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.

Factor IXa (fIXa) is a vitamin K-dependent blood coagulation factor that is essential for the amplification or “consolidation” phase of blood coagulation (1, 2). As with other blood coagulation factors (namely factors VIIa, Xa, and thrombin) fIXa is a member of the serine protease family and shares a high degree of homology with trypsin. Despite this homology, the blood coagulation enzymes differ drastically from trypsin in that their activities are profoundly modulated by the binding of various protein and non-protein cofactors. In the case of fIXa, the ability of activated factor VIII (fVIIIa), anionic phospholipid, and ionic calcium to enhance the procoagulant activity of fIXa is well documented (3–6); resulting in a 109-fold increase in activity of fIXa. The molecular details of this conversion have not been defined in total and are the subject of intense investigation by numerous groups.

The major inhibitor of fIXa in plasma is antithrombin, whose reactivity with fIXa essentially requires heparin (7–9). Heparin is known to bind to antithrombin and sterically alter its conformation to allow this serpin to react with its target (10–13). Heparin also binds to fIXa (14) allowing long chains of heparin to additionally catalyze the interaction of fIXa with antithrombin via the formation of bridged complexes where heparin acts as a “template.” Recently, we have shown that long molecular weight heparin binding to fIXa enhances reactivity of fIXa with the Kunitz-type inhibitor BPTI (15), suggesting that oligosaccharide binding could also allosterically modulate the fIXa active site region. In this study we examine in greater detail the ability of heparin to modulate fIXa reactivity toward several isolated Kunitz-type inhibitor domains. We show that the modulatory effect of heparin can be completely abrogated by mutating a single amino acid residue in the 99-loop region of the extended fIXa active site cleft outside of the heparin binding exosite.

EXPERIMENTAL PROCEDURES

Materials—Factor IXaβ, factor VIIa, factor Xla, 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 (CH3SO2O-LGR-pNA) was purchased from Diagnostica Stago (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-

Mono-Q HR 5/5 (Amersham Biosciences) in 20 mM MES, pH 6.0. In both cases the column was developed with a 0–0.5M NaCl gradient. PN2-KPI eluted at roughly 90 mM NaCl and eluting the inhibitor with 10 mM HCl, pH 2.1, 100 mM NaCl. The pH of the eluted fractions was immediately neutralized with 0.02 volumes of 2 M Tris buffer. All inhibitors were judged >95% pure by SDS-PAGE.

Recombinant human wild-type fIX and fIXK98A were isolated from 293 cell supernatants using a combination of ion-exchange and heparin affinity chromatography. A 10-fold concentration of cell supernatant was diluted 2-fold with deionized water to reduce the ionic strength before loading a 150-ml DEAE FF Sepharose (Amersham Biosciences) column equilibrated in 25 mM sodium citrate, pH 6.0, 33 mM NaCl, and 1 mM benzamidine. After loading, the column was extensively washed in the same buffer before elution of the fIX protein with a 0.033–0.4 M NaCl gradient over 10 column volumes. The fIX protein peak was identified by clotting activity, pooled, and dialyzed versus 50 mM Tris-HCl, pH 7.5, 100 mM NaCl before heparin affinity chromatography using either POROS® HE2 (Applied Biosystems) or HiPrep™ Heparin FF 16/10 (Amersham Biosciences) and eluting with a NaCl gradient. Wild-type fIX and fIXK98A both eluted as single peaks at roughly 0.46 M NaCl.

Wild-type and mutant fIX proteins were activated with the purified factor X activator from the venom of Russell’s viper, which also cleaves fIX after Arg<sup>180</sup> to generate active enzyme (fIXα). 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 fIXα, this enzyme retains 100% amidolytic activity compared with fIXαβ (activation peptide proteolytically removed) and is comparable with fIXαβ 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 fIX proteins were assayed by a standardized 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 fIXa Enzymes—Active concentrations of wild-type and mutant fIXa preparations were determined by active site titration using biotin-EGR-ck (Hematologic Technologies Inc.) essentially as described (31). Briefly, wild-type or mutant fIXa (roughly 5 μM) were incubated with 150 μM biotin-EGR-ck in 50 mM Tricine, pH 8.0, 200 mM NaCl, 10 mM CaCl<sub>2</sub>, Refolded TFPI-K1 was loaded onto the prepared trypsin-agarose column in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. The column was extensively washed with the same buffer before eluting the inhibitor with 10 mM HCl, pH 2.1, 100 mM NaCl. The pH of the eluted fractions was immediately neutralized with 0.02 volumes of 2 M Tris buffer. All inhibitors were judged >95% pure by SDS-PAGE.

