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Heparin Modulates the 99-Loop of Factor IXa

EFFECTS ON REACTIVITY WITH ISOLATED KUNITZ-TYPE INHIBITOR DOMAINS

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Reactivity of factor IXa with basic pancreatic trypsin inhibitor is enhanced by low molecular weight heparin (enoxaparin). Previous studies by us have suggested that this effect involves allosteric modulation of factor IXa. We examined the reactivity of factor IXa with several isolated Kunitz-type inhibitor domains: basic pancreatic trypsin inhibitor, the Kunitz inhibitor domain of protease Nexin-2, and the first two inhibitor domains of tissue factor pathway inhibitor. We find that enhancement of factor IXa reactivity by enoxaparin is greatest for basic pancreatic trypsin inhibitor (>10-fold), followed by the second tissue factor pathway inhibitor domain (1.7-fold) and the Kunitz inhibitor domain of protease Nexin-2 (1.4-fold). Modeling studies of factor IXa with basic pancreatic trypsin inhibitor suggest that binding of this inhibitor is sterically hindered by the 99-loop of factor IXa, specifically residue Lys98. Slow-binding kinetic studies support the formation of a weak initial enzyme-inhibitor complex between factor IXa and basic pancreatic trypsin inhibitor that is facilitated by enoxaparin binding. Mutation of Lys98 to Ala in factor IXa results in enhanced reactivity with all inhibitors examined, whereas almost completely abrogating the enhancing effects of enoxaparin. The results implicate Lys98 and the 99-loop of factor IXa in defining enzyme inhibitor specificity. More importantly, these results demonstrate the ability of factor IXa to be allosterically modulated by occupation of the heparin-binding exosite.

EXPERIMENTAL PROCEDURES

Materials—Factor IXαβ, factor VIIIa, factor XIa, and the factor X activator from Russell’s Viper venom were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Recombinant soluble tissue factor (the extracellular domain of tissue factor) was expressed and purified from bacteria as previously described (16). Factor Xa was prepared from plasma-derived factor X as previously described (17). Enoxaparin (Lovenox®) was purchased from Aventis Pharmaceuticals (Bridgewater, NJ). Purified heparin-derived oligosaccharides of 6, 10, 14, and 18 saccharide units (H6, H10, H14, and H18) were prepared and characterized essentially as described (18–20) and were a generous gift of Dr. Steven T. Olson, University of Illinois, Chicago, IL. Bovine serum albumin (Fraction V, fatty acid free) was from Calbiochem (La Jolla, CA), and ethylene glycol was from Fisher Scientific. The chromogenic substrate CBS 31.39 (CH₃SO₂-D-LGR-pNA) was purchased from Diagnostic (Parsippany, NJ). All other reagents were of the highest quality available.
Construction and Expression of Recombinant Inhibitors—

Appropriate expression clones encoded for: BPTI (59 amino acids) (21, 22), PN2-KPI (61 amino acids corresponding to residues 285–344 of Protease Nexin-2) (23), TFPI-K1 (58 amino acids corresponding to residues 50–107 of TFPI) (24), and TFPI-K2 (59 amino acids corresponding to residues 121–178 of TFPI) (24). Each construct was directionally cloned into pET11a (Novagen) and verified by sequencing. Inhibitors were expressed as inclusion bodies in Escherichia coli strain BL21(DE3). Transformed bacterial cells were first grown to log phase at 37 °C in TB media containing 50 μg/ml carbenicillin. Protein expression was induced by addition of isopropyl 1-thio-β-D-galactopyranoside to 0.5 mM (0.1 mM for TFPI-K1) and the cells were allowed to continue growing for 4 h at 37 °C.

Inclusion bodies were isolated essentially as described (25) and solubilized with 6 M guanidine HCl containing 20 mM dithiothreitol, 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA to obtain a total protein concentration of roughly 20 mg/ml. The solution was then clarified by centrifugation (16,000 × g for 30 min) and oxidative refolding (26) of each protein preparation was performed by rapid dilution into 20 volumes of buffer containing 50 mM Tris-HCl, pH 8.0, 1 M guanidine HCl, 1 mM EDTA, 2.5 mM oxidized glutathione (Sigma), and 1 mM dithiothreitol. The diluted protein solution was incubated at room temperature for 6 h with slow stirring for completion of protein refolding followed by exhaustive dialysis into an appropriate buffer for ion-exchange chromatography.

