Bachem (Bubendorf, Switzerland). The BACE inhibitor OM99-2 (Glu-Val-Asn-Mim-Ala-Glu-Phe, where Mim is a transition state mimetic), originally described by Sinha et al. (14). Recombinant human renin was purified according to legends. The progression of the fluorescence increase was measured at λemission = 520 nm with fluorescence excitation at λexcitation = 430 nm. Reaction kinetics were followed periodically for 30 min at various substrate concentrations. The detected signals were converted into moles of substrate hydrolyzed per second. Kinetic data were determined graphically from Lineweaver-Burk plots. It should be noted that data obtained by varying substrate concentrations had to be corrected for the effect of excess quenching capacity, which is typical of all the FRET substrates used here. Assays were performed at enzyme concentrations that warranted a linear progression of product formation.

Inhibitors

The BACE inhibitor P10-P4' StatVal (Lys-Thr-Glu-Ile-SerGlu-Val-Aan-Sta-Val-Ala-Glu-Phe, where Sta is a statin transition state mimetic), originally described by Sinha et al. (3), was purchased from Bachem (Bubendorf, Switzerland). The BACE inhibitor OM99-2 (Glu-Val-Asn-Mim-Ala-Glu-Phe, where Mim is a transition state mimetic), originally described by Sinha et al. (14). Recombinant human renin was purified according to legends. The progression of the fluorescence increase was measured at λemission = 520 nm with fluorescence excitation at λexcitation = 430 nm. Reaction kinetics were followed periodically for 30 min at various substrate concentrations. The detected signals were converted into moles of substrate hydrolyzed per second. Kinetic data were determined graphically from Lineweaver-Burk plots. It should be noted that data obtained by varying substrate concentrations had to be corrected for the effect of excess quenching capacity, which is typical of all the FRET substrates used here. Assays were performed at enzyme concentrations that warranted a linear progression of product formation.

Identification of Cleavage Sites Using Combinatorial Libraries

Synthesis of Targeted Peptide Libraries—The FRET substrate combinatorial libraries were synthesized on PEGA1900 (polymer of polyethylene glycol with a molecular weight of 1900 and acrylamide) resin via stepwise synthesis after standard Fmoc chemistry using HATU as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent. Fmoc-Gly-OH, Fmoc-Glu-Lucifer Yellow, and all Fmoc-protected amino acids were coupled to the amino PEGA1900 resin using HATU in the presence of diisopropylethylamine (HATU) as the condensing reagent.
The substrates are listed in the order of cleavage efficiency by BACE. An example of the structure with the decameric peptide sequence, the linker amino acids, the fluorophor, and the quencher is given in Fig. 2.

| Substrate | Sequence | Substrate type | Cleavage by BACE % of best substrate |
|-----------|----------|----------------|-----------------------------------|
| NL-D | SEVNLDAEFR | Swedish mutant APP β-cleavage site | 100 |
| EL-D | SEVEVDAEFR | Modified APP β-cleavage site | 87 |
| DA-A | VVEVDAAVTP | Artificial cleavage site at APP carboxyl terminus | 24 |
| NL-A | SEVNLAEEFR | Modified APP β-cleavage site | 16 |
| EF-P | SEVEFAAEFR | Modified APP β-cleavage site | 15 |
| KM-D | SEVMDAEEFR | Wild-type APP β-cleavage site | 9 |
| GY-E | HDSGYEVHHQ | Alternative APP β-cleavage site | 8 |
| EF-D | SEVFDAEFR | Modified APP β-cleavage site | 2 |
| P4K | SKVNLDAEFR | Modified APP β-cleavage site | 2 |
| VL-M | TSVLMAAP | Cathepsin D substrate (octameric peptide) | 2 |
| KL-V | VHIIQKLIFPA | APP α-secretase cleavage site | 1 |
| HL-V | IHPHLVIIHN | Renin substrate | 0 |

activity to the mature enzyme, a phenomenon already documented by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8 Å resolution. BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

**Validation of the Enzyme Assays**—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.

The sequences of the substrates are given in Table I. Most of the substrates cover the β-cleavage site of APP. Some were generated based on the putative specificity of β-secretase (e.g. KM-D, NL-D, or GY-E), some to confirm the results obtained by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8 Å resolution. BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