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 coding sequence was removed into the mammalian expression vector pcDNA3 (Invitrogen) and sequenced to verify the correct orientation. This construct (pFN04) was used for construction of wild-type fIX as well as PCR-based mutagenesis (28) to generate fIXK98A essentially as previously described for constructing fVII mutants (29). Expression constructs were transfected into human 293 cells using Lipofectin® (Invitrogen) and high expressing clones isolated by limiting dilution.

Protein Purifications—Purification of refolded BPTI was accomplished by ion-exchange chromatography using Mono-S HR 5/5 (Amersham Biosciences) in 20 mM Tris-HCl, pH 8.0. The column was developed with a 0–1 M NaCl gradient and BPTI eluted as a single peak at roughly 0.43 M NaCl. The specific activity of recombinant BPTI preparations was equivalent to or better than that of commercial preparations of aprotinin (not shown). Purification of refolded PN2-KPI and TFPI-K2 was accomplished by ion-exchange chromatography using Mono-Q HR 5/5 (Amersham Biosciences) in 20 mM MES, pH 6.0. In both cases the column was developed with a 0–0.5 M NaCl gradient. PN2-KPI eluted at roughly 90 mM NaCl and TFPI-K2 eluted at roughly 50 mM NaCl. Purification of refolded TFPI-K1 was accomplished by affinity chromatography over a trypsin-agarose column. Trypsin-agarose was prepared by coupling 20 mg of L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals) to 2.5 ml of Affi-Gel 10 in 100 mM MOPS, pH 7.4, 10 mM CaCl<sub>2</sub>, and 100 μg/ml leupeptin overnight at 4 °C. This was followed by blocking non-reacted sites with 1 M ethanolamine-HCl, pH 8.0.
Modulation of the fIXa 99-Loop by Heparin

Scheme Ia

\[
E + S \overset{k_i}{\underset{k_s}{\rightleftharpoons}} ES \overset{k_1}{\rightarrow} E + P + I
\]

\[
E + S \overset{k_i}{\underset{k_s}{\rightleftharpoons}} ES \overset{k_1}{\rightarrow} E + P + I
\]

Scheme Ib

\[
E \overset{k_i}{\text{I}} \rightarrow E + I^*
\]

\[
E \overset{k_i}{\text{I}} \rightarrow E + I^*
\]

FIGURE 1. Reversible slow-binding enzyme inhibition schemes. Schemes Ia and Ib both describe the reversible slow-binding inhibition of an enzyme (E) by an inhibitor (I) resulting in competitive inhibition of substrate (S) binding and the subsequent reduction in product (P) formation (33, 34). Both schemes show the formation of the Michaelis complex (ES) as described classically by \(k_i\) and \(k_s\). In Scheme Ia inhibition of enzyme occurs in two distinct steps: 1) formation of a loose E-I inhibitory complex described by \(k_i\) and \(k_s\), where \(k_i/k_s = K_i\); 2) isomerization of E to E-I* described by \(k_i\) and \(k_s\). In Scheme Ib the binding of inhibitor and isomerization of the complex are simultaneous and not distinguishable. In this case \(k_i\) and \(k_s\) represent the overall on- and off-rate constants. This situation can arise when \(k_i\) and \(k_s\) are very slow (rate-limiting) compared with \(k_i\) and \(k_s\). In both schemes the overall inhibition is described by \(K_i^*\), which is the final inhibition constant observed at equilibrium of the system.

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 compared with a standard line made using native fIXa and alkaline phosphatase-conjugated streptavidin. These were fitted with the following integrated rate equation describing slow-binding inhibition,

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

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 E-I* isomerization) were determined from progress curves above using the following relationship.

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

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

\[
k_{obs} = k_0 + k_{10} / (1 + K_i^* + S/K_m)
\]

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 \(k_{obs} \text{ versus } I\) yields a straight line, indicating conditions where \(K_i^* + S/K_m \gg 1\). Thus for Scheme Ib EI formation is insignificant and E-I* can be considered formed directly from E + I. For these cases the following linear equation is applicable for obtaining an estimate of \(k_{obs}\), the apparent second-order on-rate constant,

\[
k_{obs} = k_{10} + k_{11} / (1 + S/K_m)
\]

where the \(y\) intercept reflects \(k_{11}\) and the slope of the line is equal to \(k_{10} / (1 + S/K_m)\). Alternatively, \(k_0\) can be obtained from \(K_i^*\), which is equivalent to \(K_i^*\) in Equation 1, using the relationship \(k_0 = k_{10}/K_i^*\).
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 nM (see supplemental data). Similarly, preliminary studies indicated that preparations of isolated TFPI-K1 domain inhibited the complex of factor Vila 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 Vila-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 structure (Discovery Studio v 1.1; Accelrys Inc.) suggested a constriction of the fIXa active site in a manner that may be expected not to readily accommodate BPTI. The main site of steric hindrance seemed to be with the 99-loop of fIXa, specifically Lys<sup>98</sup> (Fig. 4). We hypothesized that heparin binding exosite residues in fIXa (14) are depicted as violet sticks; and the fIXa active site triad residues (Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup>) are depicted as red sticks.

