Glu$^{192}$ → Gln Substitution in Thrombin Yields an Enzyme That Is Effectively Inhibited by Bovine Pancreatic Trypsin Inhibitor and Tissue Factor Pathway Inhibitor*

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Modeling studies have ascribed the remarkable resistance of thrombin to inhibition by the Kunitz type inhibitors, bovine pancreatic trypsin inhibitor (BPTI) and tissue factor pathway inhibitor (TFPI), to steric inhibition by the 60-loop insertion, especially Trp$^{60}$ (in the chymotrypsin numbering system). Indeed, deletion of Pro$^{60}$, Pro$^{60}$, and Trp$^{60}$ from this loop (des-PPW) enhances BPTI inhibition ($K_{i} = 16$ nM) (Le Bonniec, B. F., Guinto, E. R., MacGillivray, R. T. A., Stone, S. R., and Esmon, C. T. (1993) J. Biol. Chem. 268, 19055–19061). Activated protein C, however, lacks an equivalent insertion loop but is nevertheless resistant to inhibition by these Kunitz inhibitors. A unique feature of thrombin and activated protein C is the presence of Glu at position 192. Substitution of Glu$^{192}$ with Gln in activated protein C dramatically enhances inhibition by BPTI and TFPI (Rezaie, A. and Esmon, C. T. (1993) J. Biol. Chem. 268, 19943–19948). We now demonstrate that thrombin E192Q (the Glu$^{192}$ → Gln mutant) is inhibited by BPTI ($K_{i} = 24$ nM) or TFPI ($K_{i} = 14$ nM) much more effectively than wild type thrombin ($K_{i} > 1$ μM for both inhibitors). A thrombin mutant having both the des-PPW deletion and E192Q substitution binds BPTI ($K_{i} = 35$ ps) and TFPI ($K_{i} = 25$ ps) even tighter. BPTI can displace dansylarginineN-(-3-ethyl-l,5-pentanediyl)amide from the active site of thrombin E192Q ($K_{i} = 19$ nM), indicating that BPTI interacts directly with the S1 binding site in thrombin. The E192Q mutation and PPW deletion contribute comparably and additively to the binding energy of thrombin with the Kunitz inhibitors. We suggest that access to the active center of thrombin is less restricted than predicted from previous studies.

Thrombin is the final enzyme in the coagulation cascade and plays a central role in normal hemostasis. This serine protease cleaves fibrinogen to yield fibrin and also cleaves a specific platelet receptor that stimulates platelet activation. Thrombin also activates several cofactors in the cascade and, when bound to thrombomodulin, activates the anticoagulant zymogen, protein C (1, 2). Thrombin is inhibited physiologically by antithrombin and several other serpins (3), but is resistant to inhibition by the Kunitz type inhibitors (4), bovine pancreatic trypsin inhibitor (BPTI) and tissue factor pathway inhibitor (TFPI) (5).

A molecular basis for the resistance of thrombin to inhibition by Kunitz inhibitors has been proposed based on the x-ray crystal structure of thrombin. The rim of the active site is shaped on each side by unique tryptophan containing surface loops, the Trp$^{60}$ and Trp$^{148}$ loop (6, 7). Modeling studies that attempt to dock BPTI with thrombin predict collision of several residues in the inhibitor, primarily Trp$^{60}$, with residues in the 60-loop, primarily Trp$^{60}$, resulting in steric hindrance that prevents access to the catalytic center of thrombin by BPTI (8, 9). Furthermore, the Trp$^{60}$ loop appears to be quite rigid (6, 10). The observation that thrombin is weakly inhibited by Kunitz type inhibitors such as BPTI (4) further supports the concept of a rigid Trp$^{60}$ loop. Consistent with the predictions from this model, deletion of the PPW motif from the Trp$^{60}$ loop results in a thrombin mutant (des-PPW) that is effectively inhibited by BPTI (11).

