Inhibition of β-site amyloid precursor protein-cleaving enzyme by a statine-based inhibitor has been studied using steady state and stopped-flow methods. A slow onset rate of inhibition has been observed under steady state conditions, and a $K_d$ of 22 nM has been derived using progress curves analysis. Simulation of stopped-flow protein fluorescence transients provided an estimate of the $K_d$ for initial inhibitor binding of 660 nM. A two-step inhibition mechanism is proposed, wherein slower “tightening up” of the initial encounter complex occurs. Two hypotheses have been proposed in the literature to address the nature of the slow step in the inhibition of aspartic proteases by peptidomimetic inhibitors: a conformational change related to the “flap” movement and displacement of a catalytic water. We compared substrate and inhibitor binding rates under pre-steady-state conditions. Both ligands are likely to cause flap movement, whereas no catalytic water replacement occurs during substrate binding. Our results suggest that both ligands bind to the enzyme at a rate significantly lower than the diffusion limit, but there are additional rate limitations involved in inhibitor binding, resulting in a $k_{on}$ of $3.5 \times 10^8$ M$^{-1}$ s$^{-1}$ for the inhibitor compared with $3.5 \times 10^9$ M$^{-1}$ s$^{-1}$ for the substrate. Even though specific intermediate formation steps might be different in the productive inhibitor and substrate binding to β-site amyloid precursor protein-cleaving enzyme, a similar final optimized conformation is achieved in both cases, as judged by the comparable free energy changes ($\Delta G$ of 2.01 versus 1.97 kcal/mol) going from the initial to the final enzyme-inhibitor or enzyme-substrate complexes.

A substantial body of evidence indicates that accumulation of insoluble plaques in the brain is an important step in the pathogenesis of Alzheimer’s disease (1). The extracellular amyloid plaques consist of aggregates of amyloid β-peptide isoforms, which are proteolytically derived from the amyloid precursor protein by two proteases, β- and γ-secretases. Until recently, the identities of these proteases have been elusive. Within the past 2 years the involvement of presenilins in the cleavage of amyloid β-protein has been identified as the proteolytic enzyme (2–5). Even though the BACE polypeptide sequence appears to be most closely related to the pepsin aspartic protease family (5, 6), the enzyme is not inhibited by pepstatin, suggesting significant differences at the active site level.

The proposed chemical mechanism for aspartic proteases involves activation of the attacking water molecule by the general base Asp-COO$^-$ with concomitant protonation of the substrate carbonyl by a general acid Asp-COOH, yielding a tetrahedral intermediate amidate hydrate (7). The statine moiety of pepstatin constitutes a tetrahedral, hydroxymethylene-isoteric replacement for the scissile peptide bond, mimicking the putative reaction intermediate and resulting in potent inhibition of aspartic proteases. Several attempts to design β-secretase inhibitors, based on this chemical mechanism, have been made recently. Incorporation of the statine moiety into the P10-P4′ peptide$^*$ representing the Swedish variant of the substrate sequence (Lys → Asn/Met → Leu at the P2-P1 positions) resulted in moderate inhibition of BACE (IC$_{50}$ ≈ 40 μM (8)). Substitution of the P1′ position Asp by Val in the same peptide resulted in a much more potent peptidomimetic inhibitor (IC$_{50}$ ≈ 30 nM (8)). Likewise, the replacement of the peptide bond between P1 and P1′ by a hydroxymethylene isostere in a substrate octapeptide, together with a Asp → Ala substitution at the P1′ position, yielded an inhibitor with an IC$_{50}$ of −1 nM (9, 10). The latter inhibitor was co-crystallized with a truncated version of BACE, and a significant number of protein-inhibitor interactions have thus been defined (11).

The interactions of eight residues of the inhibitor with BACE include four hydrogen bonds between two active site aspartates and the hydroxyl of the transition state isostere and 10 hydrogen bonds from different parts of the cleft and flap of the protein to the inhibitor backbone. Interestingly, binding of peptidomimetic inhibitors to the human immunodeficiency virus, type 1 protease involves substantial conformational changes, especially in the flap region, where backbone movements as large as 7 Å are observed (12). Considering the mechanistic similarity between BACE and human immunodeficiency virus protease, binding of peptidic inhibitors to BACE is likely accompanied by similar flap (residues VPYTQGKW) movement, and the tryptophan (Trp$^{137}$) in the flap region would be expected to provide a fluorescence probe for structural change studies. Additionally, pepstatin has been shown to be a slow and tight binding inhibitor of another

---

* 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. Tel.: 302-695-7219; Fax: 302-695-8313, E-mail: jovita.marcinkeviciene@dupontpharma.com.

