An Inhibitor Binding Pocket Distinct from the Catalytic Active Site on Human β-APP Cleaving Enzyme

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Received May 19, 2005; Revised Manuscript Received June 20, 2005

ABSTRACT: β-APP cleaving enzyme (BACE) is responsible for the first of two proteolytic cleavages of the APP protein that together lead to the generation of the Alzheimer's disease-associated Aβ peptide. It is widely believed that halting the production of Aβ peptide, by inhibition of BACE, is an attractive therapeutic modality for the treatment of Alzheimer's disease. BACE is an aspartyl protease, and there is significant effort in the pharmaceutical community to apply traditional design methods to the development of active site-directed inhibitors of this enzyme. We report here the discovery of a ligand binding pocket within the catalytic domain of BACE that is distinct from the enzymatic active site (i.e., an exosite). Peptides, initially identified from combinatorial phage peptide libraries, contain the sequence YPYF(I/L)P(L/I) and bind specifically to this exosite, even in the presence of saturating concentrations of active site-directed inhibitors. Binding of peptides to the BACE exosite leads to a concentration-dependent inhibition of proteolysis for APP-related, protein-based substrates of BACE. The discovery of this exosite opens new opportunities for the identification and development of novel and potentially selective small molecule inhibitors of BACE that act through exosite, rather than active site, binding interactions.

Alzheimer's disease (AD) is a devastating neurodegenerative disease that affects millions of elderly people worldwide; the disease affects between 6 and 10% of humans by age 65 and almost half of all people by age 85 (*1*−*3*). AD is the most common cause of nursing home admittance in the United States, resulting in enormous economic and emotional burdens on families, caregivers, and society at large. The only accepted treatment currently available for AD is acetylcholinesterase inhibitors (e.g., Aricept), which merely treat the symptoms of cognitive impairment. There is no disease-modifying treatment available for patients with AD at present.

An invariant feature of AD is the accumulation of extracellular amyloid plaques in specific regions of the brain. These plaques are composed mainly of a 38−42-amino acid peptide, termed Aβ.[1] The extracellular accumulation of this peptide, either as soluble oligomers or as insoluble plaques, is generally thought to be pathogenic for AD. The Aβ peptide is excised from the amyloid precursor protein (APP) by the sequential action of two proteases: β-site amyloid cleaving enzyme (BACE) and γ-site amyloid cleaving enzyme (γ-secretase) (*3*). Alternatively, APP can be processed to a nonpathogenic peptide, P3, by a competing set of sequential proteolyic reactions catalyzed by the enzymes α-secretase and γ-secretase. Hence, BACE is the only enzyme known to be uniquely associated with the amyloidogenic pathway of Aβ formation.

BACE is a membrane-anchored member of the aspartyl protease family of hydrolytic enzymes (*4*−*7*). Several enzymes in this family are associated with human diseases; for example, HIV protease is an established target for antiviral chemotherapy in the treatment of AIDS, and human renin is a potential target for antihypertensive therapy. Despite their high degree of biological validation as drug targets, the aspartyl proteases have generally proven to be difficult to inhibit with low-molecular weight, pharmacologically tractable molecules. Most of the aspartyl protease inhibitors in current clinical use today are peptidomimetics that target the catalytic active site of the enzyme. While these drugs have demonstrated usefulness for treating systemic viral infections, their meager ability to cross the blood−brain barrier may prove to be an obstacle to the use of such compounds for treating diseases of the central nervous system, such as AD.

In addition to the active site, some proteolytic enzymes contain additional binding pockets, termed exosites, that engage the substrate protein at locations distal to the site of hydrolytic chemistry (*8*−*12*). These binding pockets can contribute significantly to the stabilization of the enzyme−substrate binary complex by providing important structural determinants of interaction. Additionally, exosites on some proteolytic enzymes can act as allosteric regulators of enzyme

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[1] Abbreviations: BACE, human β-APP cleaving enzyme; APP, amyloid precursor protein; Aβ, amyloid-β peptide; MBP, maltose-binding protein; NMP, *N*-methylpyrrolidone; DIEA, diisopropylethylamine.

10.1021/bi050932l CCC: $30.25
Published on Web 08/06/2005

activity so that binding interactions at the exosite are transmitted, through conformational changes, to the active site to effect augmentation or diminution of the enzyme's catalytic reactivity. In some cases, molecules have been identified that bind to an exosite of a protease, and these have proven to be effective inhibitors of enzymatic activity (*10*). Hence, exosites represent an alternative target for inhibitory ligand binding to proteolytic enzymes. Because the exosites are structurally distinct from the active sites of these enzymes, the nature of the molecules that bind to the exosites can be very different from that of active site-directed inhibitors. It is possible, therefore, that in some cases the exosite could provide a more pharmacologically tractable target for small molecule interactions than does the active site of the enzyme. Finally, the structural diversity of exosites among related enzymes may be greater than for the enzyme active sites, thus potentially providing means for the development of highly selective modulators.