Based on this hypothesis, we examined a mutant of fIXa in which Lys<sup>98</sup> was mutated to Ala (fIXK98A). Wild-type and mutant forms of fIXa were expressed in human 293 cells and purified to >95% homogeneity as judged by SDS-PAGE (Fig. 5A). The fIXK98A mutant was found to retain 100% clotting activity compared with wild-type fIXa (Fig. 5B) and upon activation retained near normal amidolytic activity toward CBS 31.39 substrate (Table 1).

![Hypothetical model of the inhibited fIXa-BPTI complex](Image)

**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-BPTI<sub>11,15</sub> (PDB code 1FAK) using molecular replacement. The structure revealed potential steric hindrance between BPTI and the 99-loop of fIXa, specifically Lys<sup>98</sup> (central ball and stick structure with van der Waals radii). fIXa is depicted in orange; fIX 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 (Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup>) are depicted as red sticks.

![SDS-PAGE analysis of recombinant WT fIX and fIXK98A](Image)

**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 (○) and fIXK98A (●), which showed equivalent coagulant activity.

**TABLE 1**

| Enzyme       | K<sub>m</sub>  | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|--------------|---------------|-----------------|-----------------------------|
| WT fIXa      | 3.78 (±0.08)  | 16.5 (±0.69)   | 4.37 (±0.08) × 10<sup>3</sup> |
| fIXK98A      | 1.58 (±0.02)  | 6.23 (±0.43)   | 3.98 (±0.27) × 10<sup>3</sup> |

 Obtained by fitting the data with the Michaelis-Menton equation. Values shown are ± S.E., n = 6.

As an independent verification of k<sub>e</sub> an experiment was performed where 5 μM fIXa was incubated with 231 μM BPTI in the presence of enoxaparin for 20 min, followed by a rapid 1,000-fold dilution into the same buffer also containing enoxaparin. Timed aliquots of this diluted mixture were removed and added to CBs 31.39. From fits of the progress curves in Fig. 6, A and B, with Equation 2, secondary plots of k<sub>obs</sub> versus BPTI for WT fIXa show little or no curvature (Fig. 6C). This suggests a very high initial K<sub>e</sub> value and an indeterminate value for k<sub>e</sub>. These observations as well as the lack of change in v<sub>e</sub> with increasing inhibitor
concentrations (cf. Fig. 6, A and B) are diagnostic for Scheme lb. 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 lb) 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 $k_{obs}$ versus PN2-KPI were hyperbolic (not shown) and well described by Scheme Ia, yielding the values of $K_i$ and $k_5$ given in Table 2. The role of Lys$^{98}$ 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.
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TABLE 2
Kinetic constants for inhibition of fIXa and fIXaK98A by BPTI and PN2-KPI

| Enzyme       | Inhibitor | Exonaparin | $K_i$  | $k_a$  | $k_a$ or $k_{10}^a$ | $K_i^{* \text{calc}}^b$ |
|--------------|-----------|------------|--------|--------|-------------------|------------------------|
| WT fIXa      | BPTI      | –          | $2.1 \times 10^{-3}$ | 5.4    | $2.67 (\pm 1.50) \times 10^{-3}$ |                       |
|              |           | +          | $4.1 \times 10^{-3}$ | 23     | $1.17 (\pm 0.17) \times 10^{-3}$ |                       |
|              | PN2-KPI   | –          | $2.0 \times 10^{-2}$ | $1.3 \times 10^{-2}$ | $3.67 (\pm 2.66) \times 10^{-4}$ | $4.4 \times 10^{-6}$ |
|              |           | +          | $3.3 \times 10^{-2}$ | $1.3 \times 10^{-2}$ | $3.50 (\pm 0.67) \times 10^{-3}$ | $7.0 \times 10^{-6}$ |
| fIXaK98A     | BPTI      | –          | $1.3 \times 10^{-3}$ | $1.5 \times 10^{-3}$ | $2.33 (\pm 0.67) \times 10^{-3}$ | $1.7 \times 10^{-5}$ |
|              |           | +          | $8.8 \times 10^{-4}$ | $1.6 \times 10^{-3}$ | $1.65 (\pm 0.33) \times 10^{-3}$ | $8.2 \times 10^{-6}$ |
|              | PN2-KPI   | –          | $6.8 \times 10^{-6}$ | $1.4 \times 10^{-2}$ | $2.33 (\pm 1.00) \times 10^{-3}$ | $9.7 \times 10^{-7}$ |
|              |           | +          | $8.0 \times 10^{-6}$ | $1.4 \times 10^{-2}$ | $2.00 (\pm 0.83) \times 10^{-3}$ | $1.0 \times 10^{-6}$ |

