Inhibition of Six Serine Proteinases of the Human Coagulation System by Mutants of Bovine Pancreatic Trypsin Inhibitor*

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A series of 12 bovine pancreatic trypsin inhibitor variants mutated in the P₄ and P₃ positions of the canonical binding loop containing additional K15R and M52L mutations were used to probe the role of single amino acid substitutions on binding to bovine trypsin and to the following human proteinases involved in blood clotting: plasmin, plasma kallikrein, factors Xa and XIa, thrombin, and protein C. The mutants were expressed in Escherichia coli as fusion proteins with the LE1413 hydrophobic polypeptide and purified from inclusion bodies; these steps were followed by CNBr cleavage and oxidative refolding. The mutants inhibited the blood-clotting proteinases with association constants in the range of 10⁵–10¹⁰ M⁻¹. Inhibition of plasma kallikrein, factors Xa and XIa, thrombin, and protein C could be improved by up to 2 orders of magnitude by the K15R substitution. The highest increase in the association constant for P₃ mutant was measured for factor XIIa; P13S substitution increased the Kₐ value 58-fold. Several other substitutions at P₃ resulted in about 10-fold increase for factor Xa, thrombin, and protein C. The cumulative P₃ and P₁ effects on Kₐ values for the strongest mutant compared with the wild type bovine pancreatic trypsin inhibitor were in the range of 2.2- (plasmin) to 4,000-fold (factors XIa and Xa). The substitutions at the P₁ site always caused negative effects (a decrease in the range from over 1,000- to 1.3-fold) on binding to all studied enzymes, including trypsin. Thermal stability studies showed a very large decrease of the denaturation temperature (about 22 °C) for all P₃ mutants, suggesting that substitution of the wild type Gly-12 residue leads to a change in the binding loop conformation manifesting itself in non-optimal binding to the proteinase active site.

Blood coagulation is a series of proteolytic events resulting in clot formation. Equally important are the processes of anticoagulation and fibrinolysis that are also mediated by proteolytic enzymes. Research during the past decade has resulted in the determination of spatial structures for many of these enzymes, including thrombin, factors VIIa, IXa, and Xa, protein C, tissue plasminogen activator, and plasmin (1–6). The specificity of a particular enzyme toward its cognate sequence results from well defined subsites on the enzyme surface recognizing only the scissile peptide bond but often also more extended regions. These structural studies nicely explain earlier data on sequence-specific cleavage of natural substrates and provide a framework for drug design efforts.

Because the coagulation/fibrinolysis processes are of vital importance, they are precisely controlled. Protein inhibitors are one of the most essential regulating factors. Interestingly, the scaffold of Kunitz-type inhibitors is used both to control the natural coagulation process in human blood and to prevent blood clotting in a blood-sucking organism, the soft tick. In the former case, tissue factor pathway inhibitor (TFPI) composed of three tandemly arranged Kunitz-type domains inhibits different clotting proteinases (e.g. factor Xa at the second domain) through the canonical proteinase binding loop. On the contrary, the amino terminus of tick anticoagulant peptide, which also possesses a Kunitz-domain fold, is involved in non-canonical binding to the active site of factor Xa (7). Both TFPI and tick anticoagulant peptide were recognized as potential antithrombotic drugs (8, 9).

Bovine pancreatic trypsin inhibitor (BPTI) is the best known example of Kunitz-type inhibitors. It consists of 58 amino acid residues cross-linked by three disulfide bridges that contribute to a compact tertiary structure and remarkable thermodynamic stability (10, 11). Wild type BPTI is a powerful competitive inhibitor of several serine proteinases including trypsin, chymotrypsin, plasmin, and tissue kallikrein (12). TFPI is not a strong immunogen because specific antibodies have not been observed in humans (12). The wild type protein has been used as an antifibrinolytic agent in cardiac surgery (13) and in acute pancreatitis (12). Because of its low immunogenicity and well known mechanism of action, BPTI appears to be an ideal candidate for protein engineering studies with the aim of directing its specificity toward blocking unbalanced proteolysis. So far, several studies have shown that through semisynthetic (14, 15) or recombinant approaches (16–18) it is possible to change or enhance the BPTI inhibition spectrum. Additionally, phage display technology has been applied to generate potent inhibitors directed toward different serine proteinases based on either the BPTI sequence (19) or sequences of homologous inhibitors: amyloid β-protein precursor (20–22) and the first domain of TFPI (23, 24). Stassen et al. converted BPTI into a potent inhibitor of factors VIIa-TF, Xa, XIa and plasma kallikrein through grafting into BPTI binding loop sequences of individual TFPI domains (17).