Construction and Expression of Wild-type and Mutant fIX—
The coding sequence for wild-type fIX in pBR322 (27) was a generous gift of Dr. Earl Davie (University of Washington). The fIX cDNA sequence was removed from the plasmid expression vector pCDNA3 (Invitrogen) and sequenced to verify the correct orientation. This construct (pFN04) was used for expression of wild-type fIX as well as PCR-based mutagenesis (28) to generate fIXK98A essentially as previously described for expression of wild-type fIX as well as PCR-based mutagenesis (28) to generate fIXK98A essentially as previously described for expressing non-reacted sites with 1 M ethanolamine-HCl, pH 8.0. In both cases, the column was developed with a 0–0.5 M NaCl gradient. Wild-type fIX protein was purified away from the activated enzyme was purified away from the venom protease by subsequent heparin affinity chromatography essentially as described above using HiTrap™ Heparin HP (Amersham Biosciences). Although the activation peptide remains attached to the light chain of fIX, this enzyme retains 100% amidolytic activity compared with fIXα (activation peptide proteolytically removed) and is comparable with fIXα in kinetics of inhibition by Kunitz-type inhibitors (see “Results”). Unless indicated otherwise, the fIXα form was used in experiments.

Clotting Assays—Coagulant activities of wild-type and mutant fIXα proteins were assayed by a standard single-stage clotting assay using a Coag-a-mate XM (Organon Teknika) coagulometer, fIX-deficient plasma (George King Biomedical), and APTT Reagent (Sigma).

Reactive Site Titration of Inhibitors—The active concentration of inhibitor preparations was determined by reactive site titration essentially as described (23) using 10 nM active site-titrated trypsin (30) and S-2222 substrate (Chromogenix, Milano, Italy) to measure residual trypsin activity after a 15-min incubation period. This method assumes a 1:1 stoichiometry of inhibitor and trypsin. Amino acid analysis performed on an initial PN2-KPI preparation indicated an equivalent concentration as that determined by reactive site titration (not shown).

Active Site Titration of fIXα Enzymes—Active concentrations of wild-type and mutant fIXα preparations were determined by active site titration using biotin-EGR-ck (Hematologic Technologies Inc.) essentially as described (31). Briefly, wild-type or mutant fIXα (roughly 5 μM) were incubated with 150 μM biotin-EGR-ck in 50 mM Tricine, pH 8.0, 200 mM NaCl, 10 mM CaCl₂,
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and 30% ethylene glycol for 24 h at room temperature. Biotin-EGR-fIXa was then quantified by enzyme-linked immunosorbent assay using a goat anti-fIX polyclonal capture antibody and alkaline phosphatase-conjugated streptavidin. These were fitted with a standard line made using native fIXαβ to determine active concentrations for all fIXα enzyme preparations.

Equilibrium Enzyme Inhibition Assays—Reversible competitive inhibition of fIXα amidolytic activity at equilibrium was measured at 25°C using final concentrations of 25 nM fIXα, 5 mM CaCl2 and the indicated concentrations of enoxaparin and inhibitor in the presence of 30% ethylene glycol as previously described (15). The addition of ethylene glycol enhances the enzymatic activity of fIXα toward CBS 31.39 roughly 20-fold (32). Previous studies by us as well as control experiments performed with each inhibitor examined showed that the inclusion of ethylene glycol in the assay has no effect on the reactivity of fIXα with these inhibitors (15). Values of the final equilibrium inhibition constant (Ki,eq) were estimated as described (15, 29) using Equation 1. This equation describes simple reversible competitive inhibition where v0 is the steady state rate obtained in the absence of inhibitor, v is the steady state rate at each concentration of inhibitor, S is the experimental substrate concentration, and Km is the Michaelis constant for substrate hydrolysis.

\[
v_i = \frac{K_{i,eq}(1 + S/K_m)}{K_{i,eq}(1 + S/K_m)}
\]

(Eq. 1)