**Validation of the Enzyme Assays**—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.

The sequences of the substrates are given in Table I. Most of the substrates cover the β-cleavage site of APP. Some were generated based on the putative specificity of β-secretase (e.g. KM-D, NL-D, or GY-E), some to confirm the results obtained by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8 Å resolution. BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

**Validation of the Enzyme Assays**—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.

The sequences of the substrates are given in Table I. Most of the substrates cover the β-cleavage site of APP. Some were generated based on the putative specificity of β-secretase (e.g. KM-D, NL-D, or GY-E), some to confirm the results obtained by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8 Å resolution. BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

**Validation of the Enzyme Assays**—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.

The sequences of the substrates are given in Table I. Most of the substrates cover the β-cleavage site of APP. Some were generated based on the putative specificity of β-secretase (e.g. KM-D, NL-D, or GY-E), some to confirm the results obtained by others, implying that BACE is not a true zymogen (20). The purified BACE ectodomain crystallized and the structure was solved to 2.8 Å resolution. BACE2 was not purified to homogeneity but was shown to be almost free of other contaminating protease activities by analysis with a series of aspartic protease substrates (Fig. 3).

**Validation of the Enzyme Assays**—The FRET assay was based on dodecameric peptides containing 10 amino acids of the putative substrate and a linker amino acid at each end. The linker amino acids, lysine at the amino terminus and glutamine at the carboxyl terminus, are used to couple the fluorescent group Lucifer Yellow and the quenching group dabsyl to the substrate (Fig. 2). Because the quenching group is coupled via the ε-amino group of lysine, the peptide can still be analyzed by Edman degradation. The substrates were either used as soluble entities or immobilized to beads.
sequence element by cathepsin D or BACE when we were trying to isolate proteases with \(\beta\)-secretase activity using a mass spectrometry-based enzyme assay (17). In addition to the peptide fragment with the sequence DAEFRHIDSGYEVH-HQK with a mass of 1955 Da, two additional fragments with masses of 1338 and 1267 were identified. These masses correspond to the fragments AAVTPEERHLSK and AVT-PEERHLSK, respectively. Purified BACE generated the fragments with mass 1955 and 1267, whereas cathepsin D generated peptides with masses of 1955 and 1338. As expected, the FRET substrates were cleaved as predicted by the mass spectrometry assay. A selection of identified cleavage sites is given in Table II. They were determined by incubation of soluble substrates by the enzymes followed by separation of the fragments by reversed-phase HPLC and analysis by mass spectrometry.

All FRET substrates used in the present work have a strong excess of quenching capacity. This leads to apparently lower reaction rates (Lucifer Yellow liberated per min) at higher the substrate concentrations. The consequences are apparent substrate inhibition and underestimation of the \(K_m\) values. By correcting the quenching effect, the pair BACE and substrate DA-A showed a Michaelis-Menten-like behavior. However, BACE with the substrates KM-D and NL-D still showed a component of non-linearity, in particular at low substrate concentration. Therefore, the kinetic constants were determined graphically (Table IV). Under identical conditions, full-length BACE behaved more Michaelis-Menten-like than BACE ectodomain (data not shown). The origin of this phenomenon is not clear. The two enzyme forms also differ in terms of their stability, with the ectodomain losing activity rapidly on storage at 4 °C. To check if the two enzyme forms are comparable at all, the IC\(_{50}\) value for a number of inhibitors was measured in separate experiments with full-length BACE or the ectodomain. The inhibitors were all statin analogues of the OM99-2 and P10-P4' StatVal, covering IC\(_{50}\) values between 50 nM and 50 \(\mu\)M. The same order of potency was observed in both cases.

**Catalytic Properties of BACE**—To compare the preferred substrates and the pH optima for all aspartic proteases at hand, each enzyme was tested with seven substrates in the pH range 2–7.5 at a constant concentration of 10 \(\mu\)M (Table III). A subset of these data is shown in Fig. 3. BACE and BACE2 show a very similar substrate preference differing only with respect to the KY-E and the KL-V-substrates. Both cathepsin D and cathepsin E cleave Swedish mutant APP at the \(\beta\)-site, although with very low efficiency compared with their best substrates. Interestingly, cathepsin D cleaves the APP-derived substrates at a noticeably lower pH optimum than its preferred substrate VL-M (Fig. 3c).

