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Exosite Occupation by Heparin Enhances the Reactivity of Blood Coagulation Factor IXa

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ABSTRACT: Blood coagulation factor IXa (fIXa) is a trypsin-like serine protease with low inherent activity that is greatly enhanced in the factor X activation complex. Molecular details of the conversion of fIXa from an inactive enzyme into a fully functional procoagulant are unclear. Recent studies have identified a heparin-binding exosite in the protease domain of fIXa. Effects of exosite occupation on fIXa activity are unclear. We used the Kunitz-type inhibitor bovine pancreatic trypsin inhibitor (BPTI) to probe fIXa reactivity in the absence and in the presence of heparin. While fIXa alone was poorly reactive with BPTI ($K_i \approx 0.7$ mM), this reactivity was increased roughly 20-fold ($K_i = 37 \pm 6$ μ M) by heparin. This was reproducible with low-molecular-weight heparin (enoxaparin; $K_i = 70 \pm 12$ μ M). Surface plasmon resonance studies of the interaction between heparin and BPTI indicated an unstable interaction with very low affinity ($K_d = 172$ μ M). In contrast, kinetic studies revealed a high-affinity interaction between heparin and fIXa ($K_d = 128 \pm 26$ nM) and showed that the enhancement of BPTI inhibition of fIXa by heparin was well described by a competitive inhibition model where heparin acts as an affecter of fIXa reactivity with inhibitor. Fluorescence studies with dansyl-EGR-fIXa supported the high-affinity interaction between heparin and fIXa and suggested an altered environment in the fIXa active-site region upon heparin binding. This modulating effect of heparin was supported by the observation of a heparin-induced increase in reactivity of fIXa toward a pentapeptide substrate. When viewed together, the results imply that specific physiological exosite interactions with heparin can induce alterations in the environment of the extended fIXa active site that can result in increased reactivity.

Activated factor IX (fIXa)¹ is a vitamin K-dependent blood coagulation serine protease that is a key component of a procoagulant complex that activates factor X. Deficiency of fIXa results in the bleeding diathesis associated with hemophilia B (for reviews, see refs 1–3). Numerous studies on the kinetics of fIXa enzymatic activity have been reported for both human and bovine systems (4–7). These studies have shown that maximal procoagulant activity of fIXa requires the formation of a Ca^{2+} -dependent, phospholipid-associated enzyme–cofactor complex with factor VIIIa (a nonenzymatic protein cofactor). Detailed studies of fIXa enzymology and structure/function studies of the effects of substrate, factor VIIIa, phospholipids, and metal ions on fIXa activity are potentially confounded by the numerous protein–protein and protein–phospholipid interactions that are involved in factor X activation by the fIXa–factor VIIIa complex. Thus, a clear understanding of the detailed molecular mechanisms involved in the conversion of fIXa from an essentially inactive enzyme into a fully functional procoagulant is lacking.

The high level of enzymatic activity exhibited by the fIXa–fVIIIa–phospholipid complex seems to be observed only toward native macromolecular substrates (i.e., factor X) and not toward small peptidyl substrates (8–13). However, fIXa alone does show limited amidolytic activity that can be enhanced 30-fold in the presence of ethylene glycol and other hygroscopic compounds (14, 15). This activity has been exploited in several studies involving fIXa and allows the study of fIXa activity and regulation in greater detail than has been possible previously (16, 17).

The major physiologic inhibitor of fIXa in plasma is the serpin antithrombin, whose reactivity with fIXa is dramatically enhanced by heparin (18–20). This enhancement in reactivity has been attributed to a combination of the effect of heparin on the conformation of antithrombin (21–24), as well as on the ability of heparin to act as a template in facilitating the association of antithrombin with certain target proteases (24, 25). The heparin binding exosite has recently been identified in the protease domain of fIXa (26). Interestingly, this region is in close proximity to a proposed factor X (substrate)-binding region (27). Potential modulations of fIXa activity that may occur upon occupation of this exosite by either heparin or factor X are unclear.

While in general the low reactivity of fIXa toward small substrates is reproduced with inhibitors, fIXa has been reported to be inhibited quite efficiently by protease nexin-2 (28, 29), which contains a Kunitz-type inhibitor domain. The

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¹ Abbreviations: fIXa, factor IXa; dEGR-fIXa, active-site dansyl-labeled fIXa; BPTI, bovine pancreatic trypsin inhibitor (aprotinin); SPR, surface plasmon resonance; BSA, bovine serum albumin.

related plasma Kunitz-type inhibitor TFPI can also inhibit fIXa, though not as efficiently (30). These different reactivities are consistent with the variable degree of reactivity observed with Kunitz-type inhibitors for the different blood coagulation proteases (31–34). The limited interactions between these inhibitors and their target proteases makes them ideal candidates for screening effects on the active site of these proteases. In this study we have used bovine pancreatic trypsin inhibitor (BPTI) as a probe of the reactivity of fIXa in the absence and in the presence of heparin to examine potential modulations in fIXa activity upon occupation of the heparin-binding exosite. We report here an effect of heparin on the environment of the fIXa active-site region upon exosite occupation. This binding results in greater reactivity of fIXa and suggests a possible mechanism for regulating fIXa activity that may be partly involved in overcoming its low inherent activity.

EXPERIMENTAL PROCEDURES

Materials. Factor IXa β and dEGR-fIXa were purchased from Haematologic Technologies Inc. (Essex Junction, VT). BPTI (aprotinin) and unfractionated heparin (grade I-A from porcine intestinal mucosa) were purchased from Sigma. The molar concentration of heparin was determined from the specific activity of the preparation (180 U/mg) using the average molecular weight of the preparation as given by the manufacturer ($M_r = 18\,000$). The low-molecular-weight heparin enoxaparin (Lovenox; $M_r = 4500$) was from Aventis Pharmaceuticals (Bridgewater, NJ). Protamine sulfate was from American Pharmaceutical Partners, Inc. (Los Angeles, CA). 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 Diagnostica Stago (Parsippany, NJ), and the AT3.2 peptide (Ac-AGRSL-amide) was synthesized by New England Peptide, Inc. (Fitchburg, MA). All other reagents were of the highest quality available.

Enzyme Inhibition Assays. Reversible inhibition of fIXa amidolytic activity was measured at 25 °C using final concentrations of 25 nM fIXa, 5 mM CaCl₂, and the indicated concentrations of heparin and BPTI in HBSA buffer (20 mM Hepes–NaOH pH 7.4, 150 mM NaCl, 0.1% BSA) in the absence or in the presence of 30% ethylene glycol. In the procedure, small volumes (10–20 μ L) of heparin and BPTI were added at the appropriate concentration separately to opposite sides of an empty well of a 96-well microplate. A bolus mixture of fIXa and CaCl₂ in the presence or in the absence of ethylene glycol was then added to the well to instantaneously mix the fIXa, heparin, and BPTI. This mixture was allowed to incubate for 15 min at 25 °C before the addition of 0.1 vol CBS 31.39 substrate (1 mM final). The absorbance at 405 nm was then monitored for 30 min in a SpectramaxPLUS³⁸⁴ microplate reader set at 25 °C to determine initial rates of substrate hydrolysis. The small volume of substrate addition was intentional and necessary to minimize perturbation of the equilibrium formed between fIXa, heparin, and BPTI. In practice, no perturbation was observed based on the linear initial rates obtained. Values of initial rates were normalized to the activity obtained in the absence of BPTI (100%) to allow comparison of

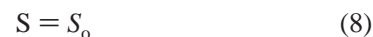
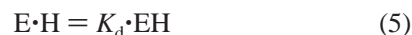
inhibition in the absence and in the presence of ethylene glycol, which produces a roughly 20-fold increase in amidolytic activity of fIXa toward this substrate.