While exosites have been reported for several enzymes of the serine and cysteine protease families, only a few examples of exosites have been reported for aspartyl proteases (*11*, *12*). In this paper, we report the discovery of an exosite within the catalytic domain of human BACE that binds small peptides in a manner that is unaffected by active site ligand occupancy. Peptides that bind in this exosite inhibit the ability of BACE to hydrolyze its natural protein substrate, APP. The size of these peptides, and the nature of the amino acids required for binding to this pocket, suggest that it may be possible to inhibit the catalytic activity of BACE by binding small, druglike molecules at this exosite.

MATERIALS AND METHODS

Expression and Purification of BACE. Expression and purification of BACE were carried out as previously described (*13*). Most of the data reported here were collected with the catalytic domain of BACE expressed in insect cells. However, all of the constructs reported by Kopcho et al. (*13*) were tested. The binding of the exosite peptides was not significantly affected by the form of BACE enzyme that was used. All peptides were synthesized using standard Fmoc chemistry. To synthesize exosite peptides with the Alexa488 label attached either to the α-amino group of the N-terminal residue or to the ω-amino group of the side chain of an α,ω-diamino acid appended to the C-terminus of the peptide, purified exosite peptides were reacted with the *N*-hydroxysuccinimidyl ester of the Alexa488 fluorophore (Molecular Probes, Eugene, OR) for 16−20 h in NMP and DIEA at a 1:1.5−2.0 ratio. The progress of the reaction was monitored by HPLC. The resulting Alexa488-labeled peptide was then purified by HPLC and characterized.

Phage Display and Panning. We used (a) random 4-, 6-, and 7-mer libraries that were constrained by disulfide bonds between fixed flanking cysteine residues and (b) random 5-, 7-, 12-, and 15-mer libraries that were unconstrained. The cyclic 7-mer and the linear 7- and 12-mer libraries were "PhD" libraries from New England Biolabs (Beverly, MA). All other libraries were constructed in the fUSE5 phage vector (*14*). Panning was carried out for three cycles against BACE. BACE was immobilized by applying it as a coat at 0.5 μg/well in four wells of Dynex Immulon 4HBX plates overnight at 4 °C in 0.1 M $NaHCO_3$ buffer (pH 9.0). Panning was carried out by standard procedures at room temperature that involved blocking wells with 2% BSA in PBS and elution with 0.1 M HCl (pH 2.2). The vector NTI alignment tool and visual inspection of sequences were employed to analyze the selected peptides.

After approximately 20−50 clones from each library had been sequenced after three cycles of selection, we prepared essentially all possible candidate clones (39 clones in total) for phage ELISA to obtain direct evidence of affinity for BACE. Eleven clones gave binding signals, and one of those clones, a 12-mer clone (NLTTYPYFIPLP, peptide 1; refer to Table 1 for a list of all the peptides that were tested), was reproducibly shown to bind specifically to BACE. We therefore sequenced additional 12-mer clones to try to find additional candidate clones. Eleven candidate clones were tested by phage ELISA, and one clone (ALYPYFLPISAK, peptide 2) exhibited specific binding to BACE. This ALYPYFLPISAK peptide is homologous to the NLTTYPYFIPLP peptide and consistent with the specific binding of both those peptides to BACE. The ALYPYFLPISAK peptide and NLTTYPYFIPLP peptide were the two most efficiently recovered clones, together constituting ∼10% of total output after three rounds of panning.

Isothermal Calorimetry. Isothermal calorimetry was performed with a VP-ITC instrument from MicroCal, Inc. (Northampton, MA). Interactions between various forms of BACE, exosite peptides 1 and 2, and the active site-directed inhibitor OM99-2 (*15*, *16*) occurred at temperatures between 25 and 37 °C, and between pH 4.5 and 7.1. The data shown in Figure 1 were collected in 25 mM NaOAc buffer (pH 5.3) containing 137 mM NaCl, 3 mM KCl, and 0.5% (v/v) DMSO, at 37 °C. BACE (expressed in CHO cells) was placed in the cell at 5 μM, and peptide 1 was used in the syringe at 166 μM, with accurate quantitation of each by absorbance. For experiments in the presence of OM99-2, the compound was added to a final concentration of 50 μM with BACE and degassed before being loaded into the cell. Complementary experiments were also carried out to demonstrate that binding of peptides 1 and 2 had little effect on the binding parameters of OM99-2 (not shown).

To collect data, a power setting of 6 μcal/s and a syringe stirring rate of 300 rpm were used. To completely define the binding isotherm, typically a 2.5−3-fold excess of peptide was added during the course of the titration. The data collection time per injection was fixed at 360 s, with a signal averaging time of 2 s. The data were analyzed using Origin 5.0 for ITC fitting into a single-binding site model to determine the binding stoichiometry (n), the dissociation constant (K_d), the enthalpy of the reaction (ΔH), the entropy of the reaction (ΔS), and the Gibbs free energy of the reaction (ΔG).