The highest substrate turnover (\(K_{\text{cat}}\)) of BACE is observed with the NL-D substrate (Table IV). The highest substrate affinity (\(K_m\)) value, however, is observed with the DA-A substrate. Both the \(V_{\text{max}}\) value observed with the NL-D substrate as well as the \(K_m\) value observed with the DA-A substrate appear less favorable than the corresponding values for cathepsin D measured with its favorite substrate VL-M.

**Substrate Specificity of BACE**—Several specific peptide libraries were designed to examine the substrate specificity of BACE at and around the \(\beta\) cleavage site in APP. Fluorogenic peptide libraries coupled to a solid phase (polymer beads) were synthesized according to Rossé et al. (24). Proteinolytic cleavage of bead-linked peptide causes the bead to fluoresce. Fluorescent beads can be manually separated from non-fluorescent beads using a fluorescent microscope. The peptide sequence associated with each fluorescent bead can be directly determined by Edman degradation. BACE ectodomain was used in these experiments after it was observed that the full-length enzyme does not cleave bead-bound substrates. Presumably full-length BACE is too large to be able to penetrate the pores of the beads. The results obtained from the evaluation of several libraries are shown in Table V. The P2-P1 library was designed to assess which amino acids other than those found in wild-type APP (Lys, Met) and Swedish mutant APP (Asn, Leu) can be accepted in the P2 and P1 positions. The Asn-Leu motif was detected several times, and Leu was also found in combination with Ghu or Asp. On the other hand, neither the Lys-Met motif nor anything resembling it was ever detected, indicating that, in our *in vitro* assay at least, wild-type APP is not a preferred substrate for BACE. The Gly-Tyr motif, corresponding to the alternative cleavage site in APP (leading to \(\alpha_\beta\)), was also never detected in these experiments.

Because 22 of 25 experiments revealed Leu as the preferred P1 residue, we generated a second library with a fixed Leu at P1 and variable amino acids at P2 and P1′ positions. Asn was found as the preferred residue in P2. From a total of 66 beads, 24 showed Asn, 14 Glu, and 12 Asp, with Gln found once. The hydroxyamino acids Thr and Ser were found at position P2 only in combination with Ser at P1′.

The relatively loose substrate specificity of BACE was fur-
The numbers were generated by experiments of the type shown in Fig. 3 using the same assay conditions; full-length BACE and all other enzymes as described under “Experimental Procedures,” substrate concentrations of 10 μM and a pH gradient from 2 to 7.5 made with 100 mM sodium citrate. The highest activity with the preferred substrate at the pH optimum was set 100%. Experimental details are given in the legend to Fig. 1. BACE-2 was assayed in the presence of 5 μM pepstatin to inhibit possible contaminating aspartic proteases. Cat, cathepsin.

### Table III

| Substrate | BACE pH 4 | BACE-2 pH 4 | Cat E pH 5 | Cat D pH 5 | Pepsin pH 4 | Napsin A pH 6.5 | Remin pH 7 |
|-----------|-----------|-------------|------------|------------|-------------|----------------|----------|
| KM-D      | 9         | 24          | 0          | 0          | 7           | 0              | 0        |
| NL-D      | 100       | 100         | 0          | 3          | 4           | 0              | 0        |
| KL-V      | 1         | 19          | 100        | 37         | 7           | 8              | 3        |
| VL-M      | 2         | 19          | 8          | 100        | 100         | 100            | 100      |
| HL-V      | 0         | 0           | 1          | 0          | –1          | 11             | 0        |
| VL-V      | 1         | 19          | 100        | 0          | 0           | 0              | 0        |
| GY-E      | 8         | 3           | 1          | 0          | 0           | 0              | 0        |
| DA-A      | 24        | 32          | –1         | 31         | 2           | 0              | 0        |

### Figure 3: Substrate specificity and pH optimum

**A** The activity of the seven aspartic proteases with the various substrates was determined using 100 mM citrate in the pH range 2–7.5. Shown are the examples of BACE (○), BACE2 (●), and cathepsin D (□). For clarity, only a selection of substrates are shown: KM-D: ●, NL-D: ○, KL-V: △, VL-M: ◇, DA-A: ▽.

**B** The high value was selected as an internal reference and set 100% (see Table III). BACE2 was tested in the absence of pepstatin, in contrast to the experiment shown in Table III. There is an increment of activity toward the substrate KL-V that can be suppressed by pepstatin and, thus, might be due at least partially to a contaminating cathepsin D-like protease. On the other hand, the activity toward the substrate KL-V could not be inhibited by pepstatin, AEBSF, aprotinin, E-64, EDTA, or leupeptin. Noteworthy is the different pH optimum of cathepsin D with respect to the different substrates.