Inhibition of fIXa activity (K_i) was determined, similar to previously described methods (32), from plots of activity versus BPTI concentration by fitting the data with eq 1, which describes a simple competitive inhibition model. In

$$v_s = v_o \frac{K_i(1 + S/K_m)}{1 + K_i(1 + S/K_m)} + C \quad (1)$$

this equation, v_o is the steady-state rate obtained in the absence of inhibitor, v_s is the steady-state rate at each concentration of inhibitor, S is the experimental substrate concentration, K_m is the Michaelis constant for substrate usage, and C is the asymptote (the activity remaining at saturating inhibitor; zero in the case of varying BPTI, but a finite number in the case of varying heparin at subsaturating levels of BPTI).

Kinetic Analysis of Heparin-Dependent fIXa Inhibition by BPTI. The effect of heparin (H) on the inhibition of fIXa (E) by BPTI (I) was analyzed according to the model depicted by Scheme 1. This model is similar to one described by Segel (35), but with heparin acting as an affecter of inhibition rather than a deinhibitor. We have made the simplifying assumption that BPTI does not inhibit fIXa in the absence of heparin. While this is not an absolute truth, the comparatively high K_i in the absence of heparin makes this a reasonable assumption and greatly simplifies the model. In this case, the associated equilibrium terms are as follows:



Heparin does not alter the kinetics of chromogenic substrate hydrolysis by fIXa (refer to Figure 1); thus, $K_S = K_S'$. Subsequently, $K_d = K_d'$ and $k_p = k_p'$, so the velocity equation for substrate hydrolysis can be written as

$$v = k_p \cdot ES + k_p \cdot EHS = k_p(ES + EHS) \quad (9)$$

with

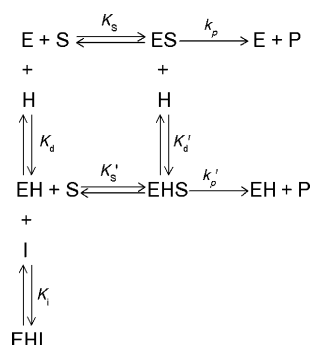
$$\frac{v}{E_o} = \frac{k_p(ES + EHS)}{E + ES + EH + EHS + EHI} \quad (10)$$

and

$$\frac{v}{V_m} = \frac{(ES + EHS)}{E + ES + EH + EHS + EHI} \quad (11)$$

Substituting the appropriate equilibrium terms for ES, EH,

Scheme 1



EHS, and EHI into eq 11 and rearranging yields

$$\frac{v}{V_m} = \frac{S}{K_s \left(1 + \frac{I}{K_i(1 + K_d/H)} \right) + S} \quad (12)$$

whose reciprocal is

$$\frac{1}{v} = \frac{K_s}{V_m S K_i (1 + K_d/H)} I + \frac{1}{V_m} \left(1 + \frac{K_s}{S} \right) \quad (13)$$

Thus, Dixon-like plots of $1/v$ versus I will yield a straight line with a slope of $\{K_s\}/\{V_m \cdot S \cdot K_i(1 + K_d/H)\}$ and a y-intercept of $(1/V_m)(1 + K_s/S)$. From eq 13, one can see that at infinite (saturating) H , the x-intercept will be $-K_i(1 + S/K_s)$, which can be used to determine the K_i if S and K_s are known.

By subsequently taking the reciprocal of the slope from eq 13 and rearranging, we obtain

$$1/\text{slope} = \frac{V_m \cdot K_i \cdot S \cdot K_d}{K_s} \frac{1}{H} + \frac{V_m \cdot K_i \cdot S}{K_s} \quad (14)$$

Based on eq 14, a secondary plot of $1/\text{slope}$ versus $1/H$ will yield a straight line with a slope of $\{(V_m \cdot K_i \cdot S \cdot K_d)\}/\{K_s\}$ and a y-intercept of $\{(V_m \cdot K_i \cdot S)\}/\{K_s\}$. Solving for the value of $1/H$ when $1/\text{slope} = 0$ reveals an x-intercept for this line of $-1/K_d$. Thus, extrapolation of the line described by eq 14 to the abscissa will yield a numeric value from which K_d can be determined. All linear and nonlinear regression procedures for analysis of inhibition data were performed using Slide-WritePlus6.0 (Advanced Graphics Software), which uses the Levenberg–Marquardt algorithm.

Fluorescence Intensity Measurements. Heparin-induced changes in the fluorescence emission intensity of dansyl-labeled fIXa (dEGR-fIXa) were measured using an SLM 8000 spectrofluorometer. Each reaction mixture contained 220 nM dEGR-fIXa in 20 mM Hepes–NaOH pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 0.2 mg/mL BSA, and 0.01% Tween 20 in a volume of 800 μ L. The sample was stirred continuously with a magnetic stirrer. The excitation and emission wavelengths were set to $\lambda_{\text{ex}} = 340$ nm and $\lambda_{\text{em}} = 540$ nm (8 nm slit widths). Initial intensity readings were taken of dEGR-fIXa alone (F_0), after which the sample was titrated with heparin. After each heparin addition, the sample was allowed to stir with slits closed for 10 min before the fluorescence intensity was recorded over a 10 s interval (F). For each experiment, a reference experiment was performed using buffer as the titrant (no heparin), and these intensities

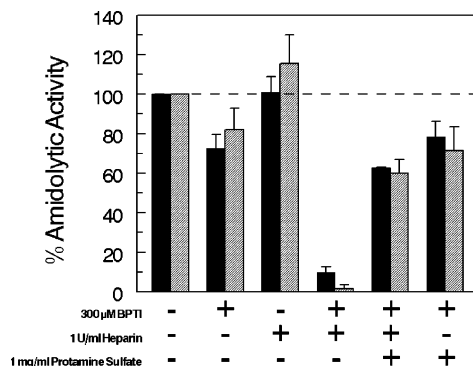


FIGURE 1: Heparin enhances fIXa inhibition by BPTI. The inhibition of fIXa amidolytic activity by 300 μ M BPTI \pm 1 U/mL (306 nM) porcine heparin was investigated using 25 nM fIXa, 5 mM CaCl₂, and 1 mM CBS 31.39 substrate as described in the Experimental Procedures. Activities were measured both in the absence (closed bars) and in the presence (hatched bars) of 30% ethylene glycol. In each case, the data were normalized to the activity obtained in the absence of inhibitor for comparison purposes. BPTI alone had little inhibitory capacity, while heparin alone showed no effect. The addition of both heparin and BPTI together resulted in roughly 90% inhibition of fIXa activity. Protamine sulfate (1 mg/mL) was able to reverse the effect of heparin but had no effect by itself over that obtained with BPTI alone. The data shown are the mean values of at least three determinations \pm the standard deviation.

were subtracted from those obtained with heparin to correct for dilution effects. Thus, the data are presented as the change in the corrected fluorescence intensity (F/F_0) at a given volume-corrected heparin concentration.

