# Allosteric Modification of Factor XIa Functional Activity upon Binding to Polyanions†

Dipali Sinha,*,‡ Karen O. Badellino,§ Mariola Marcinkiewicz,‡ and Peter N. Walsh‡,||

*The Sol Sherry Thrombosis Research Center and Departments of Medicine and Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and University of Pennsylvania, Philadelphia, Pennsylvania 19104*

*Received January 27, 2004; Revised Manuscript Received April 13, 2004*

ABSTRACT: The effects of several polyanions on the hydrolysis of the chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline (S-2366) and on the activation of factor IX by factor XIa have been investigated. Two forms of dextran sulfate ($M_r$ ∼500000 and $M_r$ ∼10000, DX10) and two forms of heparin (64 disaccharide units, $M_r$ ∼14000, and hypersulfated heparin, S-Hep, $M_r$ ∼12000) inhibited both factor XIa amidolytic activity and factor IX activation in a concentration-dependent manner. The inhibitory effect was not due to binding of either substrate by the polyanions since only a decrease in $V_{max}$ without any effect on $K_m$ was observed in kinetic assays. Steric inhibition is unlikely since the concentrations of polyanions required for inhibition of small peptide hydrolysis were lower than those required for macromolecular substrate cleavage. In contrast, an allosteric inhibitory mechanism was supported by an enhancement of the dansyl fluorescence of 5-(dimethylamino)-1-(naphthalenesulfonyl)glutamyl-glycylarginyl- (DEGR-) factor XIa observed when the fluorophore was in complex with either DX10 or S-Hep. Moreover, in the presence of a polyanion the fluorophore was far more resistant to quenching by acrylamide. These results provide compelling evidence that factor XIa binding to the polyanions, dextran sulfate and heparin, results in inhibition of the enzyme by an allosteric mechanism.

Factor XI (FXI),[1] a serine protease zymogen of 160 kDa, has an essential function as an amplifier of blood coagulation in response to vascular injury (*1*, *2*). It consists of two identical subunits (80 kDa) held together by a single disulfide bond (*3*) and circulates in plasma in complex with high molecular weight kininogen (HK) at a concentration of 30 nM (*4*). Patients with FXI deficiency, unlike those with deficiencies of factor XII (FXII), HK, or prekallikrein, suffer from abnormal hemostasis, suggesting that under normal physiological conditions FXI is not activated through the contact activation pathway (*1*, *5*−*8*). FXI has been shown to be activated by thrombin in the presence of a negatively charged surface (*1*, *2*) as well as on the activated platelet surface (*9*, *10*). FXI activation by thrombin is particularly relevant to the revised theory of blood coagulation (*1*, *2*). According to this theory thrombin generated through the tissue factor−factor VIIa (FVIIa) pathway is insufficient to produce an adequate fibrin clot, since tissue factor pathway inhibitor rapidly inactivates this pathway. Propagation of coagulation is facilitated by thrombin-catalyzed generation of FXIa, which initiates the intrinsic pathway of blood coagulation, leading to the generation of sufficient quantities of thrombin to effect hemostasis.

The role of FXIa in the formation of a fibrin clot requires its tight regulation. Several protease inhibitors that are known to inhibit FXIa are α-1-antitrypsin (*11*), C1 inhibitor (*12*, *13*), antithrombin III (*14*), and protease nexin II (PN2) (*15*−*18*). The first three inhibitors, which belong to the family of serine protease inhibitors (serpins), are characterized by association rate constants of (1.3−4.0) × $10^4$ $M^{-1}$ $s^{-1}$. Binding of FXIa to PN2, a Kunitz-type protease inhibitor released from activated platelets (*19*), is much tighter ($K_i$ ∼ 450 pM) (*15*, *17*, *18*). At the site of vessel injury activated platelets can discharge large quantities of PN2 into a nascent thrombus. This process together with the tight association constant of this inhibitor with FXIa suggests that PN2 is an important regulator of FXIa and of the procoagulant response to vascular damage. It is interesting that a negatively charged surface that potentiates activation of FXI by thrombin, FXIIa, or FXIa also enhances inactivation of FXIa by serpins and PN2. The role of heparin in the potentiation of inactivation of thrombin and factor Xa (FXa) through their major inhibitor antithrombin III has been widely studied (*20*, *21*). While a ternary complex bridging mechanism promotes inactivation of thrombin (*20*), an allosteric conformational change that recognizes an exosite on the proteinase is involved in the potentiation of FXa inactivation (*21*). As in the case of thrombin inhibition by antithrombin III in the presence of heparin, enhanced inactivation of FXIa by serpins or PN2

† This study was supported by research grants from the National Institutes of Health: HL74124, HL46213, HL64943, and HL70683 (to P.N.W.).

* To whom correspondence should be addressed. Telephone: 215-707-4458. Fax: 215-707-3005. E-mail: dipali@temple.edu.

‡ The Sol Sherry Thrombosis Research Center, Temple University School of Medicine.

§ University of Pennsylvania.

|| Departments of Medicine and Biochemistry, Temple University School of Medicine.

[1] Abbreviations: S-2366, L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline; S-2765, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginyl-*p*-nitroaniline dihydrochloride; FXI, factor XI; HK, high molecular weight kininogen; PN2, protease nexin II; DEGR-XIa, 5-(dimethylamino)-1-(naphthalenesulfonyl)glutamylglycylarginyl-factor XIa.

10.1021/bi049808c CCC: $27.50 
Published on Web 05/19/2004

in the presence of heparin has been postulated to occur by a template mechanism through colocalization of the enzyme and the inhibitor on the same heparin molecule (*22*, *23*). In these studies, however, no attempt was made to investigate any alteration in functional activity when FXIa forms a complex with a polyanion. The present study was designed to address this question and to establish whether binding to a polyanion is translated into a conformational change in the proteinase.