Binding of Alexa488-Labeled Exopeptides to BACE by Fluorescence Anisotropy. Binding studies using fluorescence anisotropy were carried out on an Aviv fluorometer at 25 °C. The excitation and emission wavelengths were set to 495 and 519 nm, with slit widths at 4 and 10 nm, respectively. Unless specified otherwise, all the binding and competition studies were carried out in 50 mM acetate buffer (pH 4.5), with a final DMSO concentration of 1%. A concentrated BACE stock was used to titrate a 300 μL solution of 10 nM labeled peptide 3, 5, or 6. The final BACE concentration ranged from 10 to 5000 nM. Fluorescence anisotropy was

Table 1: Binding and Inhibition of Enzymatic Activity for Exosite-Binding Peptides of BACE

Peptide Identifier	Sequence	% Inhibition @ 10 μM [b]	K_d (μM) [a–c]	IC_{50} (μM) [d]
1	Ac-NLTTYPYFIPLP-NH_2	77	0.8 [b] 0.38 [c]	1.9
2	Ac-ALYPYFLPISAK-NH_2		0.63 [c]	1.3
3	(Alexa)-NLTTYPYFIPLP-NH_2		1.0 [a]	
1-11	Ac-NLTTYPYFIPL-NH_2	75		3.4
1-10	Ac-NLTTYPYFIP-NH_2	56		5.1
1-9	Ac-NLTTYPYFI-NH_2	16		55
1-8	Ac-NLTTYPYF-NH_2	0		NI
1-7	Ac-NLTTYPY-NH_2	5.0		
1-6	Ac-NLTTYP-NH_2	5.0		
2-12	Ac-LTTYPYFIPLP-NH_2	76		
3-12	Ac-TTYPYFIPLP-NH_2	70		
4-12	Ac-TYPYFIPLP-NH_2	67		
5-12	Ac-YPYFIPLP-NH_2	65		6.1
6-12	Ac-PYFIPLP-NH_2	7		132
7-12	Ac-YFIPLP-NH_2	0		
8-12	Ac-FIPLP-NH_2	2.6		
9-12	Ac-IPLP-NH_2	2.5		
10-12	Ac-PLP-NH_2	2.5		
4	Ac-YPYFIPL-NH_2	71	1.2 [b]	
5	(Alexa)-YPYFIPL-NH_2		6.4 [a]	
6	Ac-YPYFIPL-(Alexa)		0.06 [a]	
5-10	Ac-YPYFIP-NH_2	37	7.7 [b]	
5-9	Ac-YPYFI-NH_2	27	21 [b]	
scrambled	Ac-LYPPYIF-NH_2	4	>100 [b]	
Y5A	Ac-**A**PYFIPL-NH_2	17		
P6A	Ac-Y**A**YFIPL-NH_2	7.2		
Y7A	Ac-YP**A**FIPL-NH_2	13		
F8A	Ac-YPY**A**IPL-NH_2	4.5		
I9A	Ac-YPYF**A**PL-NH_2	17		
P10A	Ac-YPYFI**A**L-NH_2	67		
L11A	Ac-YPYFIP**A**-NH_2	73		

[a] Measured by direction titration of BACE using FP. All FP assays were carried out at pH 4.5 and 25 °C. [b] Measured by competition of the unlabeled peptide 3 or 6 as the labeled ligand and BACE at a concentration equal to the K_d. [c] Measured directly by ITC at pH 5.3 and 37 °C, as described in the text. [d] Measured by the inhibition of BACE cleavage of the wild-type MBP−APP(547−695) fusion protein.

measured a few minutes after each addition when the change in anisotropy had plateaued.

The change in anisotropy was plotted against the BACE concentration. The K_d of labeled peptide 3, 5, or 6 and the initial (r_0) and final anisotropy (r_b) of the labeled exopeptides were calculated from curve fitting using Kaleidagraph (Synergy Software, Reading, PA). The equation used to fit the binding data is identical to eq 1 of Lai et al. (*17*).

Competition of Unlabeled Exopeptides Assessed by Fluorescence Polarization. Labeled exopeptide 3 or 6 at 10 nM was mixed with BACE at a concentration equal to the K_d of the labeled peptide to BACE (K_d = 1.0 μM and 50 nM for peptides 3 and 6, respectively). The initial anisotropy value was measured. A concentrated unlabeled peptide or compound stock was titrated into the solution of labeled peptide and BACE as described above. The final concentration of unlabeled peptide or compound ranged from 20 to 20000 nM. Fluorescence anisotropy was measured after each addition.

The anisotropy value at each concentration of unlabeled peptide (r) was converted to fractional occupancy based on the r_0 and r_b values obtained from the binding assay [$(r - r_0)/(r_b - r_0)$]. The fractional occupancy was then plotted against the concentration of the competing peptide or compound. The K_d of the competing ligand was calculated from curve fitting using Kaleidagraph. The equation used to fit the competition data is identical to eq 4 of Lai et al. (*17*).