Surface Plasmon Resonance. Binding interactions between heparin and fIXa or BPTI were measured by surface plasmon resonance (SPR) using a Biacore 3000 biosensor instrument (Biacore Inc.). Experiments were all conducted at 25 $^{\circ}$ C in 20 mM Hepes–NaOH pH 7.4, 150 mM NaCl, 5 mM CaCl₂, and 0.005% polysorbate 20 at a flow rate of 10 μ L/min. All buffer solutions were thoroughly degassed and filtered through a 0.2 μ m membrane prior to use, and all protein dilutions were made in this buffer. Porcine heparin was biotinylated by the method of O'Shannessy (36), essentially as described by Badellino and Walsh (37). The incorporation of biotin into the heparin preparation was measured using an EZ Biotin Quantitation Kit (Pierce) and yielded a molar ratio of 0.6 mol/mol. Biotin-heparin was captured onto a streptavidin sensor surface (SA chip) to a level of 7.2 RU. For equilibrium experiments, additional biotin-heparin surfaces were prepared at 265 and 824 RU to increase signals. Binding of BPTI to the prepared surfaces was measured in real time, and the surfaces were regenerated between sample injections by a 1 min injection of 5 M NaCl to remove the bound protein and regenerate the heparin surface. For each injection, the sample was passed over a reference flow cell (no heparin) followed by the heparin flow cell in series, and real-time subtraction of any nonspecific interactions was performed. The data shown indicate response differences between the reference and heparin flow cells.

The K_d value for BPTI binding to heparin was determined by equilibrium SPR experiments from plots of R_{eq} (response difference at equilibrium) versus the injected concentration of BPTI over three different heparin surface densities (see above). The data were fit to a standard hyperbolic binding isotherm by global fitting using Scientist for Windows

version 2.01 (MicroMath, Inc., Salt Lake City, UT), which uses a modified Powell algorithm (Powell's variant of the Levenberg–Marquardt algorithm) for nonlinear least-squares fitting.

Peptide Hydrolysis Assay. The peptidolytic activity of fIXa was measured at 25 °C using 1 mM pentapeptide substrate AT3.2 and 25 nM fIXa in 20 mM Hepes–NaOH pH 7.4, 150 mM NaCl, and 5 mM CaCl₂. Reactions were performed in the absence or in the presence of 309 nM (1 U/mL) heparin, 30% ethylene glycol, or both. For each reaction, aliquots were removed from the incubation at the indicated times into 0.1% TFA/H₂O and analyzed by HPLC over an Absorbosphere C₁₈ column using a 0–50% acetonitrile gradient to separate unhydrolyzed peptide substrate from the two hydrolyzed products (Ac-AGR-COOH and NH₂-SL-amide). The two product peaks generated as well as the remaining substrate peak were identified by absorbance at 215 nm by comparison to the retention times obtained in trial runs performed with trypsin. The substrate and product peaks at each time point were quantified by peak integration using the supplied HPLC software. These peak areas were normalized to either the initial substrate peak area obtained at time zero (for determining substrate remaining) or the final combined product peak areas obtained after complete hydrolysis with trypsin (for determining product generated). Time courses in the presence of ethylene glycol resulted in hydrolysis of greater than 10% of the substrate over the course of the assay. Thus, to account for potential substrate depletion effects in these cases, initial rates were estimated by fitting the data with the second-order polynomial equation $p = a + bt + ct^2$, where p is product (or substrate) at any given time, a is the y-intercept, b is the initial rate, and c is the quadratic constant describing the rate of change of the slope with time, t .

RESULTS

Heparin-Dependent Inhibition of fIXa by BPTI. Factor IXa has been found to be reactive with Kunitz-type inhibitors to varying degrees (29, 30, 38). In an initial examination of the inhibition of fIXa by the Kunitz-type inhibitor BPTI, we found that fIXa was not inhibited well by this inhibitor (Figure 1). Previous studies by us and others (14, 15) have shown that hygroscopic reagents such as ethylene glycol can enhance the amidolytic activity of fIXa. Thus, we examined the potential for ethylene glycol to enhance the reactivity of fIXa with BPTI. No enhancement of reactivity with BPTI was observed. Since fIXa is known to contain a heparin-binding exosite, we also examined the potential for heparin to alter the reactivity of fIXa. Although no significant effect of heparin was observed on fIXa amidolytic activity, we found a dramatic effect on the reactivity of fIXa with BPTI. In the presence of 1 U/mL heparin, fIXa amidolytic activity was reduced to less than 10% of its initial activity by 300 μ M BPTI. Protamine sulfate, which binds to and inactivates heparin, was able to reverse this inhibitory effect to that observed for BPTI alone. More detailed kinetic analysis of the inhibition of fIXa by BPTI in the absence and in the presence of 1 U/mL (306 nM) heparin revealed that the K_i for inhibition was reduced roughly 20-fold from ~ 0.7 mM in the absence of heparin to 37 ± 6 μ M in the presence of heparin (Figure 2). The inhibition profiles were unchanged

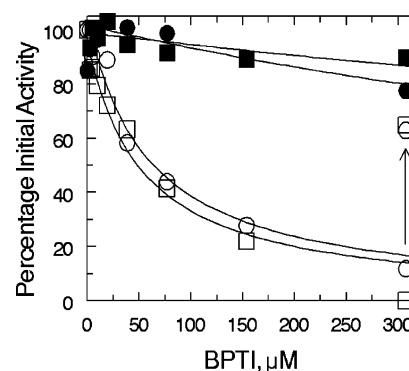


FIGURE 2: Kinetic analysis of BPTI inhibition of fIXa. The BPTI dependence of inhibition of fIXa (25 nM) was examined by varying the concentration of BPTI (indicated) in the absence (closed symbols) and in the presence (open symbols) of 1 U/mL (306 nM) heparin, and in the absence (squares) and in the presence (circles) of 30% ethylene glycol. The arrow indicates the effect of the addition of protamine sulfate (1 mg/mL) during the incubation step before substrate addition. The data ($n = 3$) were fit with eq 1 to determine values of $K_i \approx 0.7$ mM for BPTI in the absence of heparin and $K_i = 37 \pm 6$ μ M for BPTI in the presence of heparin.

in the presence of ethylene glycol. In each case, protamine sulfate was able to reverse the effect of heparin.