In this paper 12 mutants of BPTI located in the P₃ and P₄ positions (nomenclature according to Schechter and Berger

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‡ The abbreviations used are: TFPI, tissue factor pathway inhibitor; BPTI, bovine pancreatic trypsin inhibitor; NPGB, 4-nitrophenyl 4'-guanidinobenzoate; pNA, p-nitroanilide; Bz-, benzoyl-.
(25) of the binding loop segment were constructed with the aim of improving binding to several proteinases of the human blood clotting system: α-plasmin, plasma kallikrein, factor Xa, α-factor XIIa, protein C, and α-thrombin. In all the mutants the wild type Lys-15 was replaced with Arg because of its more favorable binding to the studied enzymes. Although the inhibitor contacts different serine proteinases using about seven amino acid residues of the binding loop (26), it was decided to mutate just the P1, P2, and P3 positions. Cys at P2 forms a disulfide bond with Cys-38. It is generally recognized that the unprimed side of the binding loop is energetically more essential than the prime side (27). In addition, much less substrate kinetic data is available for the prime side residues. Furthermore, our data on seven P1′ mutants showed that in BPTI-serine proteinase complexes the P1′ side chain sterically conflicts with the side chain of Ile (P1′) leading to a large decrease in the association energy (28).

**EXPERIMENTAL PROCEDURES**

**Materials**—Guadinium chloride, urea, dimethyl sulfoxide (Me2SO), N,N-dimethylformamide, methanol, and acetonitrile were purchased from Merck. Trifluoroacetic acid and cyanogen bromide were from Bachem. The other substrates: 4-nitrophenyl 4-Na, Bz-Pro-Phe-Arg-pNA were from Bachem. The other substrates: Bz-Arg-pNA, Bz-Pro-Phe-Arg-pNA, and Tos-Gly-Pro-Arg-pNA were from Sigma; H- D-Pro-Phe-Arg-pNA (S-2302) was supplied by Chro- mogenix. 4-nitrophenyl 4-Na was from Kordia and factor Xa was supplied by Roche Molecular Biochemicals. Recombinant human protein C was purified from Human Lubon (American Red Cross) (29). Bovine β-trypsin was supplied by Worthington Biochemical.

**Expression and Purification of Mutant Proteins**—All the mutants of BPTI were overexpressed as fusion proteins in Escherichia coli strain BL21 (DE3) pLysS, using the T7 promoter system (30) as described (31). The plasmids, derived from pAE4D, bearing a portion of the E. coli trp operon, which serves as a leader sequence, was prepared after site-direc- ted mutagenesis (32). All recombinant variants contained the additional mutation M52L to allow CNBr cleavage of the fusion protein. The plasmids, derived from pAED4, bearing a portion of the plasmid kallikrein and thrombin were dissolved in MilliQ water. The concentrations of both enzymes were determined using respective molar ab- sorbance coefficients ε280 = 93,280 M⁻¹ cm⁻¹ and 24,240 M⁻¹ cm⁻¹ (36). Protein C was dissolved in 50 mM Tris-HCl, 50 mM NaCl, 7.5 mM CaCl₂, pH 7.4, and its concentration was determined from the 56,640 M⁻¹ cm⁻¹ molar absorbance coefficient at 280 nm (37). The concentration of factor Xa was calculated from the Briggs-Haldane equation applying published values of catalytic parameters kₗₚ and Kₘ for the Tos-Gly-Pro-Arg-pNA substrate (38). The concentration of factor XIIa was determined from kinetic parameters kₗₚ = 15 s⁻¹ and Kₘ = 1.9 × 10⁻⁷ M for H-d-Pro-Arg-pNA (39). The following substrates were used to measure the residual enzyme activities: Tos-Gly-Pro-Arg-pNA (bovine trypsin), H-d-Pro-Phe-Arg-pNA (α-plasmin), Tos-Gly-Pro-Arg-pNA (factor Xa), H-d-Pro-Arg-pNA (α-factor XIIa), Bz-Pro-Phe-Arg-pNA (plasma kallikrein), Suc-Ala-Ala-Pro-Arg-pNA (α-thrombin), and Tos- Gly-Pro-Arg-pNA (protein C). All inhibitor concentrations were determined by titration with NPGB-standardized trypsin.

