Enhanced Plasmin Inhibition by a Reactive Center Lysine Mutant of the Kunitz-type Protease Inhibitor Domain of the Amyloid β-Protein Precursor*

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The Alzheimer’s disease related protein, amyloid β-protein precursor (AβPP), contains a domain homologous to Kunitz-type protease inhibitors (KPI). The recombinant KPI domain of AβPP is a potent inhibitor of coagulation factors XIa and IXa and functions as an anticoagulant in vitro. Here we report the expression, purification, and characterization of a reactive center lysine mutant of the KPI domain of AβPP (KPI-Lys17). An expression plasmid for the KPI-Lys17 domain of AβPP encoded amino acids 285-345 of the AβPP CDNA containing a lysine substitution at arginine 17 in the KPI domain. The secreted 61-amino acid product was purified to homogeneity and functionally characterized. The protease inhibitory properties of the KPI-Lys17 domain were compared to those of the native KPI domain of AβPP. Both KPI domains equally inhibited trypsin, chymotrypsin, and coagulation factors XIa and IXa. However, the KPI-Lys17 domain was an ~25-fold less effective inhibitor of coagulation factor XIa resulting in markedly less prolongation of the activated partial thromboplastin time compared to the native KPI domain of AβPP. On the other hand, the KPI-Lys17 domain was an ~10- and 5-fold better inhibitor of plasmin in a chromogenic substrate assay and in a fibrinolytic assay, respectively, than the native KPI domain of AβPP. Together, these studies suggest that the KPI-Lys17 domain has enhanced anti-fibrinolytic and diminished factor XIa inhibitory properties compared to the native KPI domain of AβPP.

The amyloid β-protein precursor (AβPP) is the parent molecule to the Alzheimer’s disease amyloid β-protein (1–4). AβPP can be translated from predominantly three alternatively spliced mRNA species to yield polypeptides of 695, 751, and 770 amino acids (5–7). The latter two species contain an additional insert which codes for a domain that is homologous to Kunitz-type serine protease inhibitors (KPI) (5–7). The KPI-containing isoforms of AβPP are identical to the previously described cell-secreted protease inhibitor, protease nexin-2 (PN-2) (8, 9). Messenger RNA encoding the KPI-lacking AβPP 695 isoform is found primarily in brain. However, mRNA encoding the KPI-containing AβPP 751/770 isoforms are also abundant in brain and found in most peripheral tissues, suggesting that these isoforms of AβPP may have a common function throughout the body.

Several studies have suggested a potential physiologic function for the KPI domain of PN-2/AβPP in hemostasis. For example, measurements of protease inhibition equilibrium constants revealed that PN-2/AβPP and its recombinant KPI domain are very potent inhibitors of intrinsic blood coagulation factors XIa and IXa (10–14). In this regard it is noteworthy that PN-2/AβPP is an abundant platelet α-granule protein and is secreted in high concentrations by platelets that are activated by physiologic agonists (11, 15–17). Together, these findings suggest that secreted platelet PN-2/AβPP may play a role in regulating the intrinsic blood coagulation cascade at sites of vascular injury upon release by activated platelets (10, 11, 15, 17). Fibrinolysis is another regulated proteolytic process that occurs at sites of vascular injury. Previous studies have shown that PN-2/AβPP and its recombinant KPI domain also inhibit plasmin but to a lesser extent than coagulation factors XIa and IXa (10, 12–14).

Here we report the high level expression, purification, and biochemical characterization of a reactive center lysine mutant of the KPI domain of AβPP (KPI-Lys17). The protease inhibitory, anticoagulant and anti-fibrinolytic properties of the KPI-Lys17 domain and the native KPI domain of AβPP were compared. The results of these studies indicate that the KPI-Lys17 domain has enhanced anti-plasmin and diminished factor XIa inhibitory properties compared to the native KPI domain of AβPP. These properties suggest that KPI-Lys17 may have utility as an anti-fibrinolytic agent.