## EXPERIMENTAL PROCEDURES

FXIa, FIX, FXa, and DEGR-XIa were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). High-purity recombinant FVIII was obtained as a generous gift from Baxter Healthcare Corp. (Duarte, CA). Dextran sulfate $M_r$ ~500000 (DX500), dextran sulfate $M_r$ ~10000 (DX10), and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Fractionated heparin of 64 disaccharide units (Hep 64, $M_r$ ~14000) was obtained in limited quantities from Enzyme Research Laboratories, Inc. (South Bend, IN), and was available only for certain experiments. Hypersulfated heparin (S-Hep, average $M_r$ ~12000) was from Neoparin, Inc. (San Leandro, CA). The chromogenic substrates L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline (S-2366) and *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginyl-*p*-nitroaniline dihydrochloride (S-2765) were from DiaPharma Group (Stockholm, Sweden). All other chemicals were of analytical grade or the best quality commercially available.

*Effects of Polyanions on the Cleavage of S-2366 by FXIa.* FXIa was incubated with either buffer or various concentrations of DX500, DX10, Hep 64, or S-Hep in the wells of a microtiter plate for 10 min at room temperature in a total volume of 25 μL. One hundred microliters of S-2366 (1.25 mM) was then added, and the initial rates of cleavage of the substrate were measured at 37 °C in a Thermomax microtiter plate reader (Molecular Devices, Palo Alto, CA) in the kinetics mode and plotted versus the concentrations of the polyanion using KaleidaGraph from Synergy Software, PCS Inc. (Reading, PA).

To analyze the nature of the inhibitory effect, FXIa was incubated with buffer or a fixed concentration of the polyanion to be examined for 10 min at room temperature, followed by addition of increasing concentrations of the substrate S-2366 (0.03−1.0 mM). The final concentration of FXIa in the reaction mixture (150 μL) was 3 nM. The change in absorbance at 405 nm was measured to determine the initial velocity of free *p*-nitroaniline formation. Titration curves of S-2366 hydrolysis were generated by KaleidaGraph using a nonlinear least-squares fit of data points to an equation for a rectangular hyperbola: $y = ax/(b + x)$. The same software program was also used for double reciprocal plots.

*Effect of Polyanions on the Activation of FIX by FXIa.* The effects of the polyanions on the activation of FIX by FXIa were assessed by a modification of the method described earlier (*24*, *25*). FXIa was incubated with buffer or increasing concentrations of DX10, DX500, or S-Hep for 10 min at room temperature and added to FIX in the presence of 5 mM $CaCl_2$. The final concentrations of FXIa and FIX in the reaction mixture were 1 and 250 nM, respectively. The activation was allowed to proceed for 2 min at 37 °C, and the reaction was stopped by diluting the reaction mixture by 10-fold with ice-cold TBS/BSA containing 10 mM EDTA. In the second stage of the assay, 5 or 10 μL of the diluted reaction mixture containing generated FIXa in the first stage described above was incubated with PC/PS (3:1) unilamellar vesicles in a total volume of 140 μL for 2 min at 37 °C, followed by addition of 10 μL each of freshly activated FVIII (FVIIIa) and FX, and the incubation was continued for another 3 min at 37 °C. The final concentrations of PC/PS, $CaCl_2$, FVIIIa, and FX in this mixture were 1 μM, 5 mM, 2.5 units/mL, and 400 nM, respectively. Generation of FXa was terminated by addition of 15 μL of EDTA (250 mM). In the third stage, 40 μL of the reaction mixture was mixed with 40 μL of S-2765 (0.70 mM), and the change in absorbance at 405 nm was followed on the Thermomax microtiter plate reader (Molecular Devices, Menlo Park, CA). PC/PS (3:1) unilamellar phospholipid vesicles were prepared according to the protocol described by Mayer et al. (*26*). In short, porcine brain PS and L-α-dioleoyl-PC (Avanti Polar Lipids, Alabaster, AL), stored in chloroform at 20 °C, were mixed 1:3 and, after evaporating the chloroform under $N_2$, were dissolved in benzene. The mixture was lyophilized and hydrated in aqueous buffer over a period of 30 min with occasional mixing. Vesicles thus formed were disrupted by repeated freeze/thaw cycles and finally extruded using 0.1 μm polycarbonate filters (Coster Corp., Cambridge, MA).

For kinetic analysis of the effect of a polyanion on FXIa-catalyzed activation of FIX, the same three-stage assay described above was performed using increasing concentrations (0.08−250 nM) of FIX for a fixed concentration of the polyanion under investigation, and the initial steady-state rates of S-2765 cleavage were measured by continuously monitoring absorbance at 405 nm. FIXa generated was calculated from a standard curve of FXa generation constructed using known amounts of FIXa.

To ensure that the observed inhibitory effect assessed from S-2765 hydrolysis in the third stage of the assay is not due to the binding of the polyanion to FIXa, FX, or FXa, the carryover polyanion from the first stage was neutralized with polybrene before being added to the FXa-generating reaction mixture. Addition of a 2−3-fold molar excess of polybrene over the carryover polyanion was found to generate a standard curve identical to that when FIXa alone was used. Titration curves of FIXa formation as a function of FIX concentration and the corresponding double reciprocal plots were generated by KaleidaGraph as described in the previous section.

*Effects of Polyanions on the Dansyl Fluorescence of DEGR-XIa.* Fluorescence measurements were performed on an SLM-AMINCO/Bowman Series 2 spectrofluorometer (Thermo Spectronic, Rochester, NY). Emission spectra of DEGR-XIa in the absence and presence of various concentrations of DX10 or S-Hep were recorded using an excitation wavelength of 340 nm. The excitation and emission slit widths were set at 4 nm.