Heparin action in supporting inhibition of proteases often involves a “template”-like effect (24, 25) due to its large size and multiple sites of interaction. In contrast, low-molecular-weight heparins smaller than $M_r = 5000$ have been shown not to support these template-like effects and have been used very successfully to dissect the inhibition of several blood coagulation proteases with antithrombin (39). Thus, we examined the effect of the low-molecular-weight heparin enoxaparin (Lovenox; $M_r = 4500$) in inhibition assays to test the role of template-like effects in fIXa inhibition by BPTI. Titration of fIXa with BPTI in the presence of 1 μ M enoxaparin (Figure 3A) showed inhibition of fIXa similar to that observed in the presence of porcine heparin; $K_i = 70 \pm 12$ μ M. Titration of fIXa with enoxaparin at constant BPTI (Figure 3B) indicated saturation of this effect by 300 nM ($K_{0.5} = 42 \pm 8$ nM).

Analysis of the BPTI–Heparin Interaction. Although the studies with enoxaparin did not support a template-like mechanism, in pilot studies we found that BPTI could bind weakly to a HiTrap Heparin-Sepharose column (data not shown). To further characterize this interaction and gain further insight into this potential mechanism of BPTI inhibition of fIXa, we examined the ability of BPTI to bind to heparin using surface plasmon resonance. Porcine heparin was biotinylated and captured on a streptavidin (SA) biosensor chip, and various concentrations of BPTI were passed over the prepared surface. Visual examination of the kinetics of binding of BPTI to heparin revealed that this binding interaction is characterized by extremely rapid on-and-off rates (Figure 4A), indicating an unstable interaction. Since these rapid rates precluded the accurate determination of on- and off-rate constants, the affinity of BPTI binding to heparin was determined by a series of SPR equilibrium binding experiments. Response differences were obtained at equilibrium (R_{eq} , the plateau phase of each sensorgram) for various concentrations of BPTI on three different heparin surfaces. These R_{eq} values were plotted versus BPTI concentration to yield the equilibrium binding isotherms shown

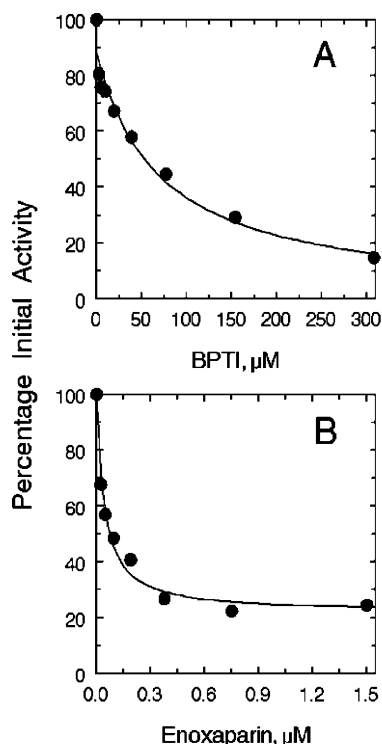


FIGURE 3: Low-molecular-weight heparin supports inhibition of fIXa by BPTI. (A) The inhibition of fIXa (25 nM) by BPTI was examined as described for Figure 2 in the presence of 1 μM enoxaparin (Lovenox) in place of full-length heparin. Fitting the data with eq 1 yielded a value of $K_i = 70 \pm 12 \mu\text{M}$. (B) The dependence of fIXa inhibition on enoxaparin was examined by varying the concentration of enoxaparin (indicated) using 300 μM BPTI. The inhibition is incomplete at the level of BPTI used here (see panel A). Fitting the data with eq 1, allowing a nonzero value of C (see the Experimental Procedures), yielded $K_{0.5} = 42 \pm 8 \text{ nM}$, which largely reflects the binding of enoxaparin to fIXa under the conditions used.

in Figure 4B. Global analysis of the data with a rectangular hyperbolic binding isotherm yielded a K_d of 172 μM and a stoichiometry of ~ 18 –20 BPTI molecules per heparin chain. This was reproducible with enoxaparin ($K_d = 177 \mu\text{M}$) but with a lower stoichiometry of 3:1 (not shown).

Kinetic Analysis of the Heparin–fIXa–BPTI Interaction. The interaction of heparin with fIXa and the potential kinetic mechanism of the heparin-dependent fIXa inhibition by BPTI were investigated by activity assays using the kinetic model described by Scheme 1. Inhibition experiments were performed at a limiting concentration of fIXa (25 nM) and different concentrations of enoxaparin with varying BPTI levels. The data were plotted in Dixon-like fashion according to Scheme 1 and eq 13 (Figure 5A). Increasing levels of enoxaparin resulted in increasing slopes, supporting increased reactivity with inhibitor as expected. The slope reached a maximal value at roughly 1 μM enoxaparin, consistent with the saturation level observed in Figure 3B. At this saturating amount of enoxaparin, the x -intercept yielded a value for K_i of roughly 52 μM , which is in excellent agreement with the K_i obtained in Figures 2 and 3A using eq 1. The slopes of the lines obtained in Figure 5A were subsequently used in a secondary double reciprocal plot of $1/\text{slope}$ versus $1/\text{enoxaparin}$, as described by eq 14 (Figure 5B). As predicted by the model, the relationship was linear, with the extrapolated x -intercept yielding a value of $K_d = 128 \pm 26 \text{ nM}$ for the

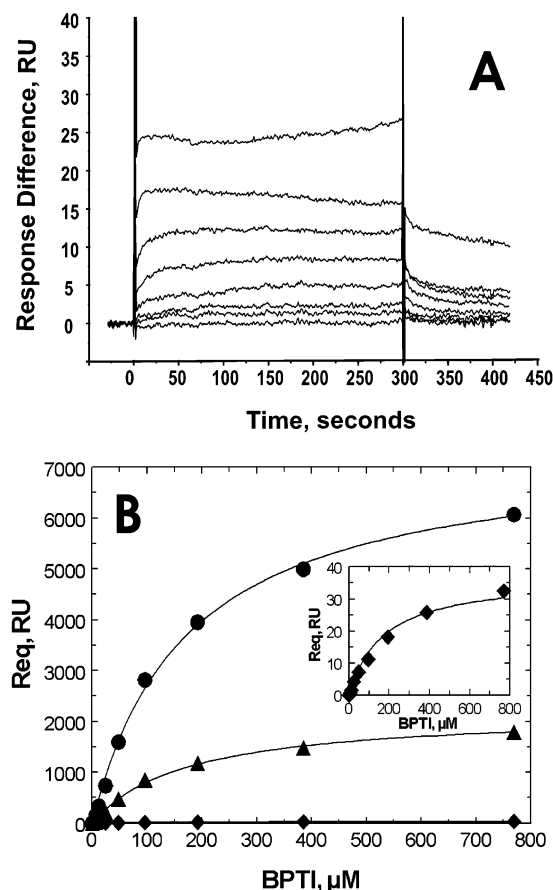


FIGURE 4: SPR studies of the BPTI–heparin interaction. The ability of heparin to bind BPTI was examined by surface plasmon resonance. (A) Increasing concentrations of BPTI (3, 6, 12, 25, 49, 99, 198, and 395 μM , bottom to top) were injected over a low-density heparin surface at a flow rate of 10 $\mu\text{L}/\text{min}$, and the binding to the surface was monitored in real time. The data have been corrected by subtracting out a zero BPTI control injection (buffer only) as well as any nonspecific binding (reference cell) to show the response difference between the experimental and reference surfaces. BPTI binding was characterized by fast kinetics and reached equilibrium (R_{eq}) within 30–60 s. Off-rates were also characterized largely by fast kinetics and showed signs of rebinding at high BPTI concentrations. (B) Equilibrium binding isotherms were generated using R_{eq} values obtained at 200 s (see panel A) for the indicated BPTI concentrations injected over three independent flow cells of different heparin densities (7.2 RU, diamonds; 265 RU, triangles; 824 RU, circles). Global fits of the data with a standard hyperbolic binding model (shown) yielded a value of $K_d = 172 \mu\text{M}$ and a stoichiometry of roughly 18 BPTI molecules per heparin chain. The inset shows the data obtained with the lowest density heparin surface in expanded form for clarity.

heparin–fIXa interaction, which was supported by SPR analysis (not shown).