The screening of a collection of truncated and mutated peptides was carried out essentially as described for the competition assay with slight modifications. Peptide 3 or 6 was used as the labeled exopeptide (K_d = 1.0 μM or 50 nM, respectively). The truncated unlabeled peptides were screened at a single concentration of 10 μM at pH 4.5. The anisotropy values detected with peptide 3 or 6 in the absence of inhibitor, i.e., truncated unlabeled peptide (r_a), and in the presence of the inhibitor (r_b) and the labeled peptide alone (r_0) were used to calculate the percent of inhibition {100[$(r_a - r_b)/(r_a - r_0)$]}. Most experiments were performed at pH 4.5 where enzymatic activity is optimal. In separate experiments, the pH was varied over the range of 4.5−5.3, as in the ITC experiments described above. The exosite peptides

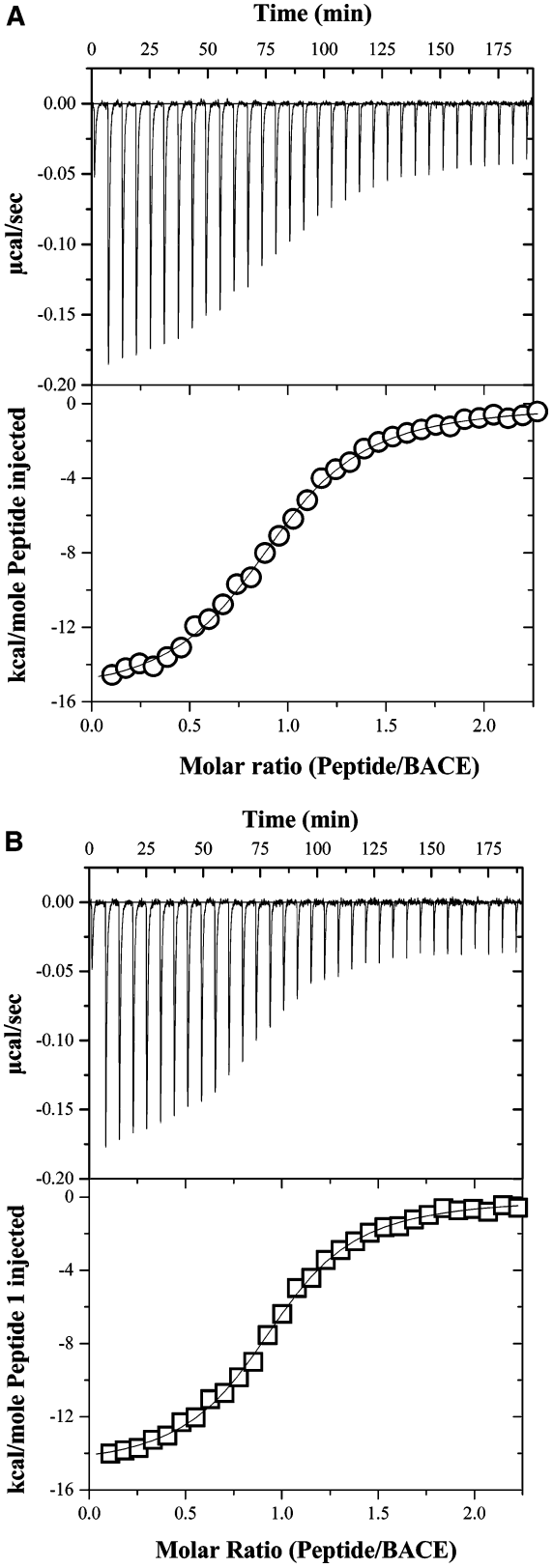

FIGURE 1: Isothermal titration calorimetry experiments demonstrating the binding affinity of BACE for peptide 1 in the absence (A) and presence (B) of 50 μM OM99-2 at pH 5.3 and 37 °C. Raw data in microcalories per second vs time (top) are shown, along with integrated raw data and the fit (bottom) to a 1:1 binding model. The best fit curve to the data in panel A shows a K_d of 0.38 ± 0.02 μM, with a ΔH of −15.9 ± 0.2 kcal/mol, and a molar ratio of 0.93 ± 0.01 peptide 1/mol of BACE. Similarly, panel B gave a best fit curve with a K_d of 0.28 ± 0.02 μM, with a ΔH of −15.0 ± 0.2 kcal/mol, and a molar ratio of 0.96 ± 0.01 peptide 1/mol of BACE.

displayed saturable binding at all pH values that were tested with a trend toward higher-affinity binding at the higher pH values. At all pH values that were tested, the presence of saturating concentrations of OM99-2 had no significant effect on exosite peptide binding to BACE.