*Quenching of Dansyl Fluorescence of DEGR-XIa.* Fluorescence quenching studies were done using the neutral quencher acrylamide. Fluorescence emission of DEGR-XIa was measured in the absence and presence of different concentrations (0−0.5 M) of acrylamide. Fluorescence at each concentration was corrected for dilution effect. Colli-

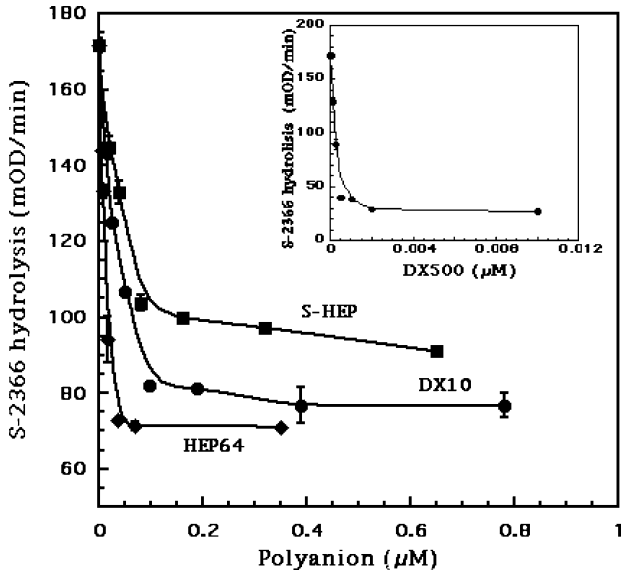


FIGURE 1: Effects of DX500, Hep 64, DX10, and S-Hep on S-2366 hydrolysis by FXIa. FXIa (20 nM) was incubated without or with various concentrations of DX500 (●) (shown in the inset), Hep 64 (◆), DX10 (●), and S-Hep (■) for 10 min at room temperature and then assayed at 37 °C for S-2366 hydrolysis. Data points represent triplicate determinations (mean ± SEM) derived from one of two experiments with identical results.

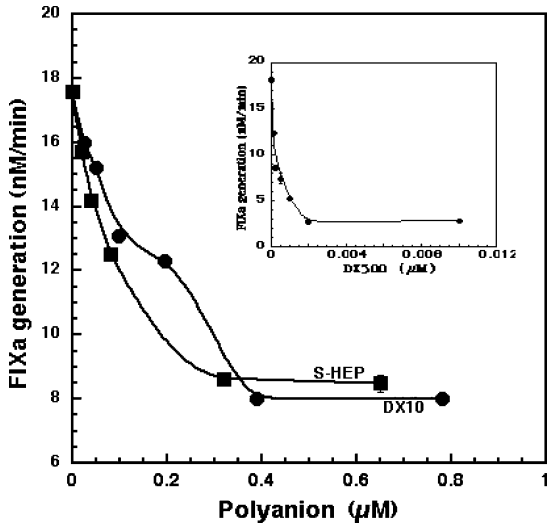


FIGURE 2: Effect of DX500, DX10, and S-Hep on the activation of FIX by FXIa. FXIa was incubated with different concentrations of DX500 (●) (shown in the inset), DX10 (●), or S-Hep (■) for 10 min before addition of the macromolecular substrate FIX; the final concentrations of FXIa and FIX were 1 nM and 250 nM, respectively. Generation of FIXa was measured by a three-stage assay described in detail in the Experimental Procedures section.

sional quenching of fluorescence is described by the Stern−Volmer equation (*27*):

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_D[Q]$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, respectively, $k_q$ is the bimolecular quenching constant, $\tau_0$ is the lifetime of the fluorescence in the absence of the quencher, and [Q] is the concentration of the quencher. The Stern−Volmer quenching constant is given by $k_q\tau_0$ or $K$. Modification of Stern−Volmer plot according to Lehrer (*28*) is given by

$$F_0/\Delta F = 1/K[Q] + 1$$

and is more useful when $\Delta F$ is very small at low quencher concentration and also when more than one kind of quencher-sensitive fluorophore is present.

## RESULTS

*Inhibition of S-2366 Cleavage by FXIa in the Presence of Polyanions.* Rates of hydrolysis of the chromogenic substrate S-2366 by FXIa in the absence and presence of different concentrations of DX500, DX10, Hep 64, and S-Hep are shown in Figure 1. All of the polyanions inhibited the hydrolysis reaction in a concentration-dependent manner, with saturable inhibition observed at polyanion concentrations greater than 5 μg/mL (∼0.1 nM DX500, ∼0.5 μM Hep 64, S-Hep, and DX10) and variable maximal percent inhibition by DX500 (83%), DX10 (56%), Hep 64 (58%), and S-Hep (48%), respectively.

*Effects of Polyanions on the Activation of FIX by FXIa.* Rates of FIXa generation by FXIa in the absence and presence of DX500, DX10, and S-Hep were investigated by a three-stage assay described in the Experimental Procedures section, and the results are shown in Figure 2. As in the case of S-2366 hydrolysis, all of the polyanions showed a concentration-dependent inhibition of FIX activation with saturable maximal inhibition by DX500 (86%), S-Hep (54%), and DX 10 (51%), respectively. Hep 64 could not be compared along with DX500, DX10, and S-Hep shown in Figure 2 because of unavailability of the reagent at the time the experiment was done. However, previously we examined Hep 64 alone in the FIX activation assay and found maximum inhibition at ∼70−80% at a concentration of 0.42 μM (data not shown). Since all four polyanions demonstrated similar inhibitory effects, we focused all of our subsequent studies at defining the mechanism of inhibition on the two polyanions with similar $M_r$ and charge characteristics, i.e., DX10 and S-Hep.

*Kinetics of S-2366 Hydrolysis by FXIa in the Presence and Absence of DX10 and S-Hep.* Since inhibition of S-2366 hydrolysis by FXIa could possibly result from binding of the polyanions to the small substrate S-2366, we examined the effects of polyanions on FXIa amidolytic activity. Rates of cleavage of S-2366 at increasing concentrations of the substrate were examined at different fixed concentrations of DX10 (Figure 3) and S-Hep (Figure 4). The insets in each figure represent double reciprocal plots. It is quite clear that both DX10 and S-Hep altered the $V_{max}$ of the hydrolysis reaction while $K_m$ (∼0.4 mM) remained unchanged. Therefore, the observed inhibition could not be the result of binding of the polyanions to S-2366.