The interaction of heparin with fIXa, and the potential for heparin to modulate the fIXa active-site region, was further examined by monitoring the fluorescence emission intensity of active-site-inhibited dansyl-labeled fIXa (dEGR-fIXa) as it was titrated with heparin. The results (Figure 6) clearly show that heparin binding to dEGR-fIXa increases the fluorescence emission intensity of the dansyl moiety of the inhibitor in the active-site cleft of fIXa. This alteration in fluorescence emission was not simply due to effects of heparin on the dansyl moiety of the inhibitor, since titration of dEGR-ck with equivalent levels of heparin produced no change in fluorescence emission intensity. The high levels

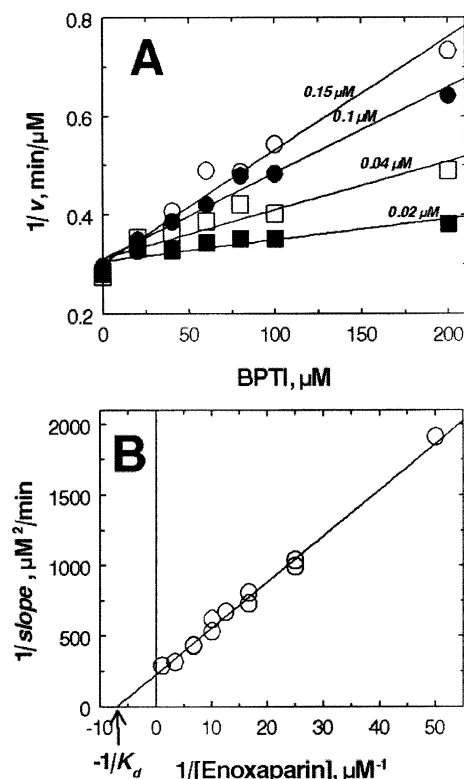


FIGURE 5: Kinetic and mechanistic analysis of the heparin-fIXa-BPTI interaction. (A) Reciprocal plots showing the effect of enoxaparin on BPTI inhibition of fIXa. Different concentrations of enoxaparin (■, 0.02; □, 0.04; ●, 0.1; and ○, 0.15 μ M) were incubated with 25 nM fIXa and varying amounts of BPTI (indicated) and assayed as described in the Experimental Procedures. The results were analyzed as described by Scheme 1 using eq 13. At saturating enoxaparin (1 μ M), the x -intercept (roughly -83μ M) is equal to $-K_i(1 + S/K_s)$. Using the experimental value of 1 mM for S and $K_s \approx K_m = 1.7$ mM under the conditions used (not shown), a value of $K_i = 52 \mu$ M was determined. This is in excellent agreement with the K_i determined in Figures 2 and 3A. (B) Double reciprocal plots of $1/\text{slopes}$ (obtained from fits of eq 13 to data from panel A) plotted versus $1/\text{enoxaparin}$ concentration as described by Scheme 1 and fitted with eq 14. Extrapolation of the fitted line to the x -intercept yielded a value of $K_d = 128 \pm 26$ nM for the heparin-fIXa interaction, consistent with the data obtained by fluorescence (Figure 6).

of fIXa required in this assay precluded the data from being used to determine a precise affinity, but the $K_{0.5}$ value of roughly 90 nM supports the results obtained above. In addition, and of greater interest, the alteration of the fluorescence emission intensity of the dye suggests alterations in the environment of the fIXa active-site region upon heparin binding.

Effect of Heparin on fIXa Peptidolytic Activity. The altered fluorescence of dEGR-fIXa upon heparin titration observed above prompted us to further examine the potential for a productive effect of heparin on the fIXa active-site region. Although heparin did not show any significant effect on the activity of fIXa toward a small tripeptidyl chromogenic substrate (cf. Figure 1), this substrate (CBS 31.39; CH₃SO₂-D-LGR-*p*NA) includes residues only on the amino-terminal side of the scissile bond and contains a non-natural D amino acid at the amino terminus. We thought it possible that effects of heparin on the active-site region of fIXa may remain undetected by this synthetic substrate but may perhaps be detectable using longer and more natural substrate sequences.

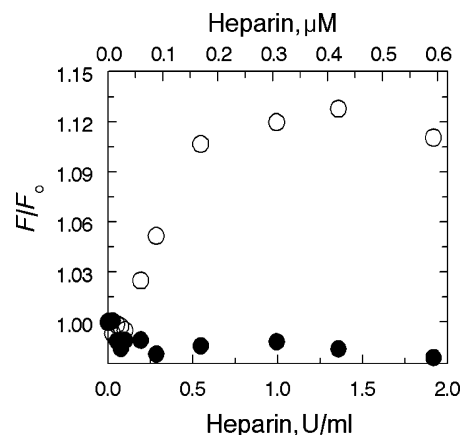


FIGURE 6: Heparin alters the environment of the extended fIXa active site. The fluorescence emission intensity of dEGR-fIXa (220 nM) was monitored at 540 nm in the presence of increasing levels of heparin (indicated). The data are presented as the ratio of the observed fluorescence to the initial fluorescence (F/F_0) and are corrected for buffer and dilution effects. Heparin caused a concentration-dependent and saturable increase in the fluorescence emission intensity of dEGR-fIXa (open circles) but not of the free label dEGR-ck (closed circles). This shows an alteration in environment of the extended fIXa active site in the vicinity of the dansyl dye and reveals nanomolar affinity for the heparin-fIXa interaction ($K_{0.5} \approx 90$ nM).

Thus, we examined the activity of fIXa toward a synthetic pentapeptide substrate (AT3.2) based on the reactive-site sequence of antithrombin, which is known to inhibit fIXa in a heparin-sensitive manner. Unlike chromogenic substrates, this pentapeptide consists of all natural amino acids (L isomers) and contains three residues on the amino-terminal side of the scissile bond as well as two residues on the carboxyl-terminal side, thus probing a wider region of the active-site cleft. Identification of the intact pentapeptide substrate and the two cleavage products was done by HPLC (Figure 7A). Although fIXa typically does not show strong reactivity with small peptide substrates, time-dependent hydrolysis of this pentapeptide was observed with fIXa alone (Figure 7B and Table 1). In the presence of saturating levels of heparin, this rate was increased roughly 2- to 6-fold. As a comparison, ethylene glycol caused a roughly 3- to 14-fold increase in rate, while the combination of ethylene glycol and heparin produced a roughly 4- to 16-fold increase in the rate of hydrolysis. No hydrolysis of this pentapeptide substrate was observed over a 24-h period in the absence of enzyme or by heparin alone.