Slow-binding Enzyme Inhibition Analysis—Kinetic parameters for slow-binding inhibition were obtained using Equations 2–5 as derived by others (33, 34) and are described in Schemes Ia and Ib (Fig. 1). In both of these schemes the non-covalent enzyme-inhibitor complex, EI, isomerizes into EI*; either after significant formation of the EI complex (Scheme Ia) or without significant accumulation of the EI complex (Scheme Ib). In both cases, the overall inhibition constant describing generation of EI* is defined as Ki,*, which is equivalent to Ki,eq obtained from equilibrium experiments (above). In cases following Scheme Ia, the parameter Ki,* can be further broken down to obtain Ks, which describes the establishment of the initial “loose” EI complex, ks and ka (see below).

Final reaction conditions were the same as described above for equilibrium studies. In these assays, however, the fIXα was preincubated with or without enoxaparin for 15 min at 25°C in the reaction mixture before the combined addition of inhibitor and substrate at time 0. The absorbance at 405 nm was then monitored for up to 30 min in a ThermoMax microplate reader (Molecular Devices) set at 25°C to monitor substrate hydrolysis using KINEMAX software (written and kindly provided by Dr. Jolyon Jesty, SUSB, Stony Brook, NY). Data for each generated curve were fitted with the following integrated rate equation describing slow-binding inhibition,

\[
A = v_i t + (v_o - v_i)(1 - e^{-k_{obs}t})/k_{obs} + A_o
\]

(Eq. 2)

where A is the absorbance at 405 nm at any time, t. Fits of progress curves with Equation 2 yield values for A_o (the initial absorbance at t = 0), v_o (the initial rate of substrate hydrolysis), v_i (the steady-state rate of substrate hydrolysis), and k_{obs} (the apparent first-order rate constant for inhibition).

For analyses using Scheme Ia, values of k_s (the reverse rate constant for EI* isomerization) were determined from progress curves above using the following relationship.

\[
k_s \text{ or } k_{10} = k_{obs}v_o/v_i
\]

(Eq. 3)

Values of k_s and initial K_i (defined as k_i/k_s) were then obtained from secondary plots of k_{obs} versus I using the following hyperbolic equation.

\[
k_{obs} = k_o + k_{obs}(1 + K_{i,eq}(1 + S/K_m))
\]

(Eq. 4)

For analyses using Scheme Ib, values of k_{10} were obtained from progress curves also using Equation 3. However, in these cases v_o does not vary with inhibitor concentration and a plot of k_{obs} versus I yields a straight line, indicating conditions where K_i (1 + S/K_m) ≫ 1. Thus for Scheme Ib EI formation is insignificant and EI* can be considered formed directly from E + I. For these cases the following linear equation is applicable for obtaining an estimate of k_s, the apparent second-order on-rate constant.

\[
k_{obs} = k_{10} + k_{obs}(1 + S/K_m)
\]

(Eq. 5)

where the y intercept reflects k_{10} and the slope of the line is equal to k_{obs}/(1 + S/K_m). Alternatively, k_s can be obtained from K_s*, which is equivalent to K_{i,eq} in Equation 1, using the relationship k_s = k_{10}/K_{s,*}.
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FIGURE 3. Support of BPTI inhibition of fIXa by short chain oligosaccharides. The ability of heparin oligosaccharides with progressively shorter chain lengths to support enhancement of fIXa reactivity with BPTI was examined using 25 nM fIXaβ, 308 μM BPTI, and the indicated concentrations of heparin-derived oligosaccharides (18–20) from 0.01 to 200 μM. Saccharide chain lengths were H16 (●), H14 (□), H10 (■), and H6 (○). Enoxaparin (H15) is shown for comparison (○). The fIXa activities were normalized to the activity of fIXa in the absence of added oligosaccharide.

Although fits with Equations 4 or 5 yield estimates of $k_6$ or $k_{10}$, respectively, the values reported herein were obtained from Equation 3 using the more accurate fits of progress curves to Equation 2 and then verified in fits with Equations 4 or 5. Experimental values of $S$ as well as experimentally determined values of $K_{eq}$ (defined in the traditional manner as $(k_2 + k_3)/k_1$ in Schemes Ia and Ib) were used as necessary in all fitting procedures. All regression procedures were performed using SlideWritePlus 6.0 (Advanced Graphics Software), which uses the Levenberg-Marquardt algorithm.