*Kinetics of FIX Activation by FXIa in the Presence and Absence of DX10 and S-Hep.* The observed inhibition of FIX activation, as in the case of the small substrate, may be the result of binding of the polyanions to FIX. This possibility was investigated by kinetic analysis of FXIa-catalyzed FIX activation in the presence and absence of the polyanions. Kinetic assays were carried out by a three-stage assay described in the Experimental Procedures section using increasing concentrations of the macromolecular substrate FIX in the presence and absence of fixed concentrations of DX10 or S-Hep. Titration curves of FIXa generation and the corresponding double reciprocal plots were generated by

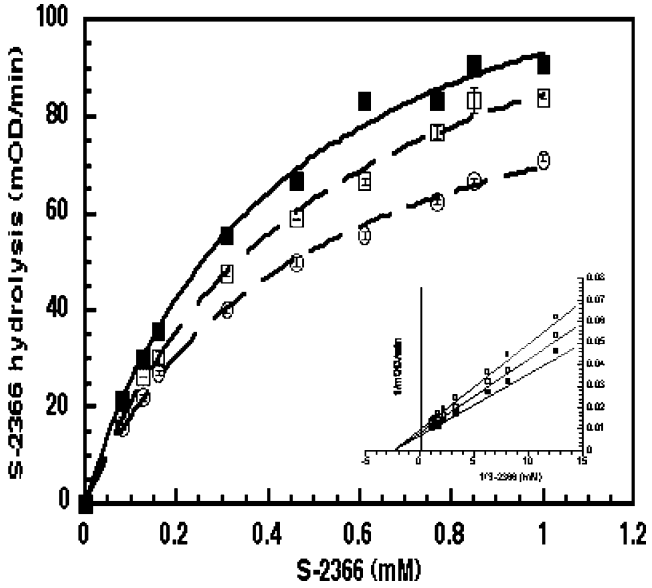


FIGURE 3: Inhibition of FXIa-induced hydrolysis of S-2366 by DX10. Hydrolysis of indicated concentrations of S-2366 by FXIa (2.6 nM) in the presence of 0 (■), 0.25 $\mu$g/mL (0.025 $\mu$M) (□), and 0.5 $\mu$g/mL (0.05 $\mu$M) (○) DX10 was measured by continuously monitoring the change in absorbance at 405 nm. Titration curves of initial rates as a function of S-2366 concentration were generated by KaleidaGraph as described in the Experimental Procedures section. Inset: double reciprocal plots of the same data points and the fitted lines.

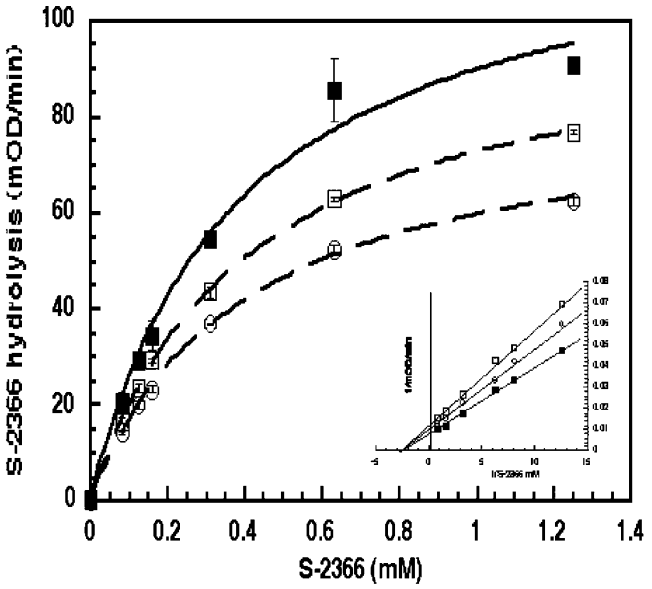


FIGURE 4: Inhibition of FXIa-induced hydrolysis of S-2366 by S-Hep. Hydrolysis of the indicated concentrations of S-2366 by FXIa (2.6 nm) in the presence of 0 (■), 0.8 $\mu$g/mL (0.067 $\mu$M) (□), and 1.6 $\mu$g/mL (0.134 $\mu$M) (○) S-Hep was measured and analyzed as described for DX10. Inset: double reciprocal plots of the same data points and the fitted lines.

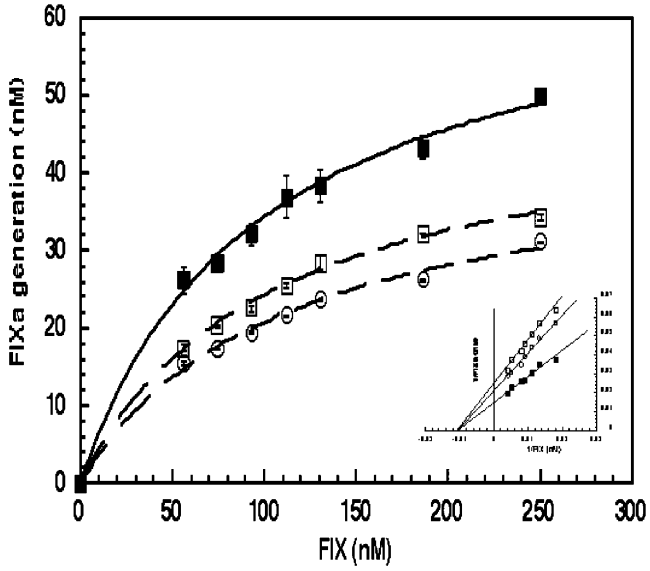


FIGURE 5: Inhibition of FXIa-induced activation of FIX by DX10. Activation of FIX by FXIa in the presence of 0 (■), 1 $\mu$g/mL (0.1 $\mu$M) (□), and 1.5 $\mu$g/mL (0.15 $\mu$M) (○) DX10 was assessed by a three-stage assay described in detail in the Experimental Procedures section. Titration curves of FIXa formation were generated by KaleidaGraph as described for S-2366 hydrolysis. Inset: double reciprocal plots and the fitted lines.