1 The abbreviations used are: BACE, β-site amyloid precursor protein-cleaving enzyme; dnp, 2,4-dinitrophenol.

2 The amino acid residues of the protease substrate have been numerically designated as Pn for residues N-terminal of the scissile bond and Pn′ for residues C-terminal of the scissile bond. The designations P1-P1′ indicates the amino acids between which hydrolysis occurs. This nomenclature system was first defined by Schechter and Berger (21).
aspartic protease, pepsin (13); hence, one might anticipate that the slow onset of inhibition is related to conformational changes accompanying inhibitor binding.

In this paper we report the kinetics of BACE inhibition by a statine-based inhibitor. These data are accompanied by stopped-flow studies of the intrinsic protein fluorescence change upon enzyme-inhibitor and enzyme-substrate complex formation, and the results are interpreted in light of the available three-dimensional structure.

**EXPERIMENTAL PROCEDURES**

**Enzyme Expression and Purification**—The cell line expressing the truncated (C-terminal His-tagged) version of human $\beta$-secretase was as described elsewhere (14). About 300–400 ml of clarified media containing secreted enzyme was dialyzed overnight into phosphate-buffered saline (Life Technologies, Inc.) and loaded onto a 30-ml nickel-nitrilotriacetic acid (Qiagen) affinity column. The column was washed initially with 60 ml of 50 mM phosphate buffer, 0.3 M NaCl, 10 mM imidazole, pH 8.0, and then with 60 ml of the same buffer, but with the imidazole concentration raised to 20 mM. The protein was eluted with a 20–250 mM linear imidazole gradient. Active fractions were pooled, dialyzed against 20 mM triethanolamine, loaded on a HR10 Mono Q column (Amersham Pharmacia Biotech) and eluted with a 0–1 M NaCl linear gradient. Active fractions exhibiting a single band on SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining were pooled and stored in 10% glycerol at $280 °C$. Protein concentration was estimated by the Bradford assay and by active site titration with the statine-based tight-binding inhibitor (Enzyme Systems Inc.); the results indicated that more than 95% of the total protein was catalytically active. These results were supported by matrix-assisted laser desorption ionization spectroscopy, suggesting that all of the protein was proteolytically activated with the prodomain being cleaved off during expression.3

**Enzyme Assays and Inhibition Studies**—Enzyme activity was monitored following the increase in fluorescence at 400 nm (excitation at 328 nm) resulting from the cleavage of the peptide: 7-methoxycoumarin-4-acyl-EVNLDAEF(K-dnp)-COOH. Assays were performed in 50 mM acetate buffer with 0.25 mg/ml bovine serum albumin, pH 4.5 at 25 °C with 2.5% Me$_2$SO (substrate solvent) present in 96-well plates. Steady-state kinetic studies were performed on a Molecular Devices SpectraMAX Gemini XS fluorescence plate reader. Data were processed using Softmax Pro 3.1.1.

Substrate concentration did not exceed 25 $\mu$M, since above this concentration a decrease in signal was observed due to the inner filter

3 M. C. Hillman, S. Sun, H. J. George, B. H. Fish, H. Chen, B. H. Selling, and R. Wynn, manuscript in preparation.
Inhibition of BACE by a Statine-based Peptide

**TABLE I**

**Kinetic parameters for two-step association of BACE with substrate and inhibitor**

The constants define the processes in the two-step association mechanism,

\[
E + A \rightleftharpoons EA \rightleftharpoons E^*A
\]

where \(A\) is either substrate or inhibitor. \(K_a^\beta\) experimental for the substrate was calculated from Equation 3, and \(K_a\) (FITSIM calculated) for the substrate was calculated from the simulated values \(k_j/k_i\).

| Parameter | Substrate (experimental) | Substrate (FITSIM calculated) | Inhibitor (experimental) | Inhibitor (FITSIM calculated) |
|-----------|--------------------------|-------------------------------|--------------------------|-------------------------------|
| \(k_1\)   | \(3.5 \times 10^5\) m\(^{-1}\) s\(^{-1}\) | \(3.2 \times 10^5\) m\(^{-1}\) s\(^{-1}\) | \(3.5 \times 10^4\) m\(^{-1}\) s\(^{-1}\) | \(5.0 \times 10^4\) m\(^{-1}\) s\(^{-1}\) |
| \(k_2\)   | 6.5 s\(^{-1}\)           | 2.7 s\(^{-1}\)                | 7.8 \times 10^4 s\(^{-1}\) | 660 nm                       |
| \(k_3\)   | 0.1 s\(^{-1}\)           | \(7.8 \times 10^4\) s\(^{-1}\) | 725 nm                   | 2.01 kcal/mol             |
| \(K_d\)   | 21.1 \(\mu\)M            | 20.3 \(\mu\)M                 | 22 nm                    | 2.01 kcal/mol             |
| \(\Delta\) | 1.97 kcal/mol            |                               |                          |                               |

