Conversion of the Alzheimer’s β-Amyloid Precursor Protein (APP) Kunitz Domain into a Potent Human Neutrophil Elastase Inhibitor*

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Site-specific mutagenesis techniques have been used to construct active site variants of the Kunitz-type protease inhibitor domain present in the Alzheimer’s β-amyloid precursor protein (APP-KD). Striking alteration of its protease inhibitory properties were obtained when the putative P1 residue, arginine, was replaced with the small hydrophobic residue valine. The altered protein was no longer inhibitory toward bovine pancreatic trypsin, human Factor XI, mouse epidermal growth factor-binding protein, or bovine chymotrypsin, all of which are strongly inhibited by the unaltered APP-KD (Sinha, S., Dovey, H. F., Seubert, P., Ward, P. J., Blacher, R. W., Blaber, M., Bradshaw, R. A., Arici, M., Mobjley, W. C., and Lieberburg, I. (1990) J. Biol. Chem. 265, 8983–8985). Instead, the P1-Val-APP-KD was a potent inhibitor of human neutrophil elastase, with a Ki = 0.8 nM, as estimated by the inhibition of the activity of human neutrophil elastase measured using a chromogenic substrate. It also inhibited the degradation of insoluble elastin by the enzyme virtually stoichiometrically. Replacement of the P1, (Ala) and P2, (Met) residues of P1-Val-MKD with the corresponding residues (Ser, Ile) from α1-proteinase inhibitor resulted in an inactive protein, underscoring the mechanistic differences between the serpins from the Kunitz-type protease inhibitor family. These results confirm the importance of the P1, arginine residue of APP-KD in determining inhibitory specificity, and are also the first time that a single amino acid replacement has been shown to generate a specific potent human neutrophil elastase inhibitor from a human KD sequence.

The Alzheimer’s disease β-amyloid precursor protein (APP) is expressed in multiple forms (for reviews see Muller-Hill and Beyreuther, 1989; Selkoe, 1989), some of which contain a 56-amino acid insert with striking homology to the Kunitz family of protease inhibitors. We, and others (Sinha et al., 1990; Kitaguchi et al., 1990), have previously described the protease inhibitory properties of the isolated Kunitz domain, which, in agreement with the presence of an arginine residue at the putative P1 position, seems primarily to be a relative specific inhibitor of a few trypsin-like serine proteases, including Factor XI, (Smith et al., 1980).

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The abbreviations used are: APP, Alzheimer’s disease β-amyloid precursor protein; KD, Kunitz-type protease inhibitor domain; BPTI, bovine pancreatic trypsin inhibitor.

A previous study (Auerswald et al., 1988) on the prototypical Kunitz domain inhibitor, bovine pancreatic trypsin inhibitor (BPTI), show the importance of the P1 residue in modulating the activity of BPTI. We therefore decided to replace the P1 arginine residue in APP-KD with selected amino acid residues to generate modified APP-KDs, using standard techniques of site-specific mutagenesis. A potent inhibitor of neutrophil elastase was obtained when the arginine side chain was replaced with that of valine. Additional alteration of the P1 and P2 residues to serine and isoleucine (as found in the plasma human neutrophil elastase inhibitor α1-proteinase inhibitor however, abolished the inhibitory activity of the P1-Val-APP-KD. We discuss the implications of these results for both the specificity determinants of APP-KD, as well as for the rational design of novel serine protease inhibitors in general.

EXPERIMENTAL PROCEDURES

Materials—Trypsin and chymotrypsin were obtained from Sigma. Epidermal growth factor-binding protein was a gift from Dr. Michael Blaber (University of California, Irvine, CA). Factor XI, was obtained from Enzyme Research Laboratories, and human neutrophil elastase and cathepsin G were from Athens Research Technology. Human mast cell chymase and rat mast cell protease 1 were a gift from Dr. Norman Schechter (University of Pennsylvania). Synthetic substrates were obtained from Bachem.

Cloning and Expression of Altered APP Kunitz Domain Inhibitors—Altered APP-KDs were produced as bacterial fusion proteins as outlined previously for the unmodified APP-KD (Sinha et al., 1990). The expression vector uses the APb promoter and gives rise to fusion proteins with an NH2-terminal leader peptide derived from the bacterial MS-2 polymerase (Seedorf et al., 1987). The described plasmid pBX9 was modified as follows: an Asp-Pro cleavage site was introduced immediately amino-terminal to the KD sequence, to allow formic acid cleavage to separate the leader peptide from the APP-KD. A stop codon behind the carboxy-terminal alanine of the KD region was also introduced. Additionally, the P1, arginine residue was changed to valine (to obtain pBX16) and the P1, P2, residues were changed to Val, Ser, Ile to obtain pBX20. All mutations were introduced by polymerase chain reaction.