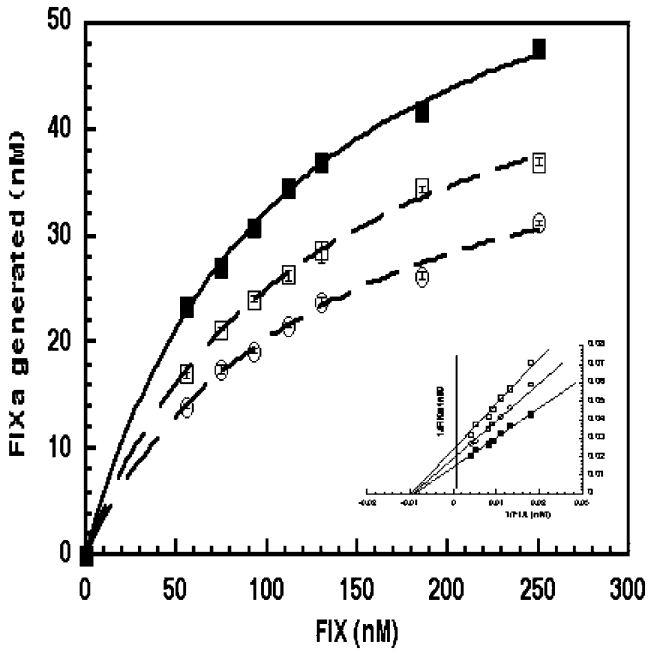


FIGURE 6: Inhibition of FXIa-induced activation of FIX by S-Hep. Activation of FIX by FXIa in the presence of 0 (■), 0.8 $\mu$g/mL (0.067 $\mu$M) (□), and 1.6 $\mu$g/mL (0.134 $\mu$M) (○) S-Hep was measured in exactly the same way as for DX10. Titration curves of FIXa formation were generated by KaleidaGraph as described for S-2366 hydrolysis. Inset: double reciprocal plots and the fitted lines.

KaleidaGraph as described for S-2366 hydrolysis and are shown in Figure 5 (DX10) and Figure 6 (S-Hep). As observed with S-2366 hydrolysis, the inhibition of FIX activation by DX10 or S-Hep was purely noncompetitive ($K_m$ ~100 nM in the absence and presence of polyanions), thus ruling out the possibility that the inhibition was the result of binding of the polyanion to FIX. These results are consistent with previous studies from our laboratory demonstrating similar kinetic parameters for FXIa-catalyzed FIX activation in the absence of polyanions (*24*, *29*).

*Comparison of Inhibitory Potency of Polyanions in S-2366 Hydrolysis and FIX Activation by FXIa.* Inhibitory potencies of DX10 and S-Hep in the hydrolysis of S-2366 and in the activation of FIX by FXIa are shown in Figure 7. If the observed inhibition of FXIa by either of these two polyanions is the result of binding of the polyanion at or near the active

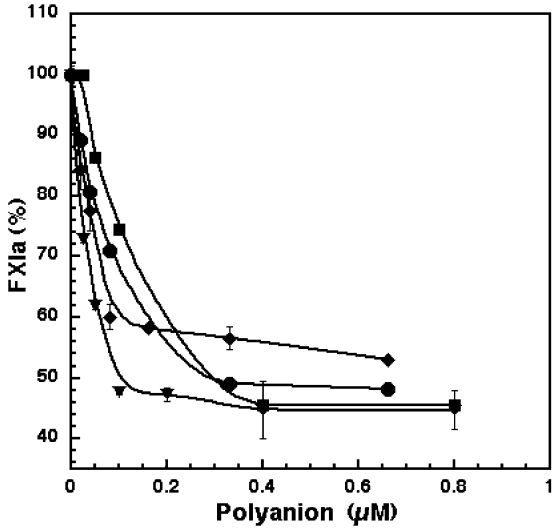


FIGURE 7: Comparison of the inhibitory effects of DX10 and S-Hep in the FXIa-mediated activation of FIX vs the hydrolysis of S-2366. FXIa-mediated hydrolysis of S-2366 and FIX activation in the presence and absence of DX10 (▼, S-2366 hydrolysis; ■, FIX activation) and S-Hep (◆, S-2366 hydrolysis; ●, FIX activation) were performed as described in Figures 1 and 2. The concentration of FXIa at a given concentration of the inhibitor was calculated, assuming that in the absence of any inhibitor to be 100%.

site of the enzyme resulting in steric hindrance, then one would expect the inhibition to be far greater for the macromolecular substrate FIX than for the small peptide substrate S-2366. The results shown in Figure 7, however, demonstrate the opposite result; i.e., each of the two polyanions exhibited inhibitory activity at slightly lower concentration in S-2366 hydrolysis than in FIX activation. Therefore, the observed inhibition of FXIa by the polyanions is not a consequence of active site sequestration by direct interactions with the polyanions.

*Effects of Binding to Polyanions on the Dansyl Fluorescence of DEGR-XIa.* Since the observed inhibition of FXIa activity is not the consequence of either binding of the polyanion to the substrates or steric inhibition by the polyanion binding at or near the active site of the enzyme, we therefore investigated whether a change in conformation around the active site is responsible for the observed inhibition. FXIa was reacted with DEGR-chloromethyl ketone to yield DEGR-XIa, an analogue of FXIa with a dansyl fluorophore attached covalently to the active site. Emission spectra of DEGR-XIa in the presence and absence of DX10 and S-Hep are shown in Figure 8. Both DX10 and S-Hep enhanced the dansyl fluorescence of DEGR-XIa, and the fluorescence enhancement was accompanied by a blue shift of 8 nm. Increasing degrees of fluorescence intensity enhancement (~4−21%) were observed with increasing concentrations (2−2.5 $\mu$M) of DX10 or S-Hep, and no further increase in fluorescence intensity was observed at polyanion concentrations greater than ~25 $\mu$g/mL (2.5 $\mu$M). It should be noted that the concentrations of DX10 and S-Hep used in these experiments were higher than those used in the functional assays of FXIa (Figures 3 and 4). The reason for this difference was that whereas 1−3 nM FXIa was required in the functional assays, the concentration of DEGR-FXIa in all fluorescence measurements was 200 nM. Since there is one dansyl group per molecule of FXIa, a higher FXIa concentration was required for an optimal response in the fluorescence experiments.

*Quenching of Dansyl Fluorescence of DEGR-XIa.* The fluorescence intensities of DEGR-XIa in the presence and absence of DX10 at different concentrations of acrylamide are shown in Figure 9A, and the corresponding reciprocal Stern−Volmer plots for the quenching are shown in Figure 9B. Whereas 75% of the dansyl fluorescence of free DEGR-XIa was quenched by 0.5 M acrylamide, only 20% quenching was observed when DEGR-XIa was in complex with dextran sulfate. When a saturating concentration of DX10 was added to the same tube containing DEGR-XIa and 0.5 M acrylamide, the fluorescence emission was potentiated to almost the same value as that of dextran sulfate-bound DEGR-XIa in the presence of 0.5 M acrylamide (Figure 8A). Similar results were obtained with S-Hep (data not shown).