BACE Enzyme Assay Monitoring Cleavage of the MBP−APP(547−695) Fusion Protein. A BACE cleavage assay that monitors the cleavage of the MBP−APP(547−695) fusion protein was carried out following the protocols described previously (*6*). All cleavage reactions were carried out at pH 4.5, where enzymatic activity is optimal.

RESULTS AND DISCUSSION

Phage display methods were used for library screening to identify peptides that bind to the catalytic domain of human BACE. Two peptides were identified in this way, which were composed of the following amino acid sequences: NLTTYPYFIPLP (peptide 1) and ALYPYFLPISAK (peptide 2). The underlined amino acids highlight a region of common sequence within these two peptides. Both peptides were then synthesized chemically and tested for their ability to bind to BACE using isothermal calorimetric measurements. Peptide 1 displayed a K_d at pH 5.3 and 37 °C of 0.38 ± 0.02 μM (Figure 1A), and peptide 2 displayed a K_d at 37 °C of 0.63 ± 0.04 μM (data not shown). Neither peptide bore any significant sequence identity to known peptidic substrates of BACE. Nevertheless, our initial hypothesis was that these peptides most likely bound within the active site cleft of the enzyme. To our surprise, however, the addition of saturating concentrations (50 μM) of the active site-directed peptide inhibitor OM99-2 (*15*, *16*) to the BACE sample had essentially no effect on the binding of either peptide 1 (Figure 1B) or peptide 2. NMR data were also consistent with the lack of displacement of OM99-2 by peptide 1 or 2 binding (not shown). Thus, OM99-2 and peptides 1 and 2 bind independently to BACE such that a ternary complex of OM99-2, BACE, and these newly discovered peptide ligands can be formed. These data suggest a novel ligand binding pocket within the catalytic domain of human BACE to which peptides 1 and 2 bind, regardless of the state of occupancy of the enzyme's active site.

To explore ligand binding to this exosite more extensively, we appended a fluorescent Alexa 488 group onto the N-terminus of peptide 1 (peptide 3; see Table 1) to develop a fluorescence polarization-based (FP) assay of binding to BACE. This assay was used to characterize the specificity of peptide binding and the minimal sequence required for interaction with BACE. All of the FP binding studies reported here were performed at pH 4.5 and 25 °C. Isothermal titration calorimetry studies determined that the affinities of peptides 1 and 2 were slightly diminished by these lower-pH and -temperature conditions. Nevertheless, we chose to study ligand binding under these conditions because they match the conditions commonly used to study BACE enzymatic activity in vitro. Figure 2 illustrates the change in peptide 3 fluorescence anisotropy that accompanies titration with the BACE enzyme. The data are fit well by a hyperbolic function that describes 1:1 binding interactions between the peptide and the enzyme with a K_d value of 1.0 μM. Fixing the concentration of peptide 3 and BACE to achieve approximately 50% occupancy, we found that we could

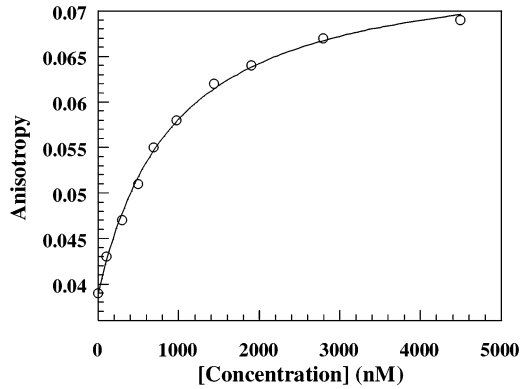

FIGURE 2: Fluorescence anisotropy of peptide 3 as a function of BACE enzyme concentration. The line drawn through the data represents the best fit to the equation for 1:1 saturable binding.

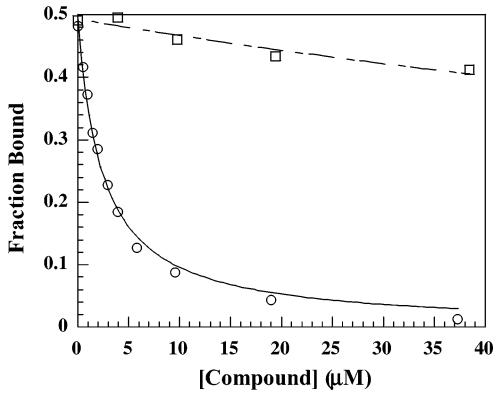

FIGURE 3: Displacement of fluorescent peptide 6 from BACE by unlabeled peptides. The unlabeled cognate peptide [peptide 4 (○)] demonstrates a concentration-dependent ability to displace peptide 6. Fitting of these data to a cubic equation (*17*) yields a K_d value for peptide 4 of 1.2 μM. In contrast, a scrambled peptide of the same size and amino acid composition as peptide 4 (□, labeled scrambled in Table 1) fails to demonstrate significant, concentration-dependent displacement of peptide 6 from BACE.

displace fully peptide 3 from BACE by titration of the unlabeled cognate peptide, peptide 1. Fitting of the binding displacement curve from this experiment yielded a K_d for peptide 1 of 0.8 μM, indicating that the addition of the Alexa 488 fluorophore to the N-terminus of this peptide had little effect on binding interactions with BACE.