Isolation and Purification of P1-Val-APP-KD—The fusion proteins were obtained as 7 M urea bacterial extracts as described previously (Sinha et al., 1990). The crude 7 M urea extract was dialyzed extensively against 1 mM formic acid and then lyophilized. The residue was dissolved in dilute (2%) formic acid and then hydrolyzed for 2 h at 108 °C in an oxygen-free atmosphere, to achieve a near-quantitative cleavage of the Asp-Pro site (Inglis, 1983). The pH of the solution was adjusted to 8.0 with NaOH, and the cleaved P1-Val-APP-KD was then affinity-purified on a column of human neutrophil elastase immobilized on Affi-Gel HZ Hydradae matrix. The bound inhibitor was eluted with 25 mM pyridine formate, pH 3.0. The protein was then purified to virtual homogeneity by reverse-phase high performance liquid chromatography, using a trifluoroacetic acid/acetonitrile solvent system. The concentration of purified P1-Val-APP-KD was determined by quantitative amino acid analysis, and the aminoterminal sequence determined by direct protein sequencing.

Assay of Protease Inhibition—Assays with chromogenic substrates were carried out in 0.1 M Tris-HCl, pH 7.5, with 0.5 M NaCl, 0.1% gelatin, and 0.05% Triton X-100. Human neutrophil elastase (60 nM) was incubated with increasing amounts of inhibitor protein (up to 1.5
× [human neutrophil elastase] for P1-Val-APP-KD, and ~5 × [human neutrophil elastase] for BX20) and residual human neutrophil elastase activity assayed with 0.25 mM MeS-AAPV-pNA. Protocols for accurate measurement of residual activities, as well for estimation of the K, value(s), were essentially as described previously (Sinha et al., 1990). Measurement of elastolysis by human neutrophil elastase was done as follows. [3H]Elastin substrate was prepared from bovine ligamentum nuchae elastin according to a previously described method (Bands et al., 1981), by reductive alkylation with [3H]sodium borohydride. The stock [3H]elastin (100 mg/ml in water) was diluted 1:100-fold with 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 1 mM CaCl2 immediately before use. Reaction mixtures were then set up, in duplicate, which contained 0.1 ml of the [3H]elastin solution, and 20 pmol of human neutrophil elastase in a final volume of 0.12 ml. The mixtures were incubated in polypropylene tubes end-over-end at 37 °C for 5 min and then either 10 or 20 pmol of P1-Val-APP-KD added with vortex mixing and the incubations continued for a further 15 or 30 min. The reactions were quenched by the addition of ice-cold trichloroacetic acid to 7%, the tubes centrifuged, and the clear supernatants were removed for scintillation counting, to assay for acid-soluble [3H]elastin fragments. Control values (obtained from samples which were quenched immediately after the 5-min preincubation period or addition of inhibitor) were subtracted from total released counts/min in each reaction mixture. "100% activity" was defined for both time points as cpm obtained without added inhibitor.

RESULTS AND DISCUSSION

A bacterial expression plasmid for the production of P1-Val-APP-KD was obtained by in vitro mutagenesis. DNA sequencing confirmed the presence of the desired mutation. Fig. 1 shows the amino acid sequence of the expressed fusion protein BX15, indicating the Asp-Pro cleavage site introduced for formic acid cleavage. This treatment, followed by affinity purification on immobilized human neutrophil elastase-AffiGel H2, gives rise to purified P1-Val-APP-KD. Table I shows the amino acid composition and amino-terminal sequence of the cleaved and affinity-purified P1-Val-APP-KD, showing excellent agreement with the predicted composition and sequence.

Table I shows the protease inhibitory specificity of P1-Val-APP-KD. No appreciable inhibition of trypsin, epidermal growth factor-binding protein, Factor XII, and chymotrypsin were obtained, even when incubated with a 4-fold molar excess of P1-Val-APP-KD. This is in direct contrast to unmodified APP-KD. Strong inhibition of human neutrophil elastase is, however, evident, the K, of the interaction, 0.8 nM, being estimated from the graph generated by plotting residual amidolytic activity against increasing inhibitor concentration (Fig. 2).

The inhibitory activity toward human neutrophil elastase was further quantitated, by measuring the effect of P1-Val-APP-KD on "ongoing" elastin hydrolysis by the protease (Fig.