DISCUSSION

The data presented clearly demonstrate that heparin enhances the reactivity of fIXa with BPTI. On the basis of previous studies, it seems reasonable to assume that the observed binding of heparin occurs at the heparin-binding exosite on fIXa (16). While the ability of BPTI to also interact with heparin at physiological pH added a great deal of complexity to the kinetic analysis, several lines of evidence suggest that the effect of heparin is due to environmental and/or structural alterations in the active-site region of fIXa as opposed to alterations in BPTI or a template-like mechanism of action (24, 25): (i) The effect of heparin on fIXa reactivity is reproduced with the low-molecular-weight heparin enoxaparin (Figure 3A). (ii) Although BPTI can bind

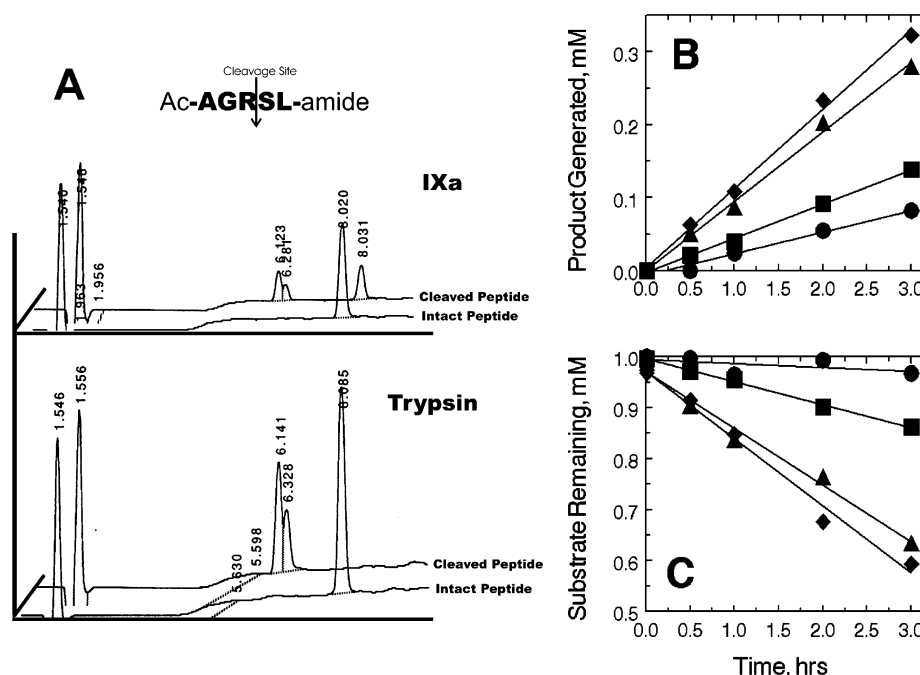


FIGURE 7: Heparin enhances fIXa peptidolytic activity. (A) Hydrolysis of 1 mM AT3.2 pentapeptide by 1 nM trypsin versus 25 nM fIXa. Typical HPLC profiles used for separation and identification of the intact (undigested) peptide substrate and the two peptidolytic products are shown for 0 and 30 min (trypsin) or 0 and 120 min (fIXa; incomplete hydrolysis). The retention times for the peaks of interest are indicated. At time zero, only the undigested substrate peak is apparent (retention time of 8 min). The substrate is digested over time to generate two products, giving rise to two new peaks at retention times 6.1 and 6.3 min. (B and C) Hydrolysis of the AT3.2 pentapeptide by 25 nM fIXa alone (circles) or in the presence of 1 U/mL heparin (squares), 30% ethylene glycol (triangles), or both (diamonds). Panel B shows the generation of products as determined by integration of product peaks over time. The data were normalized to the area obtained for 100% digestion. Panel C shows the utilization of substrate as determined by integration of the substrate peak over time. The data were normalized to the area obtained at time zero. Initial rates were determined as described in the Experimental Procedures and are given in Table 1.

Table 1. Initial Rates of AT3.2 Peptide Hydrolysis by fIXa^a

	substrate hydrolysis rate (nM/min/nM)	fold increase in rate	product generation rate (nM/min/nM)	fold increase in rate
fIXa	5.5	—	19.6	—
fIXa + heparin	30.1	5.5	31.2	1.6
fIXa + EG	74.3	13.5	63.5	3.2
fIXa + Hep + EG	87.7	15.9	72.3	3.7

^a Initial rates of AT3.2 pentapeptide hydrolysis were determined by integration of substrate and product peaks as described in Figure 7 and the Experimental Procedures. The data are normalized to the concentration of fIXa used (25 nM) to yield rates in units of nM/min per nM enzyme. Fold increases in activity by 1 U/mL heparin (Hep) and 30% ethylene glycol (EG) were calculated based on the rate obtained for fIXa alone. Heparin resulted in a 2- to 6-fold increase in hydrolysis rate, EG resulted in a 3- to 14-fold increase in hydrolysis rate, and heparin plus EG resulted in a 4- to 16-fold increase in hydrolysis rate.

to heparin, this interaction is extremely unstable, as indicated by the rapidity of the kinetics, and is of a very weak affinity compared to the measured inhibition constant and interaction of heparin with fIXa (Figure 4). (iii) Functional assays of enoxaparin-dependent inhibition of fIXa (Figure 3B) reflect the nanomolar affinity of heparin binding to fIXa as opposed to the micromolar affinity of heparin binding to BPTI, suggesting that heparin binding to BPTI is inconsequential in formation of the initial inhibitory complex. (iv) Heparin causes a change in the fluorescence emission intensity of a dansyl dye located in the extended active-site cleft of fIXa. (v) Heparin enhances the activity of fIXa toward a small pentapeptide substrate.

The latter result demonstrates a modulating effect of heparin on the extended fIXa active-site environment, although the level of the effect is significantly smaller than the effect on inhibition by BPTI. This suggests that heparin may have multiple effects, or may affect multiple elements of the fIXa–BPTI interaction, not the least of which may be the establishment of a tight inhibitory complex from the initial loose complex, as observed for the modulation of thrombin by thrombomodulin and its subsequent interaction with BPTI (32). The ability of BPTI to interact with heparin in the present study, however, confounded a similar non-equilibrium kinetic study to examine this possibility. It is important to note that, despite the evidence against a template mechanism in the heparin-dependent inhibition of fIXa by BPTI, we cannot definitively rule out the possibility that this plays some role in the overall inhibition scheme. It is interesting to speculate that the binding of heparin to BPTI may be of consequence in stabilizing the already-formed heparin–fIXa–BPTI tertiary complex. This stabilization would not be apparent in the equilibrium-type experiments performed here. Investigating this possibility is beyond the scope of the present study, however, and must await future study.