## DISCUSSION

FXIa and its isolated light chain were found to bind to heparin with similar affinities, suggesting the presence of a heparin binding site in the catalytic domain of the molecule

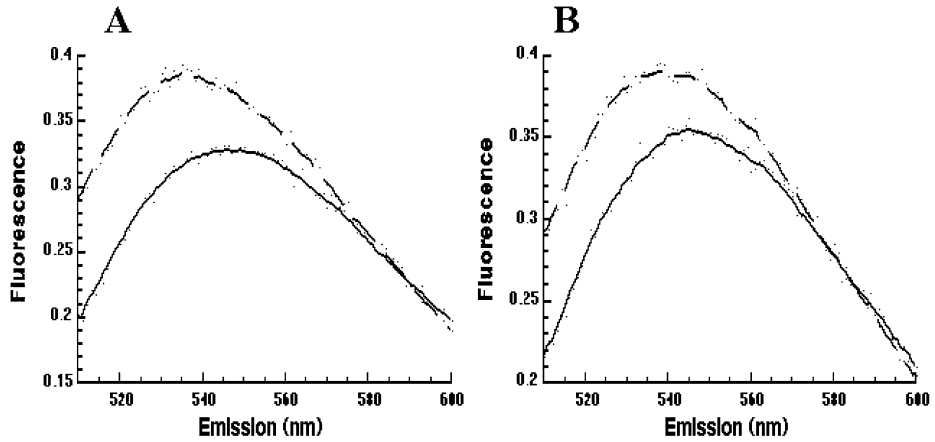


FIGURE 8: Effect of a polyanion on the dansyl fluorescence of DEGR-XIa. (A) The fluorescence emission spectra of DEGR-XIa (0.22 $\mu$M, 200 $\mu$L) in the absence (solid line) and presence of DX10 (20 $\mu$g/mL) (broken line) were recorded with excitation at 340 nm and a bandwidth of 4 nm both for excitation and emission. (B) The fluorescence emission spectra of DEGR-XIa (0.30 nM, 200 $\mu$L) in the absence (solid line) and presence of S-Hep (20 $\mu$g/mL) (broken line) were recorded with excitation at 340 nm and a bandwidth of 4 nm both for excitation and emission. The fluorescence maximum for free DEGR-XIa was at 547 nm, and that of DEGR-XIa in complex with DX10 or S-Hep was at 535 nm.

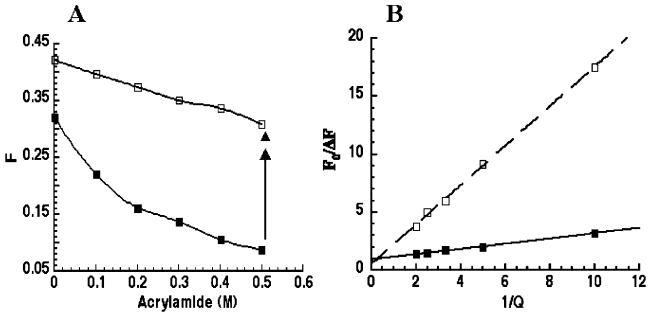


FIGURE 9: Quenching of free DEGR-XIa and dextran sulfate-bound DEGR-XIa by acrylamide. (A) Emission of dansyl fluorescence of free DEGR-XIa (■) and dextran sulfate-bound DEGR-XIa (□) was recorded in the absence and after sequential additions of acrylamide from a stock solution. The fluorescence intensity at each concentration of acrylamide was obtained after correction for dilution and plotted as a function of acrylamide concentration. The filled triangle (▲) represents the fluorescence of DEGR-XIa and 0.5 M acrylamide after addition of DX10 (20 $\mu$g/mL) (2 $\mu$M). Excitation was at 340 nm for both; the emission at 547 nm was used in the case of free DEGR-XIa, and it was 535 nm in the case of DEGR-XIa in complex with dextran sulfate. (B) Reciprocal Stern−Volmer plot of acrylamide quenching of free DEGR-XIa (■) and DEGR-XIa in complex with dextran sulfate (□).

that was later characterized by Badellino and Walsh (*30*). We therefore determined whether the functional activity of FXIa is altered upon binding to a polyanion. DX500, DX10, Hep 64, and S-Hep were found to inhibit the hydrolysis of S-2366 by FXIa in a concentration-dependent manner (Figure 1). It is interesting to note that although Hep64, DX10, and S-Hep are similar in size, the $IC_{50}$ values for the inhibition of FXIa-induced hydrolysis of S-2366 by these different polyanions were different. One explanation for the observed differences is that the affinity of FXIa for a polyanion depends on the exact nature of the charges it carries, and they are probably widely different for these three polyanions. In the case of DX500 the mechanism of inhibition is more complex because of its high $M_r$. The complex nature of inhibition is also reflected in our kinetic assays where we were unable to obtain a linear double reciprocal plot.

To investigate the mechanisms by which these polyanions inhibit FXIa, we focused our subsequent studies on DX10 and S-Hep, which have similar size and charge characteristics. Thus, a possible inhibitory mechanism takes into account the observation that the macromolecular substrate interacts with the enzyme over an extended surface that could be occluded by the polyanions. Thus, it has been shown that the heavy chain of FXIa contains a substrate binding site for FIX (*29*, *31*, *32*) whereas the active site that cleaves FIX is located within the catalytic domain. To determine whether the inhibition observed was the result of binding of the polyanions to S-2366 or to FIX or to a substrate binding site in the heavy chain of FXIa, we analyzed the mechanism of inhibition in kinetic assays (Figures 3−6). It is apparent from the double reciprocal plots utilizing both substrates that in the presence of DX10 and S-Hep the $K_m$ remained unaltered whereas the $V_{max}$ was decreased in a concentration-dependent manner by each of the two inhibitors. This result excludes binding of the polyanions to S-2366 or FIX as the cause of the observed inhibition.