In a like manner, we used displacement of peptide 3 binding to BACE to examine the affinity of a series of amino acid truncations from the N- and C-termini of peptide 1. In these experiments, the ability of an unlabeled peptide to inhibit binding of peptide 3 to BACE was tested at a single concentration of unlabeled peptide (10 μM). The results of these studies are summarized in Table 1. We found that the minimum length peptide displaying a high affinity for BACE represents the seven-amino acid sequence common to the two originally identified peptides [YPYF(I/L)P(L/I)]. Peptide 4, with the sequence YPYFIPL, represents a minimal peptide that retains good binding affinity for BACE (K_d = 1.2 μM; see Table 1). Appending an Alexa 488 fluorophore to the N-terminus of this minimal peptide increased modestly the dissociation constant for binding to BACE (peptide 5, K_d = 6.4 μM; Table 1). Surprisingly, however, moving the fluorophore to the C-terminus of this peptide (peptide 6) resulted in a significant increase in binding affinity for BACE (K_d = 0.06 μM; Table 1) and a significant increase in the change of the anisotropy value upon binding ($r_b - r_0 = 0.23$ for peptide 6, compared to 0.037 for peptide 3). This result suggests sequence specific interactions with a well-defined binding pocket on BACE that can be occupied by a combination of aromatic and aliphatic organic molecules. Despite its increased affinity for BACE, peptide 6 can still be displaced fully from the enzyme by peptide 4 in a concentration-dependent manner (Figure 3). As with peptides 1 and 2, the binding of peptide 4 or 6 to BACE is essentially unaffected by the presence of saturating concentrations of OM99-2, an active site-directed peptidic inhibitor of BACE (not shown).

That the specific amino acid sequence of peptide 4 is critical for BACE interactions is demonstrated by the fact that a scrambled version of this peptide, with an identical amino acid composition (sequence LYPPYIF; labeled "scrambled" in Table 1), was unable to displace any of the fluorescent peptides from BACE (Figure 3). Additionally, systematic replacement of each amino acid in peptide 4 with alanine (Ala) allowed us to identify the underlined residues YPYFIPL as critical determinants of binding to BACE (Table 1). Taken together, the results of the alanine scan and the scrambled peptide demonstrate that peptides 1−6 display specific interactions with a binding pocket on the BACE enzyme that is distinct from the catalytic active site. Specific interaction is also suggested by the fact that only the 12-mer phage library yielded binding peptides and that only two binding peptides were recovered, rather than a more extensive set of homologous peptides.

The binding of peptidic inhibitor OM99-2 to the catalytic active site of BACE did not significantly affect the ability of peptides 1−4 to bind at the BACE exosite. Likewise, peptide occupancy at the exosite did not perturb the affinity of OM99-2 for the active site of BACE. OM99-2 is a small peptidic inhibitor that is related to the APP sequence surrounding the β cleavage site of the Swedish mutant form of APP. We wondered how peptide occupancy at the exosite would affect the binding and cleavage of larger substrates that encompass more of the APP protein sequence. To test this, we used the MBP−APP(547−695) fusion protein in a manner similar to procedures described by Sinha et al. as a substrate for BACE and monitored proteolysis of this substrate with an ELISA (*6*). We found that peptides 1 and related peptides inhibited the BACE-mediated cleavage of the MBP−APP(547−695) fusion protein in a concentration-dependent fashion (Table 1).

As summarized in Table 1, the IC_{50} for inhibition of BACE enzymatic activity tracts with the ability of peptides to inhibit binding of the fluorescent exosite peptide 3, as quantified by the inhibition percentage of each peptide at 10 μM. If the inhibitory potency of the various peptides is a direct result of their affinity for binding to the exosite, one would expect the inhibition percentage in the FP assay to be related to the IC_{50} for inhibition of enzymatic activity by the standard Langmuir isotherm equation (*18*). This equation can be rearranged to yield an equation for the IC_{50} value as follows:

$$IC_{50} = [I]\left(\frac{100}{\%\ \text{inhibition}} - 1\right) \quad (1)$$

where [I] is the concentration of peptide used to compete with peptide 3 in the FP binding assay (10 μM) and % inhibition is the observed inhibition percentage (Table 1).