There are shown with a set of six libraries, each containing a random single amino acid at position P4, P3, P2, P1, P1’, and P2’, respectively (Table V). Again, P1 is the most restricted position with Leu as the only amino acid identified in 10 sequencing experiments. Hydrophobic side chains are preferred in the P3 position. Asp and Glu are preferred at P4 and P1’. Positions P2 and P2’, on the other hand, tolerate a variety of residues.

To verify these findings, a series of homologous substrates was synthesized (Table I). Replacement of Glu at P4 by Lys of the Swedish mutant substrate (SW) drastically reduced the efficiency to about 10% compared with the SW substrate. Replacement of the Leu at P1 by Phe reduced the cleavage preference of the enzyme for this substrate, whereas replacement of the Asn at P2 by a Glu had a relatively modest effect.

Relation to Other Proteases—The two BACE inhibitors described so far, P10-P4’ StatVal and OM99-2, contain the peptidase sequence of the APP β-cleavage site whereby the cleavage site is modified to mimic the transition state of the substrate. Both peptides inhibit BACE or BACE2 in the nanomolar range. However, the two peptides inhibit cathepsin D, cathepsin E, and pepsin as well at even lower concentrations (Table VI).

P10-P4’ StatVal is even more potent toward cathepsin D, cathepsin E, and pepsin than toward BACE, perhaps because its sequence element Sta-Val better mimics the cleavage site between two bulky hydrophobic residues. Inhibitor OM99-2 mimics the sequence Leu-Ala-Ala and is therefore close to substrates NL-A (Table I). Pepstatin, the renin inhibitor Remikiren, and the human immunodeficiency virus-protease inhibitor Saquinavir do not inhibit BACE. The least specific inhibitor appears to be pepstatin and the most specific inhibitors appear to be Remikiren and Saquinavir.

Based on these results and the substrate specificity data, the seven aspartic proteases can be grouped into three clusters, (a)
The experiments were performed with a random library of immobilized substrates and the BACE ectodomain. Similar experiments with the full-length enzyme did not reveal any cleavage, probably because the full-length enzyme is too large to enter the pores of the beads. The library type defines the amino acid positions, which were randomly occupied by all natural amino acids except cysteine. “Frequency” gives the number of beads with a particular amino acid sequence. The total number of selected and sequenced beads is calculated by adding these numbers; for instance, the number for the P2-P1 library is 25.

| Library type | Frequency | Library type | Frequency | Library type | Frequency |
|--------------|-----------|--------------|-----------|--------------|-----------|
| P2-P1        | SEVXDAEFR | P4           | SXVNLDAEFR| P1           | SEVXDAEFR |
| NL           | 9         | E            | 5         | L            | 10        |
| EL           | 6         | D            | 4         |              |           |
| DL           | 5         | G            | 1         | P1’          | SEVNLXAEFR|
| EF           | 3         |              |           | E            | 4         |
| QL           | 1         | P3           | SEXNLDAEFR| D            | 2         |
| YL           | 1         | V            | 5         | L            | 4         |
|             |           |              |           | P2’          | SEVNLXAEFR|
|             |           | I            | 2         | A            | 4         |
| P2-P1’       | SEVXLXAEFR|              |           |              |           |
| NLE          | 7         | T            | 2         | E            | 3         |
| ELD          | 7         | V            | 2         |              |           |
| DLD          | 7         |              |           | T            | 1         |
| TLS          | 6         | N            | 5         |              |           |
| NLA          | 5         | D            | 3         |              |           |
| ALD          | 4         | E            | 2         |              |           |
| NLD          | 3         | Y            | 1         |              |           |
| QLD          | 1         | M            | 1         |              |           |
| SLS          | 1         | A            | 1         |              |           |

**Table VI**

**Inhibitor profile of six aspartic proteases**

The IC_{50} values were determined in 100 mM sodium acetate buffer at a substrate concentration of 5 μM and at the optimal pH for the enzyme-substrate pair. Full-length BACE was used.