The Trp$^{60}$ loop cannot be the only determinant which prevents binding of the Kunitz-type inhibitors to serine proteases. Activated protein C, like thrombin, is resistant to inhibition by BPTI and TFPI, but activated protein C lacks a comparable Trp$^{60}$ insertion loop (activated protein C contains only a 2 residue insertion compared with trypsin). Previous studies (12) have demonstrated that substitution of Glu$^{192}$ in activated protein C with Gln results in a mutant protease that binds TFPI and BPTI with high affinity. Thrombin and activated protein C both have Glu at position 192 (in the chymotrypsin numbering system), suggesting that Glu$^{192}$ in thrombin might also contribute to resistance to inhibition by BPTI and TFPI. This concept is bolstered by the observation that in trypsin and factor Xa residue 192 is Glu (13) and that trypsin (14) and factor Xa (15) are inhibited very effectively by BPTI and TFPI, respectively. Given the key role that Glu$^{192}$ plays in regulating activated protein C interaction with the Kunitz inhibitors, we investigated the possibility that Glu at position 192 in thrombin might contribute substantially to the resistance to Kunitz inhibitors.

**EXPERIMENTAL PROCEDURES**

Human and recombinant thrombin wild type, E192Q, and des-PPW were prepared as described previously (11, 16). The cDNA for the enzyme by the Kunitz type inhibitors (4), bovine pancreatic trypsin inhibitor (BPTI) and tissue factor pathway inhibitor (TFPI) (5).

The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; TFPI, tissue factor pathway inhibitor; DAPA, dansylarginineN-(-3-ethyl-1,5-pentanediyl)amide; PPACK, H-o-Pho-Pro-Arg-chloromethyl ketone; pNA, p-nitroaniline; MOPS, 4-morpholinepropanesulfonic acid; danay, 5-dimethylaminonaphthalene-1-sulfonate.

1 The amino acid numbering system of thrombin is based on the topological alignment with the structure of chymotrypsin, suggested by Bode et al. (6).
pression of des-PPW-E192Q was constructed by substitution of the Ser1-Gly fragment of pNUT hII (des-PPW) (11) with the corresponding sequence of pNUT hII (E192Q) (16). Recombinant prethrombin 1 des-PPW-E192Q preceded by a 12 amino acid epitope of the monoclonal antibody HPC4 (17) was then expressed and thrombin des-PPW-E192Q prepared and isolated as described previously (18). Recombinant TFP1 was a generous gift from Dr. Gerald Galluppi at Monsanto Chemical Co. (St. Louis, MO). Bovine factor Xa was prepared and isolated as described previously (18). Recombinant TFP1 was a generous gift from Dr. Gerald Galluppi at Monsanto Chemical Co. (St. Louis, MO). Bovine factor Xa was prepared and isolated as described previously (18).

Thrombin E192Q was inhibited with H-o-Phe-Pro-Arg-chloromethyl ketone (PPACK-E192Q) by incubating 100 μl of 12 μM E192Q with 60 μM PPACK in 0.1 M MOPS, pH 7.0, containing 0.1 M NaCl for 30 min at room temperature. Free PPACK was removed by dialysis versus three changes of 1000 ml of 0.1 M NaCl, pH 7.5, 0.01 M azide at 4 °C. The substrates H-o-Val-Arg-pNA (Tris-HCl, pH 7.5, 0.01 M azide) and H-o-Phe-pcypol-Arg-pNA (S2238) were purchased from Chromogenix (Molndal, Sweden). The substrate H-o-hexahydroxytyrosyl-Ala-Arg-pNA (Spectrozyme-TH, number 238) and H-o-(carbobenzoxy)-Lys-Pro-Arg-pNA (Spectrozyme-PCa, number 335) and dansylarginine pNA (Spectrozyme-TH, number 4.5) were from Sigma. PPACK was obtained from Calbiochem. Diisopropyl fluorophosphate and BPTI were obtained from Sigma. PPACK was obtained from Calbiochem. Diisopropyl fluorophosphate and BPTI were obtained from Sigma. PPACK was obtained from Calbiochem. Diisopropyl fluorophosphate and BPTI were obtained from Sigma. PPACK was obtained from Calbiochem.