**Determination of Association Constants**—The association constant (Kₐ) values were determined by a modified method of Green and Work (36) described by Empie and Laskowski (40) and Otlewski and Zbyryt (41). All measurements were performed in 100 mM Tris-Cl, 150 mM NaCl, 20 mM CaCl₂, 0.05% Triton X-100, pH 8.3. Although pH 8.3 is above the optimum pH for most of the proteinases used, at this pH the Kₐ values are not perturbed by the pH of His-57, and it is a reference pH routinely used in our laboratory. To determine the association constant, increasing amounts of the inhibitor were added to a constant concen- tration of the proteinase. Kₐ determinations were done singly; for each Kₐ value 7–10 data points were measured. The enzyme concentration was chosen to fulfill the condition: 1 < [E₉] × Kₐ < 50. For the determination of low Kₐ values (10⁻¹⁰–10⁻¹² M⁻¹), the inhibitors were used at much higher concentrations than the enzyme, to force complex forma- tion. After a suitable incubation time the residual enzyme activity was measured for about 500 s by monitoring the linear release of p-nitro- nitrile with an HP 8452A diode array spectrophotometer. The recorded signal was the average value of absorbance in the 380–410-nm range corrected for the background average absorbance in the 480–510-nm range. The value of Kₐ was calculated by a 3-parameter algorithm, (E) = β[Eₙ] F, using the non-linear regression analysis program GraFit, according to the equation,

\[
E = \frac{1}{2} \left( [E_{0}] - F \cdot [I_{s}] - K_{1} \right) + \sqrt{\left( [E_{0}] + F \cdot [I_{s}] + K_{2} \right)^2 - 4 \cdot [E_{0}] \cdot F \cdot [I_{s}]} \tag{Eq. 2}
\]

where \([E_{0}]\) and \([I_{s}]\) are the total enzyme and inhibitor concentrations, respectively, \([E]\) is the residual enzyme concentration, and \(F\) is the enzyme-inhibitor equimolarity factor. In the case of weak associations (Kₐ < 10⁻¹² M⁻¹) only the 2-parameter algorithm (E) = \(\beta[E]K_{1}\) and the default value of \(F = 1\) were applied.

**RESULTS**

The BPTI molecule contains a solvent-exposed proteinase binding loop spanning the residues P₃ through P₃ of the compacted structure (Fig. 1). Thirteen mutations were installed in this segment using site-direc- ted mutagenesis. All the mutants were expressed in E. coli with an average yield of 5 mg of pure protein per 1 liter of culture. Mutant proteinases were purified on a C₄ HPLC column in both their reduced and oxidized states. Experimental molecular masses determined by electrospray spectrometry of all variants were within 1.0 atomic mass unit of the expected values.

**Stability of the Mutants**—All the mutant proteins showed similar CD spectra both in the near- and far-UV range, so no major conformational changes occurred upon installing the amino acid substitutions (data not shown). Furthermore, the thermodynamic stability of the mutants was studied by ther- mal denaturation monitored by a 223-nm CD signal. All transi- tions were well reversible, as judged by the similarity of their
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CD spectra recorded at 20 °C before and after the experiment. Differential scanning calorimetry experiments showed a ΔHcal to ΔHcal/ΔT ratio close to unity, which is indicative of a 2-state transition (data not shown). Fig. 2 shows an example of denaturation curves for the pseudo wild type BPTI (K15R, M52L) and its two P3 and P4 mutants: P13A and G12V. Table I summarizes Tdon and ΔvdH values determined for all the mutants at pH 2.0. It can easily be noticed that both the K15R and M52L mutations, which were introduced into all the mutants, did not lead to stability changes. However, all P3 and P4 mutants were systematically destabilized by about 12.5 and 22 °C, respectively.

Association with Trypsin—All the mutants were found to be functionally active, because they inhibited bovine trypsin. For all P3 mutants the association constants were very high (over 1011 M⁻¹) and could not be determined exactly (Table II). However, the P4 mutants showed large drops in their association constants with trypsin, in the range of 1300- to 4500-fold.