EXPERIMENTAL PROCEDURES

Materials—The oligonucleotides used were synthesized by Genosys. Human factors XIa, IXa, and Xa were obtained from Enzyme Research Laboratories (South Bend, IN). Bovine trypsin, bovine chymotrypsin, fibrinogen, p-nitrophenyl guanidinobenzoate, tosyl-Gly-Pro-Arg-p-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, and reagents for the activated partial thromboplastin time (APTT) were from Sigma. PyroGlu-Pro-Arg-p-nitroanilide was purchased from Helena Laboratories (Beaumont, TX). Human plasmin and carbobenzoxy-Val-Gly-Arg-4-nitroanilide were obtained from Boehringer Mannheim. Human thrombin was purchased from Calbiochem (San Diego, CA). Human urokinase was generously provided by Dr. J. Henkin of Abbott Labs (Abbott Park, IL). The recombinant native KPI domain of PN-2/AβPP was prepared and purified as described (12).
Construction of the KPI-Lys17 Expression Vector—The construction of Pichia pastoris vector, pPKI200, encoding the wild type KPI sequence has been described previously (18). To replace the reactive center arginine at position 17 with lysine, site-specific mutations were introduced by a two-step PCR mutagenesis procedure (18). The first PCR utilized a 5′ primer which includes the mutations (5′-CTGAGACTGCTCATGATTAGAATCTAGCAAGACC-3′) in the reaction is located in the AOX1 terminator region and downstream of a unique AegI site in the pkpi200 plasmid. The PKI200 plasmid served as the template for the first reaction and a 5′ primer (5′-CATAATTGCGACTGGTTCC-3′) located in the AOX1 promoter region upstream from a unique HindIII site. An aliquot of the first PCR containing a small amount of the minor long extension product was the template for the second reaction (18).

The principal product of the second PCR, a ~630-base pair fragment, was digested with AegI and HindIII digested pPKI200, and transformed into bacteria. Plasmids from individual bacterial transformants were tested initially for the presence of the diagnostic AegI restriction site and the DNA sequence to confirm the mutations and the integrity of the rest of the KPI sequence. The resulting plasmid, designated pPKI-R17K (Fig. 1), contains the promoter (5′-AOX1) and terminator (3′-AOX1) sequences of the P. pastoris methanol-inducible alcohol oxidase gene prior to transformation into the P. pastoris strain of a methanol-inducible alcohol oxidase gene and the HIS4 selectable marker. The transformation of the pPKI-R17K plasmid into P. pastoris was accomplished as described previously (19). The plasmid was linearized at the unique HindIII site within the KPI-Lys17 domain as described previously for the KPI-Lys domain (20) using the burst titrant motrypsin, and plasmin were titrated by the method of Chase and Shaw (20) with the purified native KPI domain of A. actinomycetemcomitans or the native KPI domain of A. pasteurianus.

RESULTS AND DISCUSSION

A DNA encoding the mutant KPI-Lys17 domain was prepared by site-directed mutagenesis and using PCR and the plasmid pPKI200 that encodes the native KPI domain of A. pasteurianus (Fig. 1). DNA encoding the yeast-specific secretion signal of S. cerevisiae α-mating factor prepro signal sequence was attached to the DNA encoding the KPI-Lys17 domain and incorporated into P. pastoris expression vector to generate pPKI-R17K. The recombinant plasmid pPKI-R17K (Fig. 1) was integrated into and expressed in the methylotrophic yeast P. pastoris. Transcription from the P. pastoris AOX1 promoter included in pPKI-R17K occurs at very high levels in P. pastoris cells grown in methanol providing an inducible expression system particularly designed for foreign gene expression (26). Transformed P. pastoris cells were first grown in a fermenter in the presence of methanol and then switched to methanol as the carbon source to induce expression of the KPI-Lys17 domain. The secreted KPI-Lys17 domain (>1.0 g/liter) comprised >80% of the protein in the fermentation medium.