A general expression for inhibition of an enzyme (E) by an inhibitor (I) in the presence of a substrate (S) is given by Scheme 1 (20),

\[ E + S \xrightarrow{K_m} ES \xrightarrow{k_{cat}} E + P + \alpha K_I \]

where \( k_{cat} \) is the catalytic constant and \( K_m \) is assumed to be equivalent to \( K_m \), the Michaelis constant, of the free enzyme for S and α are coefficients by which \( K_m \) and \( k_{cat} \) change, respectively, when the inhibitor occupies the enzyme. When the value of α is large enough, the inhibition appears strictly competitive. In our case, when α ~ 5×0, we considered the mechanism to be strictly competitive. In this situation, if saturation requires a large excess of the inhibitor over the enzyme can be estimated by using Equation 1 for simple competitive inhibition (11, 18).

\[ V_s = V_0/(1 + S/K_m) + 1 \]  

(Eq. 1)

where \( V_s \) is the steady state velocity of pNA release in the presence of inhibitor (I) and \( V_0 \) the velocity in the absence of inhibitor. When binding is so tight that the amount of inhibitor used is comparable with the enzyme concentration and full inhibition can be obtained, \( K_I \) can be estimated by using Equation 2 for tight binding competitive inhibition (18, 21).

\[ V_s = \left( V_0/2E_i \right)/(\left( (K_m' + I - E_i)^2 + 4K_m'\left( E_i \right)^2 \right) - (K_m' + I - E_i)) \]  

(Eq. 2)

where \( K_m' \) (the apparent inhibitor constant) is \( K_m/(1 + S/K_m) \).

When the value of \( K_m' \) is not large enough to assume competitive inhibition, \( K_m \), α and β can be estimated from a series of plots of the dependence of \( V_s \) on I and S using Equation 3 (20, 22).

\[ V_s = V_0/\left( \alpha K_m + K_I \right)/(\beta I + \alpha K_m) + \left( S + \alpha K_m \right)/(\beta I + \alpha K_m) \]  

(Eq. 3)

The α and β values depend upon the type of inhibition; when inhibition is partial competitive, \( 1 < \alpha < \beta = 1 \); when inhibition is partial noncompetitive \( \alpha = 0 < \beta = 1 \); and when inhibition is partial, \( \alpha = 0 < \beta < 1 \) (20).

Since several of the reactions studied appeared to reflect only partial inhibition, the kinetics of thrombin E192Q, des-PPW, and des-PPW-E192Q inhibition by BPTI and TFP1 were studied with several different substrate concentrations (0.05-1 μM). The values of \( K_m \) and \( k_{cat} \) for these pNA substrates were determined (in the absence of inhibitor) from the plot of initial rates of cleavage versus S by nonlinear curve fitting of the data to the Michaelis Menten equation using the Enzfitter computer program (R. J. Leatherbarrow, Elsevier-BIOSOFT, Cambridge, United Kingdom). The \( K_m \) values used in this study for S2266, S2238, and Spectrozyme-TH were 161, 45, and 4.5 μM for des-PPW; 172, 8, and 6 μM for E192Q; and 750, 190, and 34 μM for des-PPW-E192Q. The \( K_m \) of thrombin for S2266 is 160 μM.

Steady state kinetics of pNA release were studied as a function of substrate concentration at room temperature (21-24°C). Prior to the addition of the pNA substrate, the enzyme was incubated with dilutions of the Kunitz inhibitor for 1-2 h at room temperature. Time course

\[ \text{results} \]

Inhibition of Thrombin and Thrombin Mutants by BPTI and TP1—Previous studies with the PPW deletion mutant of thrombin and E192Q mutant of activated protein C suggested that both the Trp660 loop (11) and Glu99 (12) might be important in protecting thrombin from inhibition by the Kunitz inhibitors. To test this hypothesis, we prepared thrombin des-PPW, thrombin E192Q, and thrombin des-PPW-E192Q and examined their relative susceptibility to inhibition by BPTI (Fig. 1A) and TFP1 (Fig. 1B). The E192Q and des-PPW mutants were inhibited by BPTI (Fig. 1A) and TFP1 (Fig. 1B) similarly, and both mutants bound these inhibitors at least 1000 times tighter than thrombin. The combined mutation of des-PPW-E192Q resulted in a dramatic further enhancement of affinity for both inhibitors (Fig. 1, A and B).