Our results are consistent with similar studies by others and us with the related blood coagulation factors thrombin and factor VIIa (32, 40) and extend to fIXa the observation that modulation of the environment of the extended active-site region by a cofactor results in enhanced reactivity with BPTI. While we also observed an enhancing effect of heparin on the reactivity of fIXa with TFPI (increase in inhibition

from 40% to 50% by 1 μ M enoxaparin at 286 nM TFPI; not shown), the ability of the C-terminus of TFPI to bind to heparin with high affinity and the presence of three inhibitory domains in TFPI complicates interpretation of this finding. Thus, it is currently unclear if this enhancing effect is observed with other Kunitz-type inhibitors. With respect to BPTI, it has been observed for several blood coagulation proteases, including fIXa, that amino acid 192 (using chymotrypsin numbering) is important for substrate and inhibitor recognition (30, 32–34, 40, 41). While in fVIIa, fXa, thrombin, and protein C the presence of Gln at position 192 has been found sufficient to enhance inhibition by BPTI, this is apparently not the case with fIXa, which contains a Gln at this position (analogous to fXa) but requires further modulations to allow inhibition by BPTI. Thus, other regions of fIXa are likely involved and are of importance in the modulatory effect of heparin. Identification of these regions is currently under investigation.

While we found an effect of heparin on the activity of fIXa toward a pentapeptide substrate, the effect was lower than that observed with BPTI (4-fold versus 20-fold) and was not universal toward all substrates (i.e., the chromogenic substrate CBS 31.39 revealed no effect). This could be explained by heparin altering the conformation of specific extended binding pockets or loops in the active-site vicinity rather than heparin affecting the catalytic machinery of fIXa. Such alterations could result in the observed substrate/inhibitor-specific effects where small synthetic chromogenic substrates are unable to detect these perturbations while larger substrates and BPTI interact with appropriate regions to sense these changes. Thus, when taken together, the results would suggest that the effect of heparin on the environment of the extended fIXa active site results in increased reactivity of fIXa that is selective for substrate/inhibitor molecules that interact with appropriate regions in the fIXa active-site cleft. Alternatively, heparin could be enhancing fIXa interactions with select substrates/inhibitors via favorable modulation of the electrostatic environment of the active-site region. Discrimination between these possibilities must await future studies.

The nanomolar affinity observed here between fIXa and heparin (Figures 5 and 6) is comparable to that found previously by others using low-molecular-weight heparin (42). The ability of heparin to affect the environment of the fIXa active site seems to be related to the enhanced reactivity of fIXa with BPTI. Although others and we have shown that ethylene glycol and certain other hygroscopic compounds can result in a large increase in amidolytic activity of fIXa (14, 15), this effect of ethylene glycol was not observed on the reactivity of fIXa with BPTI. Thus, it would seem that the effect of heparin is distinct from that induced by ethylene glycol. This is further supported by the lack of effect of heparin on the amidolytic activity of fIXa. This is not surprising, considering the different binding regions that have been identified for these two effectors (26, 43).

The ability of heparin to alter the environment of the extended fIXa active site may shed some light on the rather large heparin dependence observed for the inhibition of fIXa by antithrombin (44). Heparin has been shown to greatly enhance the inhibition of fIXa by antithrombin (18–20). This effect has been assumed to be a result of effects of heparin on antithrombin itself, as in factor Xa inhibition (21–24),

and/or effects of bridging of antithrombin with its target, as with thrombin (24, 25). It is now apparent that an additional effect may play an equally vital role: the ability of heparin to alter the extended active-site environment of fIXa to enhance its reactivity. The actual consequences of this effect on inhibition of fIXa by antithrombin, however, are as yet unclear and are currently being investigated.

As a final point, it is of interest to note that heparin alone has been shown to inhibit factor X activation by fIXa via a noncompetitive mechanism (45). This can be explained in part by heparin and factor X sharing an exosite on fIXa (27). The recent study by Rezaie et al. supports this proposed substrate-binding region of fIXa as also being the heparin-binding site (26). The implication of this in the context of our present findings is that factor X may also act to modulate fIXa upon its interaction with this exosite, thus facilitating its own proteolysis in a form of “substrate-assisted” catalysis. Although intriguing, this possibility is beyond the scope of the present study, and its investigation must await future studies.

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REFERENCES

1. Roberts, H. R., and Tabares, A. H. (1995) in *Molecular Basis of Thrombosis and Hemostasis* (High, K. A., and Roberts, H. R., Eds.) pp 35–50, Marcel Dekker, Inc., New York.
2. Jesty, J., and Nemerson, Y. (1995) in *Williams Hematology* (Beutler, E., Lichtman, M. A., Collar, B. S., and Kipps, T. J., Eds.) pp 1227–1238, McGraw-Hill, Inc., New York.
3. Colman, R. W., Marder, V. J., Salzman, E. W., and Hirsh, J. (1994) in *Hemostasis and Thrombosis. Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., Eds.) pp 3–18, J.B. Lippincott Co., Philadelphia.
4. van Dieijen, G., Tans, G., Rosing, J., and Hemker, H. C. (1981) The role of phospholipid and factor VIIIa in the activation of bovine factor X, *J. Biol. Chem.* 256, 3433–3442.
5. Mertens, K., and Bertina, R. M. (1984) The contribution of Ca^{2+} and phospholipids to the activation of human blood-coagulation factor X by activated factor IX, *Biochem. J.* 223, 607–615.
6. Mertens, K., van Wijngaarden, A., and Bertina, R. M. (1985) The role of factor VIII in the activation of human blood coagulation factor X by activated factor IX, *Thromb. Haemost.* 54, 654–660.
7. van Dieijen, G., van Rijn, J. L. M. L., Govers-Riemslog, J. W. P., Hemker, H. C., and Rosing, J. (1985) Assembly of the intrinsic factor X activating complex—Interactions between factor IXa, factor VIIIa and phospholipid, *Thromb. Haemost.* 53, 396–400.
8. Suomela, H., Blomback, M., and Blomback, B. (1977) The activation of factor X evaluated with synthetic substrates, *Thromb. Res.* 1, 267–281.
9. Byrne, R., Link, R. P., and Castellino, F. J. (1980) A kinetic evaluation of activated bovine blood coagulation factor IX toward synthetic substrates, *J. Biol. Chem.* 255, 5336–5341.
10. McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W., and Powers, J. C. (1981) Mapping the active sites of bovine thrombin, factor IXa, factor Xa, factor XIa, factor XIIa, plasma kallikrein, and trypsin with amino acid and peptide thioesters: development of new sensitive substrates, *Biochemistry* 20, 7196–7206.
11. Lottenberg, R., Christensen, U., Jackson, C. M., and Coleman, P. L. (1981) in *Methods in Enzymology* (Lorand, L., Ed.) pp 341–361, Academic Press, New York.
12. Link, R. P., and Castellino, F. J. (1983) Kinetic properties of bovine factor IXa alpha and IXa beta toward synthetic substrates, *Biochemistry* 22, 999–1005.
13. Castillo, M. J., Kurachi, K., Nishino, N., Ohkubo, I., and Powers, J. C. (1983) Reactivity of bovine blood coagulation factor IXa-