RESULTS

Previous studies (15) by us have shown that whereas fIXa is resistant to inhibition by the Kunitz-type inhibitor BPTI, this resistance is somewhat alleviated by enoxaparin, leading to a roughly 10-fold enhancement in the equilibrium inhibition constant. To gain further insight into mechanisms of fIXa selectivity and its modulation by heparin we undertook an examination of the reactivity of fIXa with several isolated Kunitz-type inhibitor domains: BPTI, PN2-KPI, TFPI-K1, and TFPI-K2. Each of these inhibitors was expressed in E. coli using standard recombinant techniques, purified to homogeneity and quantified by reactive site titration as described under “Experimental Procedures.” The isolated PN2-KPI inhibitor domain was found to react with high affinity toward factor Xla, yielding a $K_{eq}$ of roughly 400 pm (see supplemental data). Similarly, preliminary studies indicated that preparations of isolated TFPI-K1 domain inhibited the complex of factor Vlla and soluble tissue factor with high affinity ($K_{eq} = 400$ nm) and weakly inhibited factor Xa ($K_{eq} > 1.5$ μM). Conversely, the isolated TFPI-K2 domain inhibited factor Xa with high affinity ($K_{eq} = 24$ nm) and the factor Vlla-tissue factor complex with reduced affinity ($K_{eq} = 7$ μM). These results are consistent with the expected reactivity of the isolated inhibitor domains (35, 36) and demonstrate the correct folding and inhibitor activity of the inhibitors examined.

The abilities of these isolated Kunitz inhibitor domains to inhibit fIXaβ are compared in Fig. 2. As expected, fIXa exhibited remarkable specificity toward these inhibitors despite their high homology. Of the inhibitors examined, PN2-KPI showed the highest level of reactivity ($K_{eq} = 10$ μM), followed by TFPI-K2 ($K_{eq} = 336$ μM), BPTI ($K_{eq} > 500$ μM), and TFPI-K1 ($K_{eq} > 1$ nm). Consistent with our previous observations, enoxaparin was able to enhance the reactivity of fIXa with BPTI more than 10-fold ($K_{eq} = 46$ μM). Surprisingly, however, this same level of enhancement by enoxaparin was not observed with any of the other inhibitors examined; TFPI-K2 and PN2-KPI each showed only a small, but consistent, enhancement in reactivity with enoxaparin (1.7- and 1.4-fold, respectively; $K_{eq}$ values of 203 and 7 μM) and TFPI-K1 showed no measurable enhancement in reactivity with enoxaparin.

The highly basic nature of BPTI compared with the other isolated Kunitz domains along with its ability to bind to heparin (albeit weakly; $K_d = 172$ μM (15)) raised the potential that enoxaparin, although short (15 saccharide units; H15), may retain some capacity to facilitate the interaction of BPTI with fIXa via a bridging-type mechanism. Although unlikely based on previous equilibrium kinetic studies and the level of enoxaparin used in these experiments (10 μM; or $0.06 \times K_d$ for BPTI binding versus $78 \times K_d$ for fIXa binding), this issue was examined by using increasing concentrations of enoxaparin as well as progressively smaller heparin oligosaccharides; H18, H14, H10, and H6 (18–20). As shown in Fig. 3, the typical bell-shaped profile for bridging-type mechanisms was not observed at enoxaparin concentrations ranging from 1 nm to $>100$ μM. In addition, and of greater significance, is the observation that progressively smaller oligosaccharides do not lose the ability to enhance reactivity of fIXa. These results along with previous
kinetic studies support the ability of heparin to modulate fIXa reactivity via a mechanism other than bridging, and are consistent with allosteric modulation of the fIXa protease domain.