**RESULTS**

**Steady-state Inhibition of BACE**—The statine-containing peptide has been reported to be a potent inhibitor of human brain-derived BACE, displaying an IC\(_{50}\) of 30 nm (8). We have reevaluated the inhibition of recombinant, truncated human BACE by this compound. Careful analysis of the product progress curves in the presence of this compound (Fig. 1A) revealed curvature consistent with slow onset inhibition (15). The biphasic nature of these progress curves was well modeled by Equation 1, allowing estimation of \(v_o, v_i, v_f, \) and \(k_{obs}\) at each inhibitor concentration. A plot of \(k_{obs}\) as a function of inhibitor concentration (Fig. 1B) is linear, suggesting either a single simple binding event, as in Scheme 1, or a two-step reaction mechanism (Scheme 2) for which \(K_a^\beta \ll K_i\) (see below).

\[
E + I \rightleftharpoons EI
\]

**SCHEME 1**

The values of \(k_1\) and \(k_2\) were calculated from the slope and intercept of the linear fit in Fig. 1B and were thus determined to be \((3.5 \pm 0.7) \times 10^4\) m\(^{-1}\) s\(^{-1}\) and \((7.8 \pm 4.0) \times 10^4\) s\(^{-1}\), respectively. Hence, the value of \(K_d\) obtained by the ratio \(k_3/k_2\) is 22 \(\pm\) 5 nm, in good agreement with the IC\(_{50}\) reported earlier (8).

For all of the fits in Fig. 1A, the value of \(v_o\) was non-zero, indicating that inhibition is reversible. To confirm this BACE (10 \(\mu\)M) was incubated with inhibitor (10 \(\mu\)M) for 15 min and then diluted ~2000-fold into the activity assay (final concentration of both enzyme and inhibitor was 5 nm). As seen in Fig. 2, this dilution resulted in a slow recovery of enzymatic activity that could be well fit by Equation 1, yielding an estimate of the reaction rate constant \(k_{react}\) of \((9.4 \pm 0.3) \times 10^4\) s\(^{-1}\). We note that the value of \(k_{react}\) and \(k_2\) are, within experimental error, the same; this result is consistent with fully reversible inhibition.

The statine-based inhibitor is composed of a peptide sequence similar to a known substrate of the enzyme, but the scissile bond is replaced to mimic the tetrahedral reaction intermediate of aspartyl proteases. We assume, therefore, that this inhibitor binds to the enzyme active site, with the statine isostere engaging the active site aspartate residues. We have not, however, been able to experimentally verify the competitive nature of this inhibitor because of the high \(K_m\) of the substrate, which is beyond the solubility limits of this molecule (14). Hence we could not achieve sufficient substrate saturation to discern a substrate dependence on inhibition and thus confirm competitive inhibition (16).

**Equilibrium and Pre-steady State Binding of Inhibitor to BACE**—The amino acid sequence of the catalytic domain of human BACE contains 5 tryptophan residues. When the enzyme (1 \(\mu\)M) is excited with 280 nm light, the fluorescence maximum is observed at 330 nm (data not shown), indicating that the majority of tryptophan residues in this protein experience a hydrophobic environment. The recent crystal structure of the human BACE catalytic domain complexed to a hydroxyethylene-containing peptide inhibitor reveals that there is a tryptophan residue in the “flap” region forming part of the binding pocket of the enzyme. It thus seemed likely that the fluorescence spectrum would be perturbed by ligand binding and subsequent flap movement, providing a convenient bio-
Inhibition of BACE by a Statine-based Peptide

the binding to BACE.

A

not able to estimate the course of inhibitor binding.

changes in BACE tryptophan fluorescence to follow the time

However, provide sufficient signal to follow binding interactions

formed by equilibrium methods. They do, however, provide sufficient signal to follow binding interactions by transient spectroscopic methods.

With stopped-flow instrumentation, we have used the changes in BACE tryptophan fluorescence to follow the time course of inhibitor binding.