Two other possibilities remain to explain the observed inhibitory effects: (1) the polyanions bind to or near the active site of FXIa, thereby inhibiting substrate binding to FXIa sterically, and (2) binding of FXIa to a polyanion results in obscuration of the FXIa active site by an allosteric mechanism. If steric hindrance were the sole cause for the observed inhibition, then one would expect a more pronounced inhibitory effect with a macromolecular substrate, such as FIX, than with a small peptide substrate, such as S-2366. When the inhibitory effects of the polyanions on the cleavage of S-2366 and on the activation of FIX were compared, it was apparent that hydrolysis of S-2366 was inhibited at slightly lower concentrations of the two polyanions than the FIX activation (Figure 7). Furthermore, the fact that incomplete inhibition of the enzyme by the polyanions was observed (Figure 1 and Figure 2) is more consistent with an allosteric than a steric mechanism since steric inhibition by binding of the polyanion to the active site would be expected to completely prevent access of the active site to the substrate. Therefore, it is reasonable to conclude that an allosteric effect is the most likely cause of the observed inhibitory effects.

We therefore examined the fluorescence characteristics of DEGR-XIa in order to investigate whether binding to a negatively charged surface results in an alteration in the active site configuration of the enzyme. Active site modification of serine proteases with a fluorophore (e.g., DEGR-Xa) has been successfully used to study conformational alterations in the enzyme on binding to FVa, prothrombin, phospholipid, and calcium (*33*−*35*). Examination of the fluorescence emission spectra of DEGR-XIa in the presence and absence of DX10 and S-Hep revealed that the presence of a polyanion caused an enhancement of dansyl fluorescence with a blue shift of ∼8 nm (Figure 8).

To examine whether the enhanced fluorescence observed is the result of a more hydrophobic environment acquired by the fluorophore in the presence of a polyanion, we compared quenching of free DEGR-XIa and DEGR-XIa bound to a polyanion using a collisional quenching agent. Fluorescence quenching requires molecular contact between the fluorophore and the quencher. In the case of collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state of the fluorophore. Therefore, quenching measurements can reveal the accessibility of the fluorophore to the quencher. The neutral quencher acrylamide was used in our studies since an ionic quencher (e.g., KI) could be expected to interfere with binding of FXIa to a polyanion. Figure 9A shows the quenching of the dansyl fluorescence of free DEGR-XIa and of DEGR-XIa in complex with DX10 as a function of increasing concentrations of acrylamide. Although fluorescence quenching was observed with both free and bound DEGR-XIa, the dansyl fluorescence of DEGR-XIa in the presence of a saturating concentration of DX10 was far more resistant to quenching than in its absence (Figure 9A). It is interesting to note that addition of a saturating concentration of DX10 to DEGR-XIa after quenching with 0.5 M acrylamide enhances the fluorescence to coincide nearly with the fluorescence of the DX10-bound DEGR-XIa (Figure 9A). DX500 and S-Hep-bound DEGR-XIa were also resistant to quenching by acrylamide compared to free DEGR-XIa (data not shown). A reciprocal plot of the fluorescence quenching data according to Lehrer (*28*) produced a straight line with an intercept of 1 for both free DEGR-XIa and DX10 bound-DEGR-XIa, suggesting the presence of only one class of

fluorophore in either case (Figure 9B). In the presence of a suboptimal concentration of dextran sulfate, however, the intercept was >1, indicating the presence of two classes of fluorophores, and this is clearly due to the presence of both free and dextran sulfate-bound DEGR-XIa (data not shown).

The dansyl dye is attached to the active site His residue through a three-residue tether, and even with a fully extended DEGR moiety the fluorophore would be only 7 Å from the active site (*33*). Thus the dansyl fluorophore is located near, if not at, the active center. Resistance of the fluorophore to quenching when DEGR-FXIa is in complex with a polyanion may be explained as the result either of a steric effect that does not allow the quencher to collide with the fluorophore or of acquisition by the active site of a new architecture that is les accessible. The latter and not the former possibility, however, explains all of the observed functional consequences of the enzyme when it binds to a polyanion as well as the observed fluorescence enhancement and its resistance to quenching. Therefore, we conclude that the change in the environment of the fluorophore must be the result of an alteration of the DEGR-XIa conformation upon binding to a negatively charged surface.

In conclusion, the present studies are consistent with the view that binding of FXIa to the polyanions, heparin and dextran sulfate, results in an allosteric modification of its functional activity resulting in inhibition of both small peptide and macromolecular substrate cleavage. Previous studies from our laboratory demonstrating the presence of a heparin binding site, comprising residues $^{527}$C−$^{542}$C within the FXIa catalytic domain (*30*), an exosite which is remote from the catalytic triad, are consistent with the conclusion that binding to heparin results in a conformational alteration preventing access of the active site to its substrates, thereby inhibiting FXIa activity. Interestingly, additional studies from our laboratory (*36*) indicate that FXIa binding to activated platelets does not inhibit its capacity to activate FIX and protects FXIa from inactivation by PN2. The receptor on the platelet surface for FXIa has not been identified. The physiological relevance of the present studies will require further studies including the identification of the biologically important negatively charged surface molecules exposed within the intravascular milieu that modulate the functional activity of FXIa and thereby inhibit blood coagulation. Possible candidate molecules include heparan sulfate glycosaminoglycans exposed on the plasma membranes of endothelial cells.