The expressed KPI-Lys17 domain was purified from the fermentation medium by the same procedures previously described for purification of the native KPI domain of AβPP (12). As shown in Fig. 2, the purified KPI-Lys17 domain and native KPI domain of AβPP had molecular masses of ~6.5 kDa. Amino acid sequencing of the purified KPI-Lys17 domain revealed the integral of the expressed product and showed that the amino terminus of the protein was properly processed upon secretion (data not shown). Titration experiments with the purified KPI-Lys17 domain revealed a 1:1 stoichiometry for trypsin inhibition essentially the same as for parallel titration experiments with the purified native KPI domain of AβPP (Fig. 3). Importantly, this result demonstrated that the KPI-Lys17 domain, as the native KPI domain of AβPP, was fully active, properly folded, and the disulfide bonds were in the correct orientation when expressed and secreted by the Pichia cells. This assessment was further supported by the finding that treatment of either purified KPI domain with the reducing agent dithiothreitol completely abolished inhibitory activity toward the target proteases (data not shown).

The protease inhibitory properties of the purified KPI-Lys17 domain were compared to those of purified native KPI domain of AβPP (Table I). The protease inhibition equilibrium constants obtained for the inhibition of trypsin, chymotrypsin, and coagulation factors IXa and Xa by the purified KPI-Lys17 domain were essentially the same as those obtained for the inhibition of these proteases by the purified native KPI domain of AβPP (Table I). However, the KPI-Lys17 domain was an ~25-fold less effective inhibitor of coagulation factor IXa than the native KPI domain of AβPP. This finding indicates that the arginine residue at the reactive center of the KPI domain of AβPP is important for optimal inhibition of factor IXa. On the other hand, the KPI-Lys17 domain was found to be an ~10-fold
better inhibitor of plasmin compared to the native KPI domain of AβPP (Table I). Neither KPI domain inhibited urokinase.

Since the KPI-Lys17 domain inhibited factors XIa, IXa, and Xa, we performed studies to determine if it inhibited the coagulant activity of normal human plasma (Fig. 4). The native KPI domain of AβPP at 1 μM prolonged the APTT >2-fold over pooled normal plasma as previously reported (13). In contrast, only at concentrations ~50 μM or more was the APTT prolongation >2-fold by the KPI-Lys17 domain. At 50 μM, the native KPI domain of AβPP prolonged the APTT ~15-fold. These data suggest that the KPI-Lys17 domain has diminished anticoagulant properties in vitro clotting assays compared to the native KPI domain of AβPP. Since clotting in the APTT assay involves the activity of numerous coagulation factors in the intrinsic pathway, the weaker anticoagulant activity of the KPI-Lys17 domain observed is consistent with the above finding that it is a less effective inhibitor of coagulation factor Xa than the native KPI domain of AβPP (Table I).

The KPI-Lys17 domain was found to have enhanced inhibi-
The native KPI domain of A

represent the mean measured as described under “Experimental Procedures.” The data represent the mean ± S.D. of ≥5 separate determinations. Significant differences from KPI-Lys17 and KPI; *p < 0.0004; †p < 0.02 by unpaired group t test.

| Table 1 | KPI-Lys17 domain and the native KPI domain of ABPP |
|---------|---------------------------------|
| Trypsin | K \(_m\) (mM) |
| Factor XIa | 2.7 ± 2.0 \times 10^{-10} |
| Plasmin | 2.7 ± 1.5 \times 10^{-10} |
| Chymotrypsin | 5.5 ± 2.0 \times 10^{-9} |
| Factor Xa | 6.2 ± 2.0 \times 10^{-8} |
| Urokinase | 2.3 ± 1.0 \times 10^{-7} |
| Not inhibited | Not inhibited |

KPI (mM)

Inhibition of fibrinolysis by purified native KPI domain and KPI-Lys17 domain. Fibrin microtiter plates were prepared by incubating 5.4 μM fibrinogen and 3.5 μM thrombin in a final volume of 100 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl\(_2\), in the absence or presence of increasing concentrations of the native KPI domain of ApPP (●) or the KPI-Lys17 domain (○). Then, 5 μl of 0.6 μM plasmin was added to the fibrin wells and incubated shaking for 16 h at 37°C in a humidified environment. The extent of fibrinolysis was determined as a loss in absorbance at 405 nm in a V\(_{max}\) microtiter plate reader.

Inhibitor may be useful in cardiopulmonary bypass as an alternative to aprotinin administration.

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