Characterization of the interaction of Thrombin Mutants with BPTI—To analyze the relative contribution of the Trp660 loop and Glu99 more completely, the \( K_m \) values and nature of the inhibition by BPTI and TFP1 were evaluated. BPTI inhibition of thrombin des-PPW appeared strictly competitive (\( K_m = 24.4 \pm 5 \) μM) (Fig. 2A). Incubation of thrombin E192Q with 4 μM BPTI resulted in >90% inhibition of the amidolytic activity (Fig. 2B). Fitting the data to Equation 1 resulted in \( K_m \) values that increase with decreasing substrate concentrations. Fitting the data to Scheme 1 (Equation 3) yielded a \( K_m \) of 24 ± 6 μM BPTI with \( \alpha = 2.4 \pm 0.3 \) and \( \beta = 0.15 \pm 0.015 \). One interpretation of the observed BPTI inhibition of E192Q data is that BPTI binds to thrombin E192Q and the E192Q-substrate complex and weakens the enzyme-substrate complex, resulting in at least a 6-fold decrease in the rate of catalysis. These data suggest that BPTI inhibition of E192Q hydrolysis of S2266 is more complicated and the multi complex [20] inactivation was collected over.

The kinetic mechanism of inhibition of thrombin des-PPW-E192Q was also examined. Despite retention of similar activity toward diisopropyl fluorophosphate (\( K_m \) is 4.9 ± 0.2 versus 4.0 ± 0.1 μM for thrombin), this mutant had increased \( K_m \) values for all substrates tested. Since Spectrozyme-TH was the best substrate (\( K_m = 33 \) μM), it was used for the analysis of
were preincubated for 1 h at 21-23 °C in 0.1 M NaCl, 0.06 M Tris-HCl, pH 7.5, in the presence of 1 mg/ml gelatin, prior to the addition of substrate, A, BPTI inhibition of thrombin (5 nm at 400 μm S2266 (○), E192Q (2.5 nm at 500 μm S2266), des-PPW-E192Q (■), des-PPW (2.5 nm at 500 μm S2266) (▲), and des-PPW-E192Q (0.25 nm at 100 μm Spectrozyme-TH) (●). B, TFPI inhibition of thrombin (2 nm) (○), E192Q (5 nm) (▲), des-PPW (2.5 nm), at 500 μm S2266 (▲), and des-PPW-E192Q (0.25 nm at 100 μm Spectrozyme-TH) (●). The substrate concentration used was two to three times the K_i value for the enzyme.

thrombin des-PPW-E192Q inhibition by BPTI. Inhibition was strictly competitive (Equation 2) with the K_i = 0.035 ± 0.033 nm (Fig. 2C).

The mixed-type inhibition observed with thrombin E192Q could either reflect interaction not involving the active center of thrombin or a more complex mechanism than considered in the modeling. Among the latter possibilities are allosteric effects of the substrates on the enzyme or enzyme forms that are resistant to BPTI. Therefore, other approaches were used to determine whether BPTI was interacting with the active center. We examined the ability of thrombin E192Q inhibited in the active site with PPACK (PPACK-thrombin E192Q, 50 nm) to compete with E192Q (0.5 μM) for binding to BPTI. The PPACK-thrombin E192Q did not interfere with BPTI inhibition of thrombin E192Q at concentrations of BPTI ranging from 10 to 200 nm (data not shown).