$^a$ Values are $k_{10}$ for WT fIXa with BPTI (Scheme Ib) and $k_a$ for others (Scheme Ia) $\pm$ S.D. ($n = 7$).

$^b$ Calculated from $k_{10}$, $k_a$, and $K_i^*$ using the relationship $K_i^{*\text{calc}} = k_aK_i/(k_{10} + K_i)$ (33, 34).

$^c$ Determined from refits of the data in Fig. 6C with Equation 4 using $k_a$ obtained for fIXaK98A and BPTI (see “Discussion”).

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 1EOO and 1BFC) the length of a decasaccharide is expected to be $\sim 38$ Å and that of a hexasaccharide is expected to be $\sim 23$ Å at full extension. This is compared with the measured length of the identified heparin binding site on fIXa ($\sim 35$ 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 $\mu M$ BPTI yielded results consistent with the high nanomolar affinity for enoxaparin previously observed with fIXa$\beta$ (15). No further increase in inhibition of fIXaK98A by BPTI was observed when the enoxaparin level was increased from 1 to 10 $\mu M$ (not shown). Because all experiments were conducted using 10 $\mu M$ enoxaparin, it seems reasonable to assume that fIXaK98A was saturated in these experiments.

Whereas the data for fIXa with BPTI in Fig. 6C are consistent with Scheme Ib, this scheme is essentially a simplified version of Scheme Ia with a very large value for $K_i^*$ (33, 34). Unfortunately, the $K_i^*$ values for fIXa with both inhibitors shows fairly consistent values for $k_a$ and $k_{10}$. This suggests that once formed, isomerization of the $E_1$ complex to $E_{1t}$ is essentially the same for any of the enzyme-inhibitor pairs examined. Based on this it seems reasonable to tentatively extend the value of $k_a$ to the WT fIXa-BPTI pair and re-examine the data of Fig. 6C with respect to Scheme Ia. This results in fits to Equation 4 shown in Fig. 6C as dashed lines and yields initial $K_i^*$ values of $2.1 \times 10^{-3}$ $M$ and $4.1 \times 10^{-4}$ $M$ in the absence and presence of enoxaparin, respectively. This 5-fold difference in $K_i^*$ along with $\sim 2$-fold difference in $k_{10}$ for this enzyme-inhibitor pair (Table 2) would seem to account for the $\sim 10$-fold effect of enoxaparin observed in $K_i^*$ (Table 3). Unfortunately, the rather large errors in the values of $k_a$ ($k_{10}$) 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
the interaction of fIXa with TFPI-K1, TFPI-K2, or PN2-KPI. The former shows no effect of heparin binding, whereas the latter two show only small (but reproducible) responses to heparin binding. These results would suggest that these three Kunitz inhibitor domains are not as greatly hindered as BPTI by the 99-loop of fIXa. Indeed, mutation of Lys98 enhances the inhibition by TFPI-K1 roughly 8-fold, TFPI-K2 roughly 9-fold, and by PN2-KPI only 6–7-fold. Interestingly, however, the effect of enoxaparin on fIXaK98A reactivity toward both TFPI-K1 and TFPI-K2 was unchanged, whereas that for PN2-KPI was completely abrogated. Thus, there is some disparity in the role of Lys98 and additional 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 fVIIIa and does not preclude potential effects of fVIIIa toward other inhibitors. It thus remains possible that occupation of this exosite by fVIIIa, like heparin, results in inhibitor-specific modulatory effects. Nonetheless, 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 fVIIIa interactive site on fIXa. Thus, whereas heparin binding in itself may inhibit fIXa coagulant activity via steric hindrance of fVIIIa binding, fVIIIa binding to this exosite may inversely act to allosterically modulate fIXa in an as yet undefined manner. Although previous studies found no effect of fVIIIa on fIXa inhibition by the isolated PN2-KPI domain (44), this is consistent with our observations here using enoxaparin in place of fVIIIa and does not preclude potential effects of fVIIIa toward other inhibitors. It thus remains possible that occupation of this exosite by fVIIIa, 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 fVIIIa 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 fVIIIa (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.

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