K15R Substitution—Table II shows the effect of the K15R substitution at the P1 position of BPTI measured for six human proteinases. For five of the proteinases Arg is significantly better than Lys. The largest increase of the association constants was observed for factor Xa (484-fold); the lowest increase was for factor XIII (4-fold). Three mutations (Val (8-fold), Phe (8-fold), and His (4-fold)) substantially improved binding of BPTI to factor Xa. Gly improved binding to this protease 1.5- and 1.8-fold, respectively. Other mutations were destabilizing in the range of 1.3- to 23-fold. In the case of plasmin no clear effects of the mutations at the P3 site were observed for the substitutions at the P3 site. The observed effects on protolytic properties of the side chain, such as polarity, charge, hydrophobicity, size, and branching. The observed effects on proteinases are reported in Table III. The largest effects with respect to the substitutions at the P3 site were observed for factor XIIIa. With the exception of Ile (5-fold decrease), all P3 mutants showed stronger inhibition of this proteinase than did the pseudo wild type BPTI. The largest increase (58-fold) was observed for Ser; the weakest effect, which still increased the Kₐ value, was observed for the P3 Val residue (2-fold). In the case of plasma kallikrein, the best residue was Ile (2.2-fold increase), and the worst was Asp, which decreased Kₐ by a factor of 32.

Three hydrophobic side chains (Val, Ile, and Phe) installed at the P3 site improved binding to thrombin by 4.6-, 5.7-, and 1.5-fold, respectively. Other mutations were destabilizing in the range of 1.3- to 23-fold. In the case of plasmin no clear effects of the substitution at the P3 site were observed. Two residues, His and Arg, improved binding to this protease 1.5- and 1.8-fold, respectively. The most destabilizing side chain was that of Val (5.6-fold). Three mutations (Val (8-fold), Phe (8-fold), and His (4-fold)) substantially improved binding of BPTI to factor XIIIa. Gly and Ser at the P3 site were clearly destabilizing (6.4- and 3.5-fold, respectively). For protein C the most stabilizing were β-branched side chains of Val (6-fold) and Ile (4.4-fold) but also
Arg (3.4-fold). Small side chains of Ala, Ser, and Asp destabilized the complex formation by 7.4-, 5.3-, and 3.6-fold, respectively.

Substitutions at P4—In this study the wild type Gly-12 was substituted with Ser, Val, or Phe residues. These three BPTI mutants could be oxidatively refolded, although with an average yield of 20%, compared with 80% for the P3 mutants. In addition, as described above, all P4 mutants showed much greater destabilization compared with P3 mutants (Table I).

Generally, mutations at P4 affected the association energy with the studied enzymes to a larger extent than did mutations at the P3 position. In all studied cases the effect of Gly-12 substitution led to a rather drastic lowering of $K_a$ values. The largest drops were found for trypsin (over 3 orders of magnitude), the largest effect reported in this paper. In the case of plasma kallikrein, installation of the $\beta$-branched Val residue also decreased $K_a$ by 3 orders of magnitude. Phe at P4 was almost as poor as Val. Substitution of Gly with Ser did not significantly lower the association constant. The relatively small effect of G12S substitution was also found for other enzymes studied here. The interactions of protein C and plasmin with three P4 mutants were similar to those described for plasma kallikrein; the largest (several hundred-fold) decrease of the $K_a$ value occurred in the case of Val, and a smaller but still substantial decrease (30–40-fold) occurred upon mutation to Phe.

For the remaining three proteinases (factors Xa and XIIa and thrombin) the drops in the association constants were smaller. The effects on factor Xa did not exceed 6-fold, and they were somewhat larger for factor XIIa and thrombin. In the case of thrombin, Phe at P4 was the worst residue (23-fold decrease of $K_a$).

**DISCUSSION**

In this paper the inhibition of six human proteinases involved in hemostasis and fibrinolysis (plasma kallikrein, factors Xa and XIIa, thrombin, plasmin, and protein C) by 13 mutants of BPTI was studied. In addition, bovine $\beta$-trypsin was investigated as a reference enzyme. All the P3 and P4 variants contained a fixed Arg residue at P1, because most of the studied enzymes were known to be Arg-specific.

The effect of the K15R substitution was calculated by comparing the association constant values for wild type BPTI and its Arg variant (Table II). The other mutations were installed at the P3 (nine mutations: Gly, Ala, Val, Ile, Phe, Ser, Asp, His, and Arg) and P4 (three mutations: Ser, Val, and Phe) sites.

Wild type BPTI binds to the studied human enzymes with association constants in the range of $2 \times 10^9$ M$^{-1}$ (factor Xa) to $5 \times 10^9$ M$^{-1}$ (plasmin) (Table II). Compared with the wild type, the strength of inhibition was improved for all human enzymes in the range of 2- to 4000-fold (Tables II and III). In many cases the enzymes showed opposite effects in response to the installed mutations, revealing differences in their subsite specificities.