The involvement of the active site in docking BPTI to thrombin E192Q was further confirmed by examining thrombin E192Q binding to DAPA, a fluorescent active site probe of thrombin that inhibits thrombin competitively (23). DAPA binds to the S1 specificity pocket (24). The affinity of DAPA for thrombin E192Q (K_a = 60 nm) and thrombin (K_a = 50 nm) are similar. Under our conditions, thrombin and thrombin E192Q increased DAPA fluorescence emission similarly (ΔF = 40% at 200 nm enzyme and 1 μM DAPA). Preincubation of thrombin E192Q with BPTI prevented the fluorescence enhancement in a concentration-dependent fashion (K_i = 19 ± 4 nm) (Fig. 3). In comparison, as anticipated, BPTI was relatively ineffective in displacing DAPA from the active center of wild type thrombin. These data strongly suggest that BPTI interacts with the primary specificity pocket of thrombin E192Q.

In contrast to BPTI, TFPI is involved in the regulation of blood coagulation (5), and hence it is of interest to understand why thrombin does not react with this physiological inhibitor of blood coagulation. The relative affinity of thrombin and the mutants for TFPI (Fig. 1B) was similar to that observed for BPTI (Fig. 1A). With the des-PPW mutant, inhibition of S2266 hydrolysis appears partial (K_i = 5.0 ± 0.8 nm, α = 2.7 ± 0.7 and β = 0.15 ± 0.02 (Fig. 4A). Inhibition of thrombin E192Q by TFPI is strictly competitive, K_i = 14 ± 2 nm (Fig. 4B). Inhibition of des-PPW-E192Q by TFPI is also strictly competitive, K_i = 0.025 ± 0.003 nm (Fig. 4C). As with BPTI, the K_i for TFPI of the doubly mutated thrombin is over 100-fold lower than that of either des-PPW or thrombin E192Q.

To compare the contributions of the Trp600 loop and Glu192 to the interaction with the Kunitz inhibitors, the K_i values were converted to binding energy. Since the K_i of TFPI for thrombin could not be measured with available reagents, the binding energy was calculated to be -30 kJ mol^-1. ΔG^0 was estimated to be -25.4 kJ mol^-1 for interaction with BPTI. The PPW deletion resulted in comparable changes for both inhibitors (ΔΔG^0 = -17.1 kJ mol^-1 for BPTI and -17.0 kJ mol^-1 for TFPI. The
The shift of the inhibition curve to the right caused by substrate binding. Consistent with this suggestion, in a benzamidine-thrombin crystal structure, Trp$^{60}$ was displaced at the specificity pocket, one would predict that the PPACK would stabilize a major overlap between the inhibitor and the Trp$^{60}$ loop of thrombin, primarily between Tyr$^{59}$ and Trp$^{60}$ (8). Therefore, BPTI could inhibit thrombin only if the Trp$^{60}$ loop was distorted significantly from its location in the PPACK thrombin crystal structure. This loop appears rigid based on both the B values for this loop in the PPACK thrombin crystal structure (6) and its similar conformation in the structures of thrombin docked with a variety of inhibitors (10). Since Pro is ideally suited to contact the Trp$^{60}$ which forms part of the S2 specificity pocket, one would predict that the PPACK would stabilize the conformation of the Trp$^{60}$ loop just as this loop contributes to substrate binding. Consistent with this suggestion, in a benzamidine-thrombin crystal structure, Trp$^{60}$ was displaced at least 1.5 Å from its position in the PPACK complex (26). Thus, the Trp$^{60}$ loop may adopt its structure in response to different inhibitors.