![Predicted amino acid sequence of the expressed fusion protein BX15. The formic acid-cleaved Asp-Pro bond is indicated with an asterisk. The sequence of the P1-Val-APP-KD is in boldface.](image)

![Inhibition of amidolytic activity of human neutrophil elastase by P1-Val-APP-KD.](image)
Neutrophil Elastase Inhibitor

3). 10 pmol of P1-Val-APP-KD inhibited by 37 and 57%, respectively, the ongoing elastinolytic production by 20 pmol of human neutrophil elastase over 15 and 30 min. These results are in remarkable agreement with those from the amidolytic activity assays, where this ratio (0.5:1) of P1-Val-APP-KD:human neutrophil elastase yielded a 50% inhibition. 20 pmol (1:1) of P1-Val-MKD inhibited these same reactions by 84 and 74%, respectively, over the 15- and 30-min time periods. These results demonstrate that the inhibitor is able to effectively and rapidly compete with an insoluble macromolecular substrate and to stoichiometrically bind human neutrophil elastase even when the enzyme is preincubated with the elastin. This is a stringent test of inhibitory potency, since even native α1-proteinase inhibitor, the physiological plasma inhibitor of human neutrophil elastase, is only partially effective under these conditions (Reilly and Travis, 1980). Thus, P1-Val-APP-KD is an effective and strong inhibitor of human neutrophil elastase, functioning by producing a 1:1 inhibitory complex, in two independent tests of human neutrophil elastase proteolytic activity.

The primary substrate specificity of human neutrophil elastase is directed toward Val at the P1 position (Nakajima et al., 1979), and therefore the Arg-to-Val substitution is acting to drastically lower inhibitory potency against the trypsinlike proteases and increase affinity for the neutrophil protease. The loss of inhibitory activity toward chymotrypsin in P1-Val-APP-KD would also imply, somewhat unexpectedly, that having an Arg at the P1 position is important for chymotrypsin inhibition by APP-KD, a possibility we had earlier discounted (Sinha et al., 1990).

P1 subsite-directed site-specific mutagenesis has been extensively utilized previously to alter inhibitor specificity in a wide class of protease inhibitors (Travis et al., 1985; McWherter et al., 1989; Collins et al., 1990; Patson et al., 1990). In particular, the BPTI was converted by semi-synthetic techniques into a potent human neutrophil elastase inhibitor by replacement of the P1 lysine residue with valine (Wenzel et al., 1986). However, the sequence of BPTI is only 42% identical with that of APP-KD, and it is clear that although the P1 residue is important, it is by no means the sole determinant of inhibitory specificity. For example, replacing the P1 lysine with arginine in BPTI leads to significant augmentation of human plasma kallikrein inhibition, the Kᵢ decreasing from ≈30 to 0.5 nm (Auerswald et al., 1988), which is primarily attributable to the selective recognition of arginine over lysine by the kallikrein family of trypsin-like proteases. In contrast, APP-KD, which has an arginine at the P1 position, does not detectably inhibit either human plasma kallikrein or porcine pancreatic kallikrein (Sinha et al., 1990), indicating that possibly other subsite interactions are not favorable to inhibitory complex formation. This point is underscored if the P1 (Ala) and P1' (Met) residues of the P1-Val-MKD are replaced by the corresponding residues (Ser and Ile, respectively) from the prototype human neutrophil elastase inhibitor, the serpin α1-proteinase inhibitor (Carrell et al., 1982). This version of the APP-KD was inactive as an elastase inhibitor, even when incubated at ~5 × [human neutrophil elastase] (data not shown). Caution should therefore be used in extrapolating site-specific mutagenesis results among mechanistically unrelated inhibitors. In addition, unpredictable results can be obtained even within the KD family, as our experience with chymotrypsin and kallikrein illustrate.

No crystal structure of human neutrophil elastase with a KD inhibitor is currently available. The availability of a human KD sequence which has been converted into a strong human neutrophil elastase inhibitor by a single amino acid substitution, and the development of the APP-KD crystal structure (Parenych et al., 1990; Wenzel et al., 1990; Hynes et al., 1990), may spur efforts to gain a better understanding of the interactions leading to productive inhibition of human neutrophil elastase by this class of proteins. The relatively small heat- and acid-stable nonglycosylated KD structure makes it a very attractive candidate to build novel serine protease inhibitors, especially those that effectively inhibit pathologically relevant proteases, such as human neutrophil elastase. Site-specific mutagenesis, including sites removed from the P1 position, could be a powerful tool to generate such inhibitors once the fundamentals of the interactions are well understood.

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REFERENCES


FIG. 3. Inhibition of ongoing elastinolytic activity of human neutrophil elastase by P1-Val-APP-KD.