To gain further insight into the potential mechanism of heparin modulation of fIXa, we prepared a rudimentary hypothetical model of the fIXa-BPTI complex using available crystal structures of BPTI (37), fIXa (38), and the fVIIa-BPTI complex (39). Using the latter structure as a template, superimposition of the fIXa structure over the fVIIa-BPTI complex model was constructed using Discovery Studio software (Accelrys) based on known crystal structures of fIXa (PDB code 1RFN) and fVIIa-BPTI11,15 (PDB code 1FAK) using molecular replacement. The structure revealed potential steric hindrance between BPTI and the 99-loop of fIXa, specifically Lys98 (central ball and stick structure with van der Waals radii). BPTI is depicted in orange; fIXa is depicted in green with the 99-loop in blue; the heparin binding exosite residues in fIXa (14) are depicted as violet sticks; and the fIXa active site triad residues (Ser195, His57, and Asp102) are depicted as red sticks.

FIGURE 4. Hypothetical model of the inhibited fIXa-BPTI complex. A hypothetical fIXa-BPTI complex model was constructed using Discovery Studio software (Accelrys) based on known crystal structures of fIXa (PDB code 1RFN) and fVIIa-BPTI11,15 (PDB code 1FAK) using molecular replacement. The structure revealed potential steric hindrance between BPTI and the 99-loop of fIXa, specifically Lys98 (central ball and stick structure with van der Waals radii). BPTI is depicted in orange; fIXa is depicted in green with the 99-loop in blue; the heparin binding exosite residues in fIXa (14) are depicted as violet sticks; and the fIXa active site triad residues (Ser195, His57, and Asp102) are depicted as red sticks.

FIGURE 5. A, SDS-PAGE analysis of recombinant WT fIX and fIXK98A. Five μg of purified WT fIX (lane 1) and fIXK98A (lane 2) were loaded onto a 12% polyacrylamide gel under reducing conditions for SDS-PAGE. The resulting gel was stained with Coomassie Brilliant Blue G-250. Molecular weight markers are indicated on the left. B, clotting activity of fIXK98A versus WT fIX. Standard single-stage clotting assays were performed on recombinant WT fIX (C) and fIXK98A (○), which showed equivalent coagulant activity.

TABLE 1
Kinetic parameters for CBS 31.39 hydrolysis by WT fIXa versus fIXaK98A

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (M)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT fIXa</td>
<td>3.78 ± 0.08 × 10⁻³</td>
<td>16.5 ± 0.69</td>
<td>4.37 ± 0.08 × 10³</td>
</tr>
<tr>
<td>fIXaK98A</td>
<td>1.58 ± 0.02 × 10⁻³</td>
<td>6.23 ± 0.43</td>
<td>3.98 ± 0.27 × 10³</td>
</tr>
</tbody>
</table>

Obtained by fitting the data with the Michaelis-Menten equation. Values shown are ± S.E., n = 6.
concentrations (cf. Fig. 6, A and B) are diagnostic for Scheme Ib. The data of Fig. 6C were thus fitted with Equation 5 to obtain estimates for the apparent second-order rate constant; \( k_9 = 5.4 \text{ M}^{-1} \text{s}^{-1} \) without enoxaparin and \( 23 \text{ M}^{-1} \text{s}^{-1} \) with enoxaparin. The value obtained above for \( k_9 \) (\( k_{10} \) in Scheme Ib) is consistent with the values obtained for \( k_{10} \) using Equation 5; \( k_{10} = 1.3 \times 10^{-3} \text{ s}^{-1} \) without enoxaparin and \( 1.2 \times 10^{-3} \text{ s}^{-1} \) with enoxaparin.

A similar slow-binding kinetic analysis was performed with WT fIXa and PN2-KPI. In contrast to what was observed with BPTI, secondary plots of \( k_{\text{obs}} \) versus PN2-KPI were hyperbolic (not shown) and well described by Scheme Ia, yielding the values of \( K_i \), \( k_5 \), and \( k_6 \) given in Table 2. The role of Lys98 and the 99-loop of fIXa in inhibition of fIXa by both BPTI and PN2-KPI were next examined using fIXaK98A. This mutant fIXa showed increased reactivity toward all of the inhibitors examined (Table 3). More importantly, the enhancing effect of enoxaparin was substantially reduced when compared with WT fIXa. Detailed analysis of the slow-binding kinetics of inhibition of fIXaK98A by BPTI revealed that the major difference compared with WT fIXa was the enhanced formation of the initial enzyme-inhibitor complex, as seen visually by the variation in initial rate (\( v_0 \)) of progress curves (Fig. 7, A and B) and the hyperbolic nature of secondary plots (Fig. 7C). Although enoxaparin significantly enhanced the reactivity of both WT fIXa and fIXaK98A toward BPTI (Table 2), the effect on the mutant was much reduced and was completely abrogated for its inhibition by PN2-KPI.