When a 6 μM (final concentration) solution of the enzyme was mixed with 5, 10, or 15 μM solutions of the inhibitor under pre-steady-state conditions, an increase in fluorescence signal was observed (Fig. 3A). Higher inhibitor concentrations were not experimentally attainable due to the solubility limits of the inhibitory peptide. Hence, pseudo-first order conditions ([I] ≫ [E]) could not be achieved. Decreasing the enzyme concentrations resulted in insufficient signal amplitude. For these reasons in the concentration range of 5–15 μM no dependence of $k_{obs}$ on inhibitor concentration was observed; hence we were not able to estimate $k_1$ and $K_d$. Therefore, the same data were fit to a simple second order reaction ($A + B \rightarrow C$) using the program KINSIM, to obtain estimates of $k_1$ and $k_2$. Initially, fitting was optimized by incremental adjustment of values of individual rate constants by visual inspection. Once reasonable fits of the experimental data were obtained, final parameter adjustment was made by use of the program FITSIM (Table I). The average value of the second order rate constant ($k_1$) thus obtained from the binding transients was $(5.0 \pm 1.4) \times 10^4 \text{M}^{-1} \text{s}^{-1}$, which is in good agreement with the value of $k_1$ obtained from the measurements of steady-state inhibition (Fig. 1B, $(3.5 \pm 0.7) \times 10^4 \text{M}^{-1} \text{s}^{-1}$). In contrast, however, the average value of $k_2$ from these experiments was $(3.3 \pm 2.5) \times 10^{-2} \text{s}^{-1}$, some 43-fold faster than the corresponding rate constant for inhibitor dissociation obtained from steady-state measurements $(7.8 \pm 4.0) \times 10^{-3} \text{s}^{-1}$. The $K_d$ estimated from these data by the ratio of $k_2/k_1$ is thus $660 \text{ nM}$.

The discrepancy between the dissociation rate constants determined from pre-steady-state binding and steady-state inhibition measurements suggest a two-step inhibition mechanism, according to Scheme 2 (see above). The stopped-flow measurements essentially report on formation of the initial encounter complex, $E*I$, while steady-state inhibition data reflect formation of the final complex, $E^*I$. The similarity of the measured association rates for the two sets of experiments suggest that a common step is rate-limiting to both processes and must therefore be associated with initial binding of the inhibitor. The steady-state data do not directly reflect the two-step nature of the $E*I$ formation, because the intermediate species $E*I$ does not accumulate under our experimental conditions ([I] ≪ $K_d$).

Pre-steady-state Substrate Binding to BACE—Considering the general ligand interactions with the active site of BACE, we wondered if substrate binding might also proceed through a two-step mechanism like that seen for the inhibitor. To address this we measured the binding of the substrate to the enzyme under pre-steady-state conditions, on a time scale where substrate cleavage and product dissociation is negligible. For these studies we used the 9-residue peptide substrate described under “Experimental Procedures.” This peptide represents the region proximal to the scissile bond of the Swedish mutant amyloid precursor protein substrate and contains a dnp chromophore appended to the ε-amino group of the lysyl residue at P5’. The absorbance spectrum of dnp overlaps with the emission spectrum of the tryptophan residues in BACE. Hence, binding of the peptide to the enzyme should result in quenching of the proximal tryptophan fluorescence. Indeed, when the enzyme (3 μM) and substrate (5–40 μM) are mixed, a reduction in fluorescence signal is observed, as illustrated in Fig. 3B. No recovery of fluorescence intensity is observed over the time course studied (5 s), suggesting that cleavage and product dissociation occur at a rate less than 0.2 s$^{-1}$.

The diminution of fluorescence seen in Fig. 3B could not be adequately described by a single exponential process. Rather, the time course was best fit to a double exponential model. Similar data were obtained at a number of substrate concentrations ranging from 5 to 40 μM. These two kinetic phases could correspond to a two-step substrate binding mechanism, similar to that proposed for the inhibitor, as represented by Scheme 3.

$$
E + S \rightleftharpoons ES \rightleftharpoons E^*S
$$

**Scheme 3**

If so, one would expect that for the first phase, corresponding to $ES$ formation, $k_{obs1}$ would display a linear dependence on substrate concentration, with slope and $y$ intercept values equal to $k_1$ and $(k_2 + k_3 + k_4)$ (18). The second phase, corre-
Inhibition of BACE by a Statine-based Peptide

In the absence of crystallographic data for the apoenzyme, it is speculative to correlate the slow step during inhibition with either a conformational change or displacement of a catalytic water. Our comparison of the kinetic data for the substrate and inhibitor indicate, however, that most likely both factors are contributing. Flap movement (conformational change) involved in the binding of both ligands could account for the much slower than diffusion-limited association kinetics, although additional rate limitations occur in the case of the inhibitor, as suggested by our data. This additional limitation could be attributed to the displacement of a catalytic water, since it does not occur during substrate binding; however, more detailed studies would be needed to address this hypothesis further.