- beta, factor Xa-beta, and factor XIa toward fluorogenic peptides containing the activation site sequences of bovine factor IX and factor X, *Biochemistry* 22, 1021–1029.
14. Neuenschwander, P. F., McCollough, J., McCallum, C. D., and Johnson, A. E. (1997) A Conformational Change in the Active Site of Blood Coagulation Factor IXa is Associated with an Increase in Activity Upon Treatment with Ethylene Glycol, *Thromb. Haemost. Suppl.*, 428.
 15. Stuerzebecher, J., Kopetzki, E., Bode, W., and Hopfner, K. P. (1997) Dramatic enhancement of the catalytic activity of coagulation factor IXa by alcohols, *FEBS Lett.* 412, 295–300.
 16. Yang, L. K., Manithody, C., and Rezaie, A. R. (2002) Localization of the heparin binding exosite of factor IXa, *J. Biol. Chem.* 277, 50756–50760.
 17. Sheehan, J. P., and Phan, T. M. (2001) Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex by an allosteric mechanism, *Biochemistry* 40, 9480–9490.
 18. Rosenberg, J. S., McKenna, P. W., and Rosenberg, R. D. (1975) Inhibition of human factor IXa by human antithrombin, *J. Biol. Chem.* 250, 8883–8888.
 19. Kurachi, K., Fujikawa, K., Schmer, G., and Davie, E. W. (1976) Inhibition of bovine factor IXa and factor Xa (beta) by antithrombin III, *Biochemistry* 15, 373–377.
 20. DiScipio, R. G., Kurachi, K., and Davie, E. W. (1978) Activation of human factor IX (Christmas factor), *J. Clin. Invest.* 60, 1528–1538.
 21. Villaneuva, G. B., and Danishefsky, I. (1977) Evidence for a heparin-induced conformational change on antithrombin III, *Biochem. Biophys. Res. Commun.* 74, 803–809.
 22. Villaneuva, G. B. (1984) Predictions of the secondary structure of antithrombin III and location of the heparin binding site, *J. Biol. Chem.* 259, 2531.
 23. Olson, S. T., and Bjoerk, I. (1992) Role of protein conformational changes, surface approximation and protein cofactors in heparin-accelerated antithrombin-proteinase reactions, *Adv. Exp. Med. Biol.* 313, 155–165.
 24. Olson, S. T., Bjoerk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin–proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement, *J. Biol. Chem.* 267, 12528–12538.
 25. Griffith, M. J. (1982) Kinetics of the heparin-enhanced antithrombin III/thrombin reaction. Evidence for a template model for the mechanism of action of heparin, *J. Biol. Chem.* 257, 7360–7365.
 26. Yang, L., Manithody, C., and Rezaie, A. R. (2002) Localization of the Heparin Binding Exosite of Factor IXa, *J. Biol. Chem.* 277, 50756–50760.
 27. Chang, J. L., Jin, J. P., Lollar, P., Bode, W., Brandstetter, H., Hamaguchi, N., Straight, D. L., and Stafford, D. W. (1998) Changing residue 338 in human factor IX from arginine to Alanine causes an increase in catalytic activity, *J. Biol. Chem.* 273, 12089–12094.
 28. Schmaier, A. H., Dahl, L. D., Hasan, A. A. K., Cines, D. B., Bauer, K. A., and Van, N. W. E. (1995) Factor IXa inhibition by protease nexin-2/amyloid Beta-protein precursor on phospholipid vesicles and cell membranes, *Biochemistry* 34, 1171–1178.
 29. Schmaier, A. H., Dahl, L. D., Rozemuller, A. J. M., Roos, R. A. C., Wagner, S. L., Chung, R., and Van, N. W. E. (1993) Protease nexin-2/amyloid Beta protein precursor. A tight-binding inhibitor of coagulation Factor IXa, *J. Clin. Invest.* 92, 2540–2545.
 30. Hsu, Y. C., Hamaguchi, N., Chang, Y. J., and Lin, S. W. (2001) The distinct roles that Gln-192 and Glu-217 of factor IX play in selectivity for macromolecular substrates and inhibitors, *Biochemistry* 40, 11261–11269.
 31. Rezaie, A. R., and Esmon, C. T. (1993) Conversion of glutamic acid 192 to glutamine in activated protein C changes the substrate specificity and increases reactivity toward macromolecular inhibitors, *J. Biol. Chem.* 268, 19943–19948.
 32. Neuenschwander, P. F., and Morrissey, J. H. (1995) Alteration of the substrate and inhibitor specificities of blood coagulation factor VIIa: Importance of amino acid residue K192, *Biochemistry* 34, 8701–8707.
 33. Rezaie, A. R., and Esmon, C. T. (1995) Contribution of residue 192 in factor Xa to enzyme specificity and function, *J. Biol. Chem.* 270, 16176–16181.
 34. Rezaie, A. R., and Esmon, C. T. (1996) Molecular basis of residue 192 participation in determination of coagulation protease specificity, *Eur. J. Biochem.* 242, 477–484.
 35. Segel, I. H. (1975) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York.
 36. O'Shannessy, D. J., Dobersen, M. J., and Quarles, R. H. (1984) A novel procedure for labeling immunoglobulins by conjugation to oligosaccharide moieties, *Immunol. Lett.* 8, 273–277.
 37. Badellino, K. O., and Walsh, P. N. (2001) Localization of a heparin binding site in the catalytic domain of factor XIa, *Biochemistry* 40, 7569–7580.
 38. Delaria, K. A., et al. (1997) Characterization of placental bikunin, a novel human serine protease inhibitor, *J. Biol. Chem.* 272, 12209–12214.
 39. Olson, S. T., and Chuang, Y. J. (2002) Heparin activates antithrombin anticoagulant function by generating new interaction sites (exosites) for blood clotting proteinases, *Trends Cardiovasc. Med.* 12, 331–338.
 40. Rezaie, A. R., He, X., and Esmon, C. T. (1998) Thrombomodulin Increases the Rate of Thrombin Inhibition by BPTI, *Biochemistry* 37, 693–699.
 41. Guinto, E. R., Ye, J., Le, B. B. F., and Esmon, C. T. (1994) Glu192→Gln substitution in thrombin yields an enzyme that is effectively inhibited by bovine pancreatic trypsin inhibitor and tissue factor pathway inhibitor, *J. Biol. Chem.* 269, 18395–18400.
 42. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) The binding of low molecular weight heparin to hemostatic enzymes, *J. Biol. Chem.* 255, 10073–10080.
 43. Sichler, K., Banner, D. W., D'Arcy, A., Hopfner, K. P., Huber, R., Bode, W., Kresse, G. B., Kopetzki, E., and Brandstetter, H. (2002) Crystal structures of uninhibited factor VIIa link its cofactor and substrate-assisted activation to specific interactions, *J. Mol. Biol.* 322, 591–603.
 44. Bedsted, T., Swanson, R., Chuang, Y. J., Bock, P. E., Bjork, I., and Olson, S. T. (2003) Heparin and calcium ions dramatically enhance antithrombin reactivity with factor IXa by generating new interaction exosites, *Biochemistry* 42, 8143–8152.
 45. Barrow, R. T., Parker, E. T., Krishnaswamy, S., and Lollar, P. (1994) Inhibition by heparin of the human blood coagulation intrinsic pathway factor X activator, *J. Biol. Chem.* 269, 26796–26800.

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