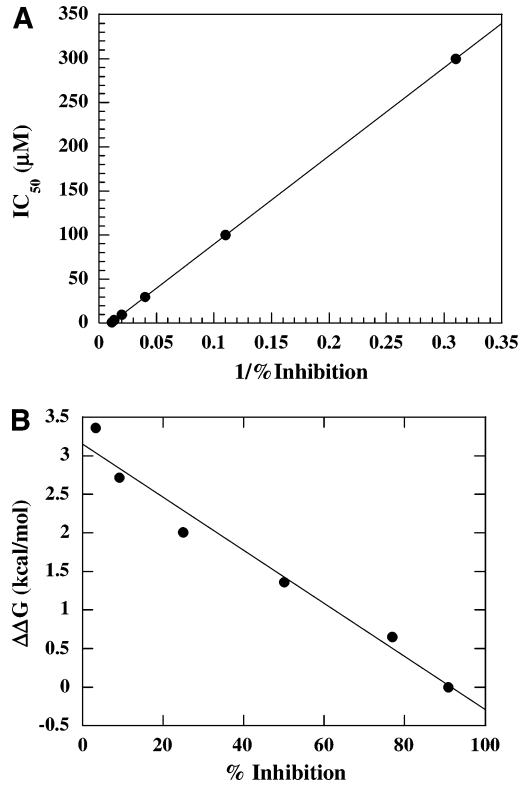

FIGURE 4: (A) Correlation plot of IC_{50} (micromolar) for inhibition of BACE catalytic activity and the reciprocal of % inhibition at 10 μM for competition with peptide 3 in the FP binding assay. The line drawn through the data is the theoretical relationship between these values based on eq 1. (B) Correlation plot of $\Delta\Delta G_{binding}$ for inhibition of BACE catalytic activity and % inhibition for competition with peptide 3 in the FP binding assay. The line drawn through the data is the least squares best fit to a linear equation.

Thus, from eq 1 we would expect the IC_{50} to be a linear function of the reciprocal of % inhibition, with the *y*-intercept equal to -10 (i.e., $-[I]$) and a slope equal to 1000 (i.e., 100[I]). Figure 4A illustrates the correlation between the experimentally obtained values of IC_{50} and % inhibition for six exosite peptides spanning a range of inhibitory potencies. The line drawn through the data represents the theoretical linear relationship expected from eq 1. The agreement between the experimental data and the theoretical expectation is remarkable, especially considering that the peptides compete with different ligands in the FP (i.e., here they compete against peptide 3) and enzymatic [i.e., here they compete against the MBP−APP(547−695) fusion protein] assays.

The IC_{50} value for inhibiting BACE enzymatic activity is also related to the K_i for inhibitory binding of the peptides to BACE, and this dissociation constant is in turn related to the free energy of binding. The relationship between IC_{50} and K_i can, however, depend on a number of factors (*18*). Nevertheless, because the IC_{50} values for all of the peptides were determined under a constant set of assay conditions, the ratio of the IC_{50} values for any two peptides can be related to the change in free energy of binding, according to the following equation (*18*):

$$\Delta\Delta G_{binding} = RT \ln\left(\frac{IC_{50}^{X}}{IC_{50}^{1}}\right) \quad (2)$$

where IC_{50}^{X} is the IC_{50} value for peptide *X* and IC_{50}^{1} is the IC_{50} for a standard peptide, in our case, peptide 1. The change in binding free energy thus calculated from the inhibition data for enzyme activity should be inversely proportional to the peptide's ability to compete with the fluorescently labeled peptide 3 for BACE binding, if binding affinity for the exosite relates directly to enzyme inhibition. In Figure 4B, we illustrate the correlation between $\Delta\Delta G_{binding}$ and inhibition percentage for the same six peptides as in Figure 4A. Consistent with our expectation, the data are described well by an inverse linear relationship. Thus, it appears that peptide occupancy at the exosite of BACE is sufficient to block the proteolysis of protein-based substrates of BACE. At present, we cannot define the inhibition modality for the exosite peptides. It is clear from the isothermal calorimetry and fluorescence anisotropy binding data (vide supra) that these peptides form binary complexes with the free enzyme species, as expected for inhibitors that conform to either competitive or noncompetitive modes of inhibition (*18*). Whether the peptides are also capable of forming a ternary complex with the ES species, and thus are noncompetitive with respect to the substrate, is unclear from the data that are currently available.

To our knowledge, this is the first report of an exosite associated with BACE. Exosites are well-known for other proteases, including members of the serine- and cysteine-protease families (see, for example, ref *8*). In these cases, the exosites are known to contribute to the overall binding energy of substrate interactions. A few examples have also been reported for aspartyl proteases, including pepsin and γ-secretase (*11*, *12*). Other enzymes that act on macromolecular substrates, such as protein kinases, are also known to utilize exosites as key substrate binding determinants (*9*). Inhibitors, including peptides and small molecules, have been identified that target some of these enzymes through exosite interactions (*10*). Because the structure of an exosite is distinct from the enzyme active site, these ligand binding pockets offer opportunities to design novel inhibitors that do not rely on traditional structural components (e.g., transition state analogues) to engage key active site components of the enzyme. Hence, exosite-directed inhibitors may offer unique opportunities to develop potent inhibitors of enzymes such as BACE, with a high degree of selectivity over other enzymes that share similar active site structures and mechanisms (e.g., other human aspartyl proteases). The nature of the critical determinants for binding to the BACE exosite was defined through the combination of amino acid truncations and Ala scanning (vide supra). It was found that the critical components of the exosite binding peptides were composed of aromatic and aliphatic amino acids. It was further demonstrated that judicious positioning of a polycyclic aromatic group (i.e., Alexa 488) at the C-terminus of a minimal peptide significantly increased the binding affinity for BACE. Our results suggest that the exosite binding pocket on BACE can be occupied by druglike organic molecules that lend themselves to therapeutic use.