**DISCUSSION**

Previous studies by us have demonstrated that heparin binding to fIXa enhances fIXa reactivity with BPTI (15). Whereas in that study unfractionated heparin was found to have a slightly greater effect, enoxaparin retained most of the ability to enhance fIXa reactivity.
**TABLE 2**

Kinetic constants for inhibition of fIXa and fIXaK98A by BPTI and PN2-KPI

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>Exonaparin</th>
<th>$k_{i0}$</th>
<th>$k_{e}$</th>
<th>$k_{i}$ or $k_{e0}$</th>
<th>$K_{i0}^*$</th>
<th>$K_{e0}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT fIXa</td>
<td>BPTI</td>
<td>–</td>
<td>$2.1 \times 10^{-3}$</td>
<td>5.4</td>
<td>$2.67 \times 10^{-3}$</td>
<td>1.17</td>
<td>$2.66 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>$4.1 \times 10^{-3}$</td>
<td>$2.0 \times 10^{-2}$</td>
<td></td>
<td>$3.50 \times 10^{-3}$</td>
<td>$7.0 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>fIXaK98A</td>
<td>BPTI</td>
<td>–</td>
<td>$3.3 \times 10^{-3}$</td>
<td>$1.3 \times 10^{-2}$</td>
<td></td>
<td>$2.33 \times 10^{-3}$</td>
<td>$1.7 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>$8.8 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-2}$</td>
<td></td>
<td>$2.33 \times 10^{-3}$</td>
<td>$8.2 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>PN2-KPI</td>
<td>–</td>
<td>$8.6 \times 10^{-3}$</td>
<td>$1.4 \times 10^{-2}$</td>
<td></td>
<td>$9.7 \times 10^{-7}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>$8.0 \times 10^{-3}$</td>
<td>$1.4 \times 10^{-2}$</td>
<td></td>
<td>$9.7 \times 10^{-7}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>

*Values are $k_{i0}$ for WT fIXa with BPTI (Scheme Ib) and $k_{e0}$ for others (Scheme Ia) $/ S.D.$ ($n \approx 7$).

The conclusion that heparin can modulate the active site of fIXa is supported in the present study by several observations, not the least of which is the ability of short chain oligosaccharides to retain the ability to enhance fIXa reactivity with BPTI. Whereas the shortest oligosaccharide examined (H6) requires higher concentrations than do H10–H18, the higher IC_{50} for H6 is consistent with the reduced binding energy one may expect for a small oligosaccharide whose projected length would contact only two thirds of the heparin binding exosite: based on available crystal structures (Protein Data Bank codes 1BFC and 1EOO), the length of a decasaccharide is expected to be ~38 Å and that of a hexasaccharide is expected to be ~23 Å at full extension. This is compared with the measured length of the identified heparin binding site on fIXa (~35 Å) based on mutational studies (14).

With respect to the inhibition of fIXa by BPTI, two main conclusions may be drawn from these studies: 1) fIXa residue Lys^98 is in part responsible for protecting fIXa from inhibition by BPTI, likely via steric hindrance. Removal of this steric obstruction by mutation of Lys^98 results in greater inhibition of fIXa by BPTI (26-fold enhancement). 2) Heparin binding to fIXa in part counteracts the steric protection provided by Lys^98. Mutation of Lys^98 results in a reduction in the ability of heparin to further enhance reactivity of fIXa with BPTI (roughly 2-fold effect for fIXaK98A compared with >10-fold effect with WT fIXa). The lack of complete abrogation of the effect of heparin suggests that other as yet undefined factors also play a role in BPTI inhibition of fIXa and its response to heparin. This is likely due to the movement of more than simply Lys^98 and may or may not involve other residues in the 99-loop or even the entire loop.