The Thrombin Mutants and Factor Xa Appear to Bind to the Same Kunitz Domain of TFPI—TFPI has three tandem Kunitz domains and is a slow tight binding inhibitor of factor Xa (15). Factor Xa interacts with the second Kunitz domain, whereas the tissue factor-factor VIIa complex interacts with the first domain (25). To determine the TFPI domain responsible for interaction with the thrombin mutants, we carried out competition experiments with factor Xa (Fig. 5A). Specifically, 50 nM bovine factor Xa was preincubated with 1 nM thrombin E192Q at varying TFPI concentrations, and the residual activity of thrombin E192Q was measured with 52238 (50 nM) bovine factor Xa was preincubated with 1 nM thrombin E192Q at 1000 (A), 500 (B), 200 (C), and 100 (D) μM S2266. The inhibition data were fitted to Equation 1, and the average $K_i$ at different substrate concentrations was reported here. C, inhibition of 0.25 nM des-PPW-E192Q at 200 (O), 100 (A), 50 (O), 37.5 (I), and 18.7 (W) μM Spectrozyme-TF. The data were fitted to Equation 2.

**Discussion**

In modeling studies, docking BPTI with thrombin resulted in a major overlap between the inhibitor and the Trp$^{60}$ loop of thrombin, primarily between Tyr$^{59}$ and Trp$^{60}$ (8). Therefore, BPTI could inhibit thrombin only if the Trp$^{60}$ loop was distorted significantly from its location in the PPACK thrombin crystal structure. This loop appears rigid based on both the B values for this loop in the PPACK thrombin crystal structure (6) and its similar conformation in the structures of thrombin docked with a variety of inhibitors (10). Since Pro is ideally suited to contact the Trp$^{60}$ which forms part of the S2 specificity pocket, one would predict that the PPACK would stabilize the conformation of the Trp$^{60}$ loop just as this loop contributes to substrate binding. Consistent with this suggestion, in a benzamidine-thrombin crystal structure, Trp$^{60}$ was displaced at the specificity pocket, one would predict that the PPACK would stabilize the conformation of the Trp$^{60}$ loop just as this loop contributes to substrate binding. Consistent with this suggestion, in a benzamidine-thrombin crystal structure, Trp$^{60}$ was displaced at

![Graph showing competition of BPTI and DAPA binding to E192Q.](image)

**Fig. 3.** Competition of BPTI and DAPA binding to E192Q. Thrombin (O) and E192Q (•) at 200 μM were preincubated with increasing concentrations of BPTI for 1 h at 21-23°C prior to the addition of 1 μM DAPA. Re-equilibration was allowed to occur for 10 min. BPTI enzyme binding and fluorescence measurements were performed in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mg/ml polyethylene glycol-6000. Thrombin-dependent fluorescence was obtained following background correction due to DAPA fluorescence. The solid lines were obtained by fitting the data to Equation 1.

The contributions of the dual mutation were additive ($\Delta G^o = -17.6$ kJ mol$^{-1}$ for BPTI and $\Delta G^o = -14.5$ kJ mol$^{-1}$ for TFPI). The contributions of the dual mutation were additive ($\Delta G^o = -33.7$ kJ mol$^{-1}$ for BPTI and $\Delta G^o = -30.0$ kJ mol$^{-1}$ for TFPI) indicating the Glu$^{192}$ and the PPW portion of the Trp$^{60}$ loop block inhibitor interaction by different mechanisms.

**Fig. 4.** Inhibition of des-PPW, E192Q, and des-PPW-E192Q by TFPI. Steady state velocities were determined as a function of TFPI concentration. A, inhibition of 2.5 μM des-PPW at 1000 (A), 600 (O), 350 (•), 250 (O), 125 (O), and 50 (O) μM S2266. The inhibition data were fitted to Equation 3 to obtain the $K_i$ (at $V_{max} = 12.4 \pm 0.4$ min$^{-1}$) $B$, inhibition of 5 nM E192Q at 1000 (O), 500 (O), 400 (A), 200 (O), and 100 (D) μM S2266. The inhibition data were fitted to Equation 1, and the average $K_i$ at different substrate concentrations was reported here. C, inhibition of 0.25 nM des-PPW-E192Q at 200 (O), 100 (A), 50 (O), 37.5 (I), and 18.7 (W) μM Spectrozyme-TF. The data were fitted to Equation 2.