At present, we have no data to address whether exosite occupancy is sufficient to block BACE-mediated cleavage of APP in vivo. If, however, this proves to be the case, the newly identified exosite offers an attractive alternative target for small molecule inhibitors of BACE activity. Further

studies will determine the utility of such an approach for the development of chemotherapeutic agents for the treatment of Alzheimer's disease.

ACKNOWLEDGMENT

We are grateful to Y. Zhang, Marcia Wood, and Joseph Cook for technical assistance.

REFERENCES

1. Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics, *Science 297*, 353−356.
2. Selkoe, D. J. (1997) Alzheimer's disease: Genotypes, phenotypes, and treatments, *Science 275*, 630−631.
3. Olson, R. E., Copeland, R. A., and Seiffert, D. (2001) Progress towards testing the amyloid hypothesis: Inhibitors of APP processing, *Curr. Opin. Drug Discovery Dev. 4*, 390−401.
4. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, 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., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, *Science 286*, 735−741.
5. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity, *Nature 402*, 533−537.
6. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., and John, V. (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain, *Nature 402*, 537−540.
7. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein, *Proc. Natl. Acad. Sci. U.S.A. 97*, 1456−1460.
8. Krishnaswamy, S., and Betz, A. (1997) Exosites determine macromolecular substrate recognition by prothrombinase, *Biochemistry 36*, 12080−12086.
9. Horiuchi, K. Y., Scherle, P. A., Trzaskos, J. M., and Copeland, R. A. (1998) Competitive inhibition of MAP kinase activation by a peptide representing the α C helix of ERK, *Biochemistry 37*, 8879−8885.
10. Maun, H. R., Eigenbrot, C., and Lazarus, R. A. (2003) Engineering exosite peptides for complete inhibition of factor VIIa using a protease switch with substrate phage, *J. Biol. Chem. 278*, 21823−21830.
11. Das, C., Berezovska, O., Diehl, T. S., Genet, C., Buldyrev, I., Tsai, J. Y., Hyman, B. T., and Wolfe, M. S. (2003) Designed helical peptides inhibit an intramembrane protease, *J. Am. Chem. Soc. 125*, 11794−11795.
12. Ng, K. K., Petersen, J. F., Cherney, M. M., Garen, C., Zalatoris, J. J., Rao-Naik, C., Dunn, B. M., Martzen, M. R., Peanasky, R. J., and James, M. N. (2000) Structural basis for the inhibition of porcine pepsin by *Ascaris* pepsin inhibitor-3, *Nat. Struct. Biol. 7*, 653−657.
13. Kopcho, L. M., Ma, J., Marcinkeviciene, J., Lai, Z., Witmer, M. R., Cheng, J., Yanchunas, J., Tredup, J., Corbett, M., Calambur, D., Wittekind, M., Paruchuri, M., Kothari, D., Lee, G., Ganguly, S., Ramamurthy, V., Morin, P. E., Camac, D. M., King, R. W., Lasut, A. L., Ross, O. H., Hillman, M. C., Fish, B., Shen, K., Dowling, R. L., Kim, Y. B., Graciani, N. R., Collins, D., Combs, A. P., George, H., Thompson, L. A., and Copeland, R. A. (2003) Comparative studies of active site-ligand interactions among various recombinant constructs of human β-amyloid precursor protein cleaving enzyme, *Arch. Biochem. Biophys. 410*, 307−316.
14. Scott, J. K., and Smith, G. P. (1990) Searching for peptide ligands with an epitope library, *Science 249*, 386−390.
15. Ghosh, A. K., Shin, D., Downs, D., Koelsch, G., Lin, X., Ermolieff, J., and Tang, J. (2000) Design of potent inhibitors for human brain memapsin 2 (β-Secretase), *J. Am. Chem. Soc. 122*, 3522−3523.
16. Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A. K., Zhang, X. C., and Tang, J. (2000) Structure of the Protease Domain of Memapsin 2 (β-Secretase) Complexed with Inhibitor, *Science 290*, 150−153.
17. Lai, Z., Auger, K. R., Manubay, C. M., and Copeland, R. A. (2000) Thermodynamics of p53 binding to hdm2(1−126): Effects of phosphorylation and p53 peptide length, *Arch. Biochem. Biophys. 381*, 278−284.
18. Copeland, R. A. (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists*, Wiley, New York.

BI050932L