## REFERENCES


1. Gailani, D., and Broze, G. J., Jr. (1991) Factor XI activation in a revised model of blood coagulation, *Science 253*, 909−912.
2. Naito, K., and Fujikawa, K. (1991) Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces, *J. Biol. Chem. 266*, 7353−7358.
3. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) Location of the disulfide bonds in human coagulation factor XI: the presence of tandem apple domains, *Biochemistry 30*, 2056−2060.
4. Saito, H., and Goldsmith, G. H., Jr. (1977) Plasma thromboplastin antecedent (PTA, factor XI): a specific and sensitive radioimmunoassay, *Blood 50*, 377−385.
5. Alexander, B., Goldstein, R., Landwehr, G., and Cook, C. D. (1951) Congenital SPCA deficiency: a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions, *J. Clin. Invest. 30*, 596.
6. Bolton-Maggs, P. H., Young Wan-Yin, B., McCraw, A. H., Slack, J., and Kernoff, P. B. (1988) Inheritance and bleeding in factor XI deficiency, *Br. J. Haematol. 69*, 521−528.
7. Ragni, M. V., Sinha, D., Seaman, F., Lewis, J. H., Spero, J. A., and Walsh, P. N. (1985) Comparison of bleeding tendency, factor XI coagulant activity, and factor XI antigen in 25 factor XI-deficient kindreds, *Blood 65*, 719−724.
8. White, C. A., Rees, T. D., and Hurt, W. C. (1986) Factor XII (Hageman factor) deficiency in a periodontal surgery patient, *J. Oral Med. 41*, 105−107.
9. Baglia, F. A., and Walsh, P. N. (1998) Prothrombin is a cofactor for the binding of factor XI to the platelet surface and for platelet-mediated factor XI activation by thrombin, *Biochemistry 37*, 2271−2281.
10. Oliver, J. A., Monroe, D. M., Roberts, H. R., and Hoffman, M. (1999) Thrombin activates factor XI on activated platelets in the absence of factor XII, *Arterioscler. Thromb. Vasc. Biol. 19*, 170−177.
11. Scott, C. F., Schapira, M., James, H. L., Cohen, A. B., and Colman, R. W. (1982) Inactivation of factor XIa by plasma protease inhibitors: predominant role of alpha 1-protease inhibitor and protective effect of high molecular weight kininogen, *J. Clin. Invest. 69*, 844−852.
12. Schapira, M., Scott, C. F., and Colman, R. W. (1981) High molecular weight kininogen protects human plasma kallikrein and factor XIa against inactivation by plasma protease inhibitors, *Trans. Assoc. Am. Physicians 94*, 190−197.
13. Wuillemin, W. A., Eldering, E., Citarella, F., de Ruig, C. P., ten Cate, H., and Hack, C. E. (1996) Modulation of contact system proteases by glycosaminoglycans. Selective enhancement of the inhibition of factor XIa, *J. Biol. Chem. 271*, 12913−12918.
14. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin, *J. Biol. Chem. 255*, 10081−10090.
15. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity, *Nature 331*, 530−532.
16. Scandura, J. M., Zhang, Y., Van Nostrand, W. E., and Walsh, P. N. (1997) Progress curve analysis of the kinetics with which blood coagulation factor XIa is inhibited by protease nexin-2, *Biochemistry 36*, 412−420.
17. Smith, R. P., Higuchi, D. A., and Broze, G. J., Jr. (1990) Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein, *Science 248*, 1126−1128.
18. Van Nostrand, W. E., Wagner, S. L., Farrow, J. S., and Cunningham, D. D. (1990) Immunopurification and protease inhibitory properties of protease nexin-2/amyloid beta-protein precursor, *J. Biol. Chem. 265*, 9591−9594.
19. Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S., and Cunningham, D. D. (1990) Protease nexin-II (amyloid beta-protein precursor): a platelet alpha-granule protein, *Science 248*, 745−748.
20. Olson, S. T., and Bjork, I. (1991) Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects, *J. Biol. Chem. 266*, 6353−6364.
21. Olson, S. T., Bjork, 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.
22. Zhang, Y., Scandura, J. M., Van Nostrand, W. E., and Walsh, P. N. (1997) The mechanism by which heparin promotes the inhibition of coagulation factor XIa by protease nexin-2, *J. Biol. Chem. 272*, 26139−26144.
23. Badellino, K. O., and Walsh, P. N. (2000) Protease nexin II interactions with coagulation factor XIa are contained within the Kunitz protease inhibitor domain of protease nexin II and the factor XIa catalytic domain, *Biochemistry 39*, 4769−4777.
24. Sinha, D., Marcinkiewicz, M., Gailani, D., and Walsh, P. N. (2002) Molecular cloning and biochemical characterization of rabbit factor XI, *Biochem. J. 367*, 49−56.

25. Sun, M. F., Zhao, M., and Gailani, D. (1999) Identification of amino acids in the factor XI apple 3 domain required for activation of factor IX, *J. Biol. Chem. 274*, 36373−36378.
26. Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure, *Biochim. Biophys. Acta 858*, 161−168.
27. Lakowicz, J. R. (1999) Quenching of Fluorescence, in *Principles of Fluorescence Spectroscopy*, 2nd ed., pp 237−265, Kluwer Academic/Plenum, New York.
28. Lehrer, S. S., and Leavis, P. C. (1978) Solute quenching of protein fluorescence, *Methods Enzymol. 49*, 222−236.
29. Sinha, D., Seaman, F. S., and Walsh, P. N. (1987) Role of calcium ions and the heavy chain of factor XIa in the activation of human coagulation factor IX, *Biochemistry 26*, 3768−3775.
30. 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.
31. Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1991) Identification and chemical synthesis of a substrate site for factor IX on coagulation factor XIa, *J. Biol. Chem. 266*, 24190−24197.
32. Sun, Y., and Gailani, D. (1996) Identification of a factor IX binding site on the third apple domain of activated factor XI, *J. Biol. Chem. 271*, 29023−29028.
33. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) The active site of blood coagulation factor Xa. Its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex, *J. Biol. Chem. 262*, 12953−12961.
34. Krishnaswamy, S. (1990) Prothrombinase complex assembly. Contributions of protein−protein and protein-membrane interactions toward complex formation, *J. Biol. Chem. 265*, 3708−3718.
35. Krishnaswamy, S., Jones, K. C., and Mann, K. G. (1988) Prothrombinase complex assembly. Kinetic mechanism of enzyme assembly on phospholipid vesicles, *J. Biol. Chem. 263*, 3823−3834.
36. Baird, T. R., and Walsh, P. N. (2002) The interaction of factor XIa with activated platelets but not endothelial cells promotes the activation of factor IX in the consolidation phase of blood coagulation, *J. Biol. Chem. 277*, 38462−38467.

BI049808C