It is important to note that the reduction in the effect of heparin with the fIXaK98A mutant is not merely due to reduced heparin binding because fIXaK98A retained the ability to bind to heparin-Sepharose and eluted at the same salt concentration as WT fIXa during purification procedures. In support of this, preliminary experiments performed by titrating fIXaK98A with enoxaparin in the presence of 100 μM BPTI yielded results consistent with the high nanomolar affinity for enoxaparin previously observed with fIXaβ. 

The results with BPTI are in contrast to Scheme Ia. This results in fits to Equation 4 shown in Fig. 6C as dashed lines and yields initial $K_{i0}$ values of 2.1 × 10^{-3} μM and 4.1 × 10^{-4} μM in the absence and presence of enoxaparin, respectively. This 5-fold difference in $K_{i0}$ along with ~2-fold difference in $k_{i0}$ (Table 2) for this enzyme-inhibitor pair (Table 2) would seem to account for the ~10-fold effect of enoxaparin observed in $K_{i0}^*$ (Table 3). Unfortunately, the rather large errors in the values of $k_{i0}$ preclude definitive conclusions concerning potential effects of enoxaparin on this rate constant.

Regardless, these results are consistent with a very weak initial interaction of fIXa with BPTI. Enoxaparin binding to the heparin binding exosite in fIXa at least in part acts to allosterically modulate the 99-loop of fIXa in a manner that facilitates this initial interaction. The results with BPTI are in contrast to...
Thus, there is some disparity in the role of Lys98 and additional domains are correct. Taken as a whole, the results here would seem to imply that either the connecting regions of TFPI are important for its activity toward fIXa or other structural/topological elements are involved. Nonetheless, it is likely that the TFPI-K2 domain is the inhibitor domain of TFPI most likely responsible for interaction with fIXa. Although the isolated third Kunitz repeat of TFPI was not examined in this study, this domain is likely not an active inhibitor domain per se (41).

The ability of heparin to allosterically modulate the active site of fIXa demonstrates the ability of fIXa to respond to binding at this exosite. This observation is made more interesting by the observation by Sheehan et al. (42, 43) that the heparin binding exosite in part represents a fVIIa interactive site on fIXa. Thus, whereas heparin binding in itself may inhibit fIXa coagulant activity via steric hindrance of fVIIa binding, fVIIa binding to this exosite may inversely act to allosterically modulate fIXa in an as yet undefined manner. Although previous studies found no effect of fVIIa on fIXa inhibition by the isolated PN2-KPI domain (44), this is consistent with our observations here using enoxaparin in place of fVIIa and does not preclude potential effects of fVIIa toward other inhibitors. It thus remains possible that occupation of this exosite by fVIIa, like heparin, results in inhibitor-specific modulatory effects. Alternatively, the modulating effect of heparin on the 99-loop may be simply due to heparin-specific electrostatic forces that are introduced by heparin binding in close proximity to Lys98 (cf. Fig. 4). These forces may or may not be mimicked by fVIIa binding. Further clarification of these issues must await future studies.

The allosteric modulation of fIXa by enoxaparin is somewhat reminiscent of the effect of thrombomodulin on the interaction of thrombin with BPTI, where binding of thrombomodulin alters the conformation of one of the specificity loops (60-loop) at the mouth of the active site of thrombin, resulting in enhanced reactivity with BPTI (45). Interestingly, in that study the chondroitin sulfate moiety of thrombomodulin (which binds to the heparin binding site on thrombin; anion binding exosite 2) further enhanced the inhibition of an E192Q mutant of thrombin but not wild-type thrombin. Comparison of the sequence of fIXa with thrombin reveals that fIXa contains a Gln at position 192, similar to the thrombin E192Q mutant and fXa. In addition, previous studies have revealed that mutation of the homologous residue in fVIIa (Lys192) to Gln enhances its reactivity with BPTI (29), and that mutation of Gln192 in fIXa altered its reactivity toward TFPI (40). Neither of these studies examined the potential effect of heparin on reactivity with these Kunitz inhibitors. These studies are intriguing and seem to implicate residue 192 in potential heparin responsiveness along with the 99-loop. The investigation of potential interplay between residue 192 and the 99-loop of fIXa in its response to heparin binding is beyond the scope of the present study, however, and must await future studies.