| Enzyme | Substrate (5 μM) | pH | OM99-2 | P10-P4/StatVal | Pepstatin | Remikiren | Saquinavir |
|--------|------------------|----|--------|----------------|-----------|-----------|-----------|
|        |                  |    | μM     | μM             | μM        | μM        | μM        |
| BACE   | NL-D             | 4.5| 0.08   | 0.2            | 80        | >100      | >100      |
| BACE2  | NL-D             | 4.5| 0.12   | 0.3            | 10        | >100      | >100      |
| Cathepsin E | KL-V  | 4.5| 0.01   | 0.01           | 0.0007    | 5         | 7         |
| Pepsin | VL-M             | 4.5| 0.06   | 0.006          | 0.002     | 38        | 17        |
| Cathepsin D | VL-M | 5.5| 4      | 0.04           | 0.0007    | 1         | 0.2       |
| Renin  | HL-V             | 7  | >100   | >100           | >100      | 0.013     | >100      |

BACE and BACE2, (b) cathepsin D, cathepsin E, napsin A, and pepsin and (c) renin. Group a prefers leucine at P1 and accepts polar or even acidic residues at P2 and P1'. Position P3 is mostly occupied by a hydrophobic residue, preferentially a valine residue. Group b prefers bulky hydrophobic residues at positions P2, P1, and P1', and group c, represented by renin, appears to be very specific, with the angiotensinogen cleavage motif the only known substrate. Groups a and c appear to be the most distant enzymes with respect to pH optimum, substrate specificity, and cross-reactivity of the inhibitors. In this respect it is perhaps unsurprising that not a single BACE inhibitor was found in a library containing more than 1800 renin inhibitors.4

**DISCUSSION**

The results of the present investigation can be summarized as follows: (a) BACE exhibits very poor kinetic constants when assayed with soluble decameric peptides deriving from the APP β-cleavage site (b) BACE accepts a wide variety of peptidic substrates and, in contrast to other mammalian aspartic proteases, prefers acidic or polar residues at the P2 and P1' positions (c) an alternative substrate, DA-A, which is unrelated to the β-secretase cleavage site in APP, was identified.

BACE exhibits very low catalytic efficiency toward its most preferred substrate (K_{cat}/K_{m} = 0.002 for NL-D). The catalytic efficiency of cathepsin D, for instance, with the best substrate tested here, is 250-fold higher (K_{cat}/K_{m} = 0.5 for VL-M). Similar values for BACE have been reported by other groups using a variety of BACE constructs expressed in diverse cellular expression systems and with other FRET substrates (5, 23, 25, 26). The poor performance of BACE is therefore unlikely to be because of the quality of the protein. It may be that APP is not the only substrate for BACE *in vivo* and that a better, as yet unidentified substrate, would give a much higher K_{cat}/K_{m} value. However, the peptidic substrate analysis described here indicates that the Swedish mutant APP peptide, NL-D, already contains an optimal sequence of amino acids in the P4-P2' positions so it seems unlikely that a better substrate exists. There may be factors other than the peptide sequence itself that contribute to the catalytic efficiency of BACE. Our data show that the KM-D peptide, which derives from the BACE cleavage site in wild-type APP, is an extremely poor substrate, but a number of publications document that BACE cleaves in vivo and in vitro (1, 3, 6, 7). The fact that BACE does cleave wild-type APP *in vivo* may have to do with the juxtaposition of these two proteins in the luminal membrane of the ER-Golgi system. Both the catalytic domain of BACE and the β-secretase cleavage site in APP face into the lumen, and this may provide the optimal orientation for substrate-protease interaction, allowing for more efficient substrate binding and cleavage. A recent publication showing that the transmembrane domain of BACE is essential for efficient interaction of APP and BACE supports this interpretation (27).

The data presented above also indicate that BACE can accept a wide variety of peptidic substrates. Compared with other mammalian aspartic proteases, BACE is more like cathepsin D (relatively unspecific) than renin (highly specific) in terms of substrate specificity. However, BACE clearly has a different

---

4 H. Dobeli, unpublished data.
substrate selectivity from cathepsin D, preferring polar or acidic amino acids in the P2 and P1’ positions; cathepsin D and indeed all other mammalian aspartic proteases prefer hydrophobic amino acids in these positions. A more problematic issue for drug developers might be the extended substrate pocket in BACE. Any attempt to decrease the size of the inhibitor OM99-2 resulted in a gradual loss of inhibitory potency regardless of whether amino-terminal or carboxyl-terminal amino acids were deleted.3

The limited substrate and inhibitor analysis undertaken here shows that BACE and BACE2 are very similar, at least with respect to their ability to digest the BACE substrates VL-M and KL-V (Table III and Fig. 3), these substrates being cleaved at a low level by BACE2 but not by BACE. Despite these subtle differences between BACE and BACE2 in vitro, a recent publication suggests that BACE2 might not actually cleave at the β-secretase site at all in vivo, with the enzyme preferring to cleave further downstream at a second site proximal to the α-secretase cleavage site in APP (28). The reason for this is unclear but it suggests that it might be possible to develop BACE inhibitors that might act selectively in vivo.