The largest positive effects on the association energy were due to the K15R mutation at the P4 site. Except for plasmin and trypsin, all other proteinases studied here contain Ala-190 at the bottom of their S1 specificity pocket (Table II). Conse-
quently, as it was originally noticed for tissue plasminogen activator (5), the S1 pockets of these enzymes are slightly larger, with one less hydrogen bond partner for P1 Lys, thus favoring Arg at this position. The result of the K15R substitution on factor Xα, a 485-fold increase (Table II), is in agreement with the $k_{cat}/K_m$ values for oligopeptide substrates, which show a 50–260-fold preference for Arg (38). Natural plasma inhibitors of factor Xα (antithrombin III and the second domain of TFPI), as well as the natural factor Xα protein substrate prothrombin, contain an Arg at their P1 sites. Additionally, it is worth noticing that potent inhibitors of factor Xα isolated from exogenous sources (antistasin and ghilanten) also contain an Arg residue at the P1 site (42). Site-directed mutagenesis of antistasin showed a 20-fold preference for Arg over Lys (43), and the K15R mutation in human pancreatic secretory trypsin inhibitor led to a 100-fold improvement of inhibition (44).

Similarly, there is an agreement between our results (Table II) and oligopeptide kinetic data for human plasma kallikrein, which show a 15–50-fold preference for Arg over Lys (43), and the K15R mutation in human pancreatic secretory trypsin inhibitor led to a >100-fold improvement of inhibition (44).

Two hydrophobic substitutions at P4 (G12V and G12F) were chosen to probe the binding properties of the S4 aryl binding site, which is structurally defined for factor Xα and thrombin (2, 3). The S4 pocket of protein C is more polar and flanked by Thr-99 and Asn-174 (4), and this pocket was tested with Ser. For other proteinases the S4 site is not well defined. Gly-12 is strongly conserved among Kunitz-type inhibitors and, together with three disulfide bridges and the residues Phe-33, Gly-37, and Phe-45, shows the critical locations in the BPTI fold (48). Inspection of the crystal structure of BPTI reveals that the main chain ($\phi = 88.6^\circ$ and $\psi = -178.5^\circ$) angles of Gly-12 in free BPTI (49) cannot be adopted by non-Gly residues without conformational adjustment. Both the poor yield of oxidative refolding and the $20^\circ\text{C}$ decrease of $T_{den}$ values observed for P4 mutants suggest a significant conformational change in free mutant structure upon replacement of Gly-12.

The effects at the P4 site were deleterious for all the studied enzymes, but to different extents. The P4 effect was very strong for trypsin; for all the mutants the $K_a$ value dropped more than 3 orders of magnitude. Interestingly, for enzymes with the aryl binding site (factor Xα and thrombin), the decreases due to the G12V mutation were only about 6-fold, compared with e.g. 1,000-fold for plasma kallikrein, 500-fold for plasmin, or 230-fold for protein C. This might indicate that the deleterious effect on the loop conformation is partially compensated for by a favorable interaction of the phenyl ring with the S4 pocket.

Significant destabilization of the inhibitor molecule and a lack...
of positive effects on proteinase inhibition were discouraging to further mutational studies.

Compared with the effects on P₄, nine substitutions at the P₃ position provided differential data on proteinase binding. For example, with the exception of Ile, all the mutations at position P₃ resulted in a substantial improvement of association with factor XII (Table III). The most favorable single substitution, P13S, yielded a 58-fold increase of the association constant, which is in agreement with $k_{cat}/K_m$ data on $p$-nitroanilide substrates (50). Such comparisons should be taken with caution, because substrate kinetic (and probably also thermodynamic effects on inhibitor binding) data are dependent on the structural context of the mutated site. For example, when Phe is present at P₃ in tripeptide substrates, Pro→Ser substitution at P₃ increases $k_{cat}/K_m$ 97-fold, whereas when Gly is present at P₃ the effect is only 3.6-fold (50).

Three hydrophobic mutations introduced at the P₃ site (Val, Ile, Phe) improved binding of BPTI to thrombin severalfold (Table III), in agreement with the X-ray structure of human α-thrombin inhibited by d-Phe-Pro-Arg chloromethylketone, which shows that the S₃ site overlaps with the aryl binding site (51). According to the model, the presence of positive effects on proteinase inhibition were discouraging to further mutational studies.

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