A least 1.5 Å from its position in the PPACK crystal complex (26). Thus, the Trp$^{60}$ loop may adopt its structure in response to different inhibitors.

The present study brings into question the concept of the extremely rigid loop as the sole or even primary basis for the Kunitz inhibitor specificity. At least three possibilities could account for inhibition of thrombin E192Q by BPTI. In the first, the Trp$^{60}$ loop is inherently more flexible or in a different conformation in free thrombin than is predicted based on the PPACK crystal structure. In the second, BPTI forms strongly complementary bonds, including those with Glu$^{192}$ that allow the inhibitor to dock and force the Trp$^{60}$ loop into a different conformation. In the third, the E192Q mutation dramatically influences the mobility of the Trp$^{60}$ loop. The first possibility is supported by the observation that E192Q substitution is all
thrombin (16). If the Trp$^{602}$ loop were perturbed, one would assume that the S2 specificity pocket would be altered and that the catalytic competence would be significantly diminished. Second, in the PPACK-thrombin crystal structure (6), Glu$^{192}$ is at least 7 Å from Trp$^{602}$, and the side chain is flexible at the base of the active site. Therefore, Glu$^{192}$ can at most only indirectly influence the stability of the Trp$^{602}$ loop. Thus, it would appear that the most likely explanation for the ability of BPTI to interact with thrombin E192Q is that in active thrombin the Trp$^{602}$ loop is either flexible enough to provide access to the specificity pocket of thrombin or alternatively exists in a stable conformation distinct from that determined from the crystal structure of thrombin-inhibitor complexes.

Exactly how Glu$^{192}$ increases BPTI affinity for thrombin must await structural analysis of the complex, but some potential mechanisms can be proposed based on the interactions of this residue with BPTI in trypsin. In the trypsin-BPTI complex, Glu$^{196}$ is involved in hydrogen bonding with several residues of BPTI as well as a water molecule in the active site (27, 28). In the case of both activated protein C (12) and thrombin, the Glu to Gln substitution is all that is necessary to allow effective inhibition by BPTI and TFPI, suggesting that the resulting interactions are major contributors to tight binding.

Little is known about the three-dimensional structure of TFPI. From the data presented here, it would appear that the structural basis for TFPI binding to target proteases may be similar to that of BPTI. TFPI, however, contains three tandem Kunitz inhibitor domains (5). Factor Xa is inhibited by binding to the second Kunitz domain (25). The first Kunitz domain appears to inactivate VIIa-tissue factor complexes. Both thrombin E192Q and activated protein C E192Q (12) also interact with the second Kunitz domain.

One perplexing problem is that low level residual activity is often detected with the thrombin mutants in the presence of very high levels of the inhibitors. The kinetic data suggest that the inhibitor-enzyme complex may be able to bind the chromogenic substrate and catalyze cleavage with impaired efficiency. Alternatively, traces of stable conformers or proteolysis products of thrombin exist in these preparations that account for low level residual activity. The preparations appear homogeneous by gel electrophoresis, but the residual activity is low. The possibility of multiple relatively stable conformations is supported by the studies (29) that sodium ions convert thrombin into a conformation with greater catalytic activity toward small substrates. It is therefore possible that the apparent partial mixed type inhibition is a reflection of stable distinct conformers in the preparation. These conformer populations may be perturbed to a different extent by different substrates. For instance, TFPI is a complete inhibitor of E192Q when S2266 is used as a substrate (Fig. 4), but not with S2238 (Fig. 5). Despite these complications, that the inhibitors interact with the active center of thrombin seems nearly certain based on the structure of the inhibitors, the observation that the active site inhibited thrombin mutant does not compete with the active enzyme for binding to the inhibitor, and the ability of TFPI to displace the fluorescent active site inhibitor DAPA.

These studies suggest that Glu$^{192}$ is as important as the surface loops blocking inhibition by Kunitz inhibitors. We infer from these results that in solution, the Trp$^{602}$ loop is likely to be more flexible than predicted from the crystal structure.

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