Acknowledgment—We thank Dr. Manfred Broichhaus, Dr. Eric Kitas, Dr. Georg Schmid, Veronique Horny, Daniela Hägin, Daniel Mona, Heidi Ortolf, Nicole Soder, Dr. Hans-Werner Lahn and Urs Röthlisberger for supporting us in this work.

REFERENCES

1. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mandia, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Bier, A., L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Trenan, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741

2. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T., Chapman, C., Schneider, K., Ratcliffe, S. J., Tattersall, D., Testa, T., Southan, C., Ryan, D. M., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (2000) Mol. Cell. Neurosci. 16, 609–619.

3. Schauer-Vukasinovic, V., Bur, D., Kitas, E., Schlatter, D., Rosse, G., and Taramelli, R. (2000) FEBS Lett. 469, 59–64.

4. Ghosh, A. K., Shin, D., Downs, D., Koelsch, G., Lin, X., Ermolieff, J., and Tang, J. (2000) J. Am. Chem. Soc. 122, 3522–3523.

5. Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A. K., Zhang, X. C., and Tang, J. (2000) Science 289, 150–153.

6. Bennett, B. D., Babu-Khan, S., Loeloff, R., Louis, J. C., Curran, E., Citron, M., and Varghese, J. (2000) J. Biol. Chem. 275, 21099–21106.

7. Roser, G., Rueng, R., Page, M. G., Schauer-Vukasinovic, V., Gilger, T., Lahm, H. W., and Huhnzer, P., and Schallert, D. (2000) J. Comb. Chem. 2, 461–466.

8. Marcinkeviciene, L., Luo, Y., Graciani, N. R., Combs, A. P., and Copeland, R. A. (2001) J. Biol. Chem. 276, 37712–37717.

9. Fleerems, J. W., Dominguez, D. I., Plets, E., Sorensen, L., Taylor, N. A., Multhaupt, G., Kraezaert, K., Annaert, W., and De Groover, B. (2000) J. Biol. Chem. 276, 4211–4217.

10. Capell, A., Steiner, H., Wille, M., Kaiser, C., Walter, J., Lammich, S., Multhaupt, G., and Haase, C. (2000) J. Biol. Chem. 275, 30849–30854.

11. Benjannet, S., Elagoz, A., Wickham, L., Mamarbachi, M., Munzer, J. S., Basak, A., Lazure, C., Cronish, J. A., Sodisso, S., Chellet, F., Chretien, M., and Seidah, N. G. (2000) J. Biol. Chem. 275, 1097–10887.

12. Shi, X. F., Chen, E., Yin, C. K., Na, S., Garsky, V. M., Lai, M. T., Li, Y. Z., Plachetka, M., Register, R. B., Sardana, M. K., Tang, M. J., Tiberius, J., Wood, T., Shafer, J. A., and Gardell, S. J. (2001) J. Biol. Chem. 276, 10366–10373.

13. Benjannet, S., Elagoz, A., Wickham, L., Mamarbachi, M., Munzer, J. S., Basak, A., Lazure, C., Cronish, J. A., Sodisso, S., Chellet, F., Chretien, M., and Seidah, N. G. (2000) J. Biol. Chem. 275, 1097–10887.

14. Marcinkeviciene, L., Luo, Y., Graciani, N. R., Combs, A. P., and Copeland, R. A. (2001) J. Biol. Chem. 276, 37712–37717.

15. Ermolieff, J., Loy, J. A., Koelsch, G., and Tang, J. (2000) Biochemistry 39, 12450–12456.

16. Yan, R., Han, P., Miao, H., Greengard, P., and Xu, H. (2001) J. Biol. Chem. 276, 36788–36796.

17. Yan, R., Munzner, J., Shuck, M., and Bienkowski, M. (2001) J. Biol. Chem. 276, 34019–34027.