Down-regulation of Factor IXa in the Factor Xase Complex by Protein Z-dependent Protease Inhibitor*

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Protein Z-dependent protease inhibitor (ZPI) was recently identified as a serpin that potently inhibits activated blood coagulation factor X (FXa) (EC 3.4.21.6) in a manner dependent on protein Z, Ca2+, and phospholipids (1–3). ZPI is estimated to be present at 3.8 ng/ml (53 nm) in plasma, forms non-covalent complexes with protein Z in plasma, and has an apparent mobility of 72 kDa on SDS-PAGE (3, 4). ZPI is 25–35% homologous to human serpins (2) and 70% homologous to rat liver serpins (3) (EC 3.4.21.22), we investigated whether ZPI might inhibit FIXa in the FXase complex, as well as FXa in the prothrombinase complex. If so, we wondered whether FXIIa might protect FIXa from ZPI inhibition, as FVa protects FXa from ZPI inhibition, and whether protein Z might be required for inhibition of FXa. The homologous prothrombinase and FXase complexes work in tandem to generate thrombin (EC 3.4.21.5), and a physiologic inhibitor of both complexes could be particularly significant.

EXPERIMENTAL PROCEDURES

Plasma-derived Proteins and Reagents—ZPI was purified from plasma as described (1). Protein Z was purified from citrated plasma by barium adsorption and elution followed by DEAE-Sephacel (Amersham Biosciences) chromatography, in which protein Z was the last protein eluted in a salt gradient (23). Protein Z was >90% homogeneous as judged by SDS-PAGE. Concentrations of protein Z and ZPI were estimated using A_{280} = 1.2 or 1.0, respectively, for 0.1% solutions (1, 23). FX was purified and activated as described (24), and recombinant FVIII was obtained as a gift from Dr. Roger Lundblad of Baxter, Inc, Anaheim, CA. Fx, FX, FII, protein Z, and thrombin were purchased from Enzyme Research Laboratories, South Bend, IN. Thrombin substrate CBS 34.47 (H-D-cyclohexyl-Gly-aminobutyryl-Arg-p-nitroanilide) was purchased from Diagnostica Stago, Asnières, France; FXa substrate

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2 The abbreviations used are: ZPI, protein Z-dependent protease inhibitor; rZPI, recombinant ZPI; BSA, bovine serum albumin; F, factor; HBS, Heps-buffered saline, pH 7.4.
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S-2765 (N-α-Z-D-Arg-Gly-Arg-p-nitroanilide) was from DiaPharma, West Chester, OH, and FIXa substrate Pefluor FIXa 3688 (H-(D)-Leu-phenylglycinol-Ang-aminomethylcoumarin) was from Centerchem, Inc, Norwalk, CT. Phospholipids were from Sigma; NaB₃H₄ was from Amersham Biosciences; sheep anti-protein Z was from The Binding Site, Birmingham, UK; and ascites containing monoclonal antibody A against FIX was a gift from Dr. Ted Zimmerman and Jim Roberts of The Scripps Research Institute. The anti-FIX was purified by 35% saturated ammonium sulfate precipitation followed by dialysis and Hi-load Q Sepharose chromatography. CM5 chips for surface plasmon resonance binding studies were from Biacore, Inc., Piscataway, NJ.

Recombinant ZPI—Due to the modest concentration of ZPI in plasma and the large number of purification steps required, recombinant (r) ZPI was prepared. A 1.4-kb cDNA was cloned from human liver cDNA from two individuals using 5′ primers beginning at the signal peptide sequence and 3′ primers extending beyond the stop codon and a high fidelity proofreading DNA polymerase (Invitrogen) in a polyermase chain reaction. The product was introduced into the vector pcDNA3.1 Topo (Invitrogen) to transform competent Escherichia coli cells. DNA was prepared from selected colonies and subjected to restriction digestion to verify insertion size, and the sequence was then verified. Plasmid DNA was purified for transfection into transformed human kidney HK293 cells using liposome-mediation (Superfect, Invitrogen). Optimal conditions were determined using transient expression, and then stably transfected cells were prepared. Immunoblotting of conditioned medium allowed selection of cells with the highest expression level (1–2 μg/ml). Several 1-liter batches of conditioned serum-free medium were prepared, treated with 1 mM Pefabloc protease inhibitor (Roche Diagnostics) and frozen until purification procedures were performed.

For purification, conditioned medium was concentrated 10-fold using Amicon YM-30 membrane filtration (Millipore Corp., Bedford MA) and subjected to heparin-Sepharose chromatography at pH 6.3 as described for plasma ZPI followed by either MonoQ or MonoS chromatography (Amersham Biosciences) using an NaCl gradient at pH 6.3 (1).

FXa Activity Measurements—To assess the effect of ZPI on FXa activity, prothrombinase assays were performed with 0.5 nM FXa, 25 μM phospholipids, 5 mM CaCl₂, 0.6 μM prothrombin with or without 20 PM FVa in Hepes-buffered saline (HBS) containing 0.5% BSA (25). Briefly, variable ZPI and protein Z were preincubated for 5 min with all components except prothrombin; prothrombin was added, and subaliquots were taken over time and quenched in EDTA prior to the addition of thrombin substrate; the linear rate of thrombin formation was determined in the presence or absence of ZPI/protein Z. To monitor inhibition of FIXa by ZPI, FX was tritiated by reductive alkylation of carbohydrate groups that reside primarily in its activation peptide region, using NaB₃H₄ as described (26) followed by chromatography on a Sephacryl-300 column. The product retained 80% of its clotting activity as measured in FX-deficient plasma, contained 57,000 cpm/μg of FX, and was stored in small aliquots at −70 °C. Subaliquots (50 μl) of FXase incubation mixtures containing labeled and unlabeled FX were taken over time and mixed with 50 μl of cold 0.13% BSA. Cold 15% trichloroacetic acid (50 μl) was added and mixed and allowed to stand on ice for 2 min before centrifugation at 9,000 × g for 5 min. An aliquot of the trichloroacetic acid-soluble fraction containing released ³H activation peptide was counted in scintillation fluid. Counts in inhibited mixtures were expressed as a percentage of the controls containing no ZPI/protein Z.

FXa-based Clotting Activity—To monitor inhibition of FIXa by ZPI in a plasma milieu, a FXa-based two-stage clotting assay was developed. FXa (10 nM) was incubated in a volume of 50 μl for 3 min at 37 °C with 125 μM phospholipid vesicles, 6 mM CaCl₂, 1.5 mg/ml fibrinogen, 1.0% BSA-HBS ± ZPI ± protein Z. Prewarmed 1% BSA-HBS (52.5 μl), 10 μl of 60