Acknowledgments—We thank Dr. Steven T. Olson for the generous gift of heparin-derived oligosaccharides (H6, H10, H14, and H18), Dr. Earl W. Davie for the factor IX cDNA clone, and Dr. Jolyon Jesty for the ThermoMax microplate reader control software, KINEMAX.
Modulation of the fIXa 99-Loop by Heparin

REFERENCES

SUPPLEMENTAL DATA
HEPARIN MODULATES THE 99-LOOP OF FACTOR IXA: EFFECTS ON REACTIVITY WITH ISOLATED KUNITZ-TYPE INHIBITOR DOMAINS

Table S1. Amino Acid Sequence Alignment of Kunitz-type inhibitor domains

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<tr>
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<tr>
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Conserved amino acids with BPTI are shaded. Reactive site loops (thin boxes) and stabilizing loops (thick boxes) are held in close proximity by a disulfide bond between Cys14 and Cys38 (indicated). The P1 reactive site residues are in bold.

Figure S1. SDS-PAGE analysis of isolated Kunitz inhibitor domains. Approximately 5–10 µg of each purified inhibitor was loaded onto a 12% polyacrylamide gel for electrophoresis. Lane 1 – BPTI; lane 2 – PN2-KPI; lane 3 – TFPI-K1; and lane 4 – TFPI-K2. The gels were each stained with Coomassie Brilliant Blue. Molecular weight markers are indicated on the left.
SUPPLEMENTAL DATA

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Figure S2. Quality and activity check of PN2-KPI. Factor XIA (1 nM; Haematologic Technologies, Inc.) was incubated at room temperature for 15 min with the indicated concentrations of reactive-site titrated PN2-KPI in 50 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.1 % BSA. The chromogenic substrate S-2366 (Chromogenix) was added to 1 mM and the initial rate of amidolytic activity was recorded at 405 nm. Reaction rates obtained (mOD/min) were converted to percentage activity and plotted as a function of inhibitor concentration. The data were fit with an equation describing tight-binding inhibition where $IC_{50} = K_i (1+S/K_m) + 0.5[E]$ (34) to yield a $K_{ieq}$ of roughly 400 pM using a value of $K_m$=0.4 mM.
SUPPLEMENTAL DATA
HEPARIN MODULATES THE 99-LOOP OF FACTOR IXA: EFFECTS ON REACTIVITY WITH ISOLATED KUNITZ-TYPE INHIBITOR DOMAINS

Figure S3. Quality and activity check of TFPI-K1 and TFPI-K2. A. Inhibition of the complex of factor VIIa and soluble tissue factor. The indicated concentrations of TFPI-K1 (●) or TFPI-K2 (○) were incubated with 5 nM factor VIIa (Haematologic Technologies, Inc.), 100 nM recombinant soluble tissue factor (16) and 5 mM Ca$^{2+}$ in 20 mM Hepes-NaOH pH 7.4, 100 mM NaCl and 0.1% BSA for 15 minutes at room temperature as previously described (24). The chromogenic substrate Chromozym-tPA (Roche Diagnostics) was then added to 1 mM and the initial rate of amidolytic activity was monitored at 405 nm. Reaction rates obtained (mOD/min) were converted to percentage activity and plotted as a function of inhibitor concentration. Fits of the data with Equation 1 yielded $K_{\text{eq}}$ values of 400 nM for TFPI-K1 and ~7 μM for TFPI-K2. B. Inhibition of factor Xa. The indicated concentrations of TFPI-K1 (●) or TFPI-K2 (○) were incubated with 5 nM factor Xa and 5 mM Ca$^{2+}$ in 20 mM Hepes-NaOH pH 7.4, 100 mM NaCl and 0.1% BSA for 15 minutes at room temperature. The chromogenic substrate S-2222 (Chromogenix) was then added to 0.5 mM and the initial rate of amidolytic activity was monitored at 405 nm. Reaction rates (mOD/min) were converted to percentage activity and plotted as a function of inhibitor concentration. Fits of the data with Equation 1 yielded $K_{\text{eq}}$ values of >1.5 μM for TFPI-K1 and 24 nM for TFPI-K2.