REFERENCES

1. Small, D. H., and McLean, C. A. (1999) J. Neurochem. 73, 443–449
2. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 398, 513–517
3. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashi, A., and Tang, J. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 1456–1460
4. Yuan, R., Blenkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brasher, J. R., Stratman, N. T., Mathews, W. R., Buhle, A. E., Catter, D. B., Tomasselli, A. G., Parodi, L. A., Heinricson, R. L., and Gurney, M. E. (1999) Nature 402, 533–537
5. Vassar, R., Bennett, B. D., Busb-Bhan, S., Kain, S., Maenada, E. A., Denis, P., Teplov, B. D., Ross, S., Amaranta, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenshon, S., Lie, J., Jorotschna, M. A., Bion, A. L., Cueran, E., Burgess, T., Louis, J.-C., and Collins, F. (1999) Science 296, 735–741
6. Bennett, B. D., Khan, S. B., Loeloff, R., Louis, J.-C., Cueran, E., Citron, M., and Vassar, R. (2000) J. Biol. Chem. 275, 20647–20651
7. Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D., and Davies, B. D. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7009–7013
8. Sinha, S., Anderson, J. P., Barbouf, R., Bass, G. S., Caccavello, R., Davis, D., Davis, M., devey, H. F., Prigon, N., Hong, J., Jacobsen-Cranak, K., Wetwi, N., Keim, P., Kropas, J., Lieberberg, I., POWER, M., Tan, H., Tatsuno, H., Tung, J., Schenk, D., Seubertt, P., and Suome, S. M. (1999) Nature 402, 537–540
9. Ghosh, A., Shin, D., Downs, D., Koelsch, G., Lin, X., Ermolielff, J., and Tang, J. (2000) J. Am. Chem. Soc. 122, 3521–3522
10. Ermolielff, J., Loy, J., Koelsch, G., and Tang, J. (2000) Biochemistry 39, 12450–12456
11. Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A., Zhang, X., and Tang, J. (2000) Science 290, 150–153
12. Miller, M., Schneider, J., Sathyamurayana, R. K., Teth, M. V., Marshall, G. R., Clavson, L., Selk, L., Kent, S. B. H., and Wlodawer, A. (1989) Science 246, 1149–1152
13. Cho, Y.-K., Rehbultz, K. L., and Northrop, D. (1994) Biochemistry 33, 9637–9642
14. Malamuriti, K., Kope, L., Marciniekiewicz, J., Copeland, R. A., and Rosenberg, T. L. (2001) J. Med. Chem. 44, 619–626
15. Jennings, M. W., and Fass, C. T. (1988) Adv. Enzymol. 61, 1–26
16. Tian, W. X., and Tsou, C. L. (1982) Biochemistry 21, 1026–1032
17. Rodriguez, E., Debuck, C., Deckman, I. C., Soud-Abu, H., Rausch, F. M., and Meek, T. D. (1995) Biochemistry 34, 3573–3583
18. Johnson, K. A. (1992) The Enzymes (Sigman, D. S., ed) Vol. XX, Third Ed., pp. 2–60, Academic Press, Inc., San Diego, CA
19. Brouwer, A. C., and Kirsch, J. F. (1982) Biochemistry 21, 1302–1307
20. Rich, D. H. (1995) J. Med. Chem. 38, 8556–8560
21. Schechter, L., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162

\[ \Delta G = RT \ln \left( \frac{k_{\text{obs2}}}{k_{\text{obs1}}} \right) \]  

(5. Eq.)

where \( R \) is the ideal gas constant and \( T \) is temperature (in Kelvin). Using this equation the \( \Delta G \) calculated for the transition \( EI \rightarrow E^*I \) is 2.01 kcal/mol. When the same calculation is performed for the transition \( ES \rightarrow E^*S \), a value of 1.97 kcal/mol is obtained. The close agreement between these values strongly implies that a common set of structural changes are induced to optimize substrate as well as inhibitor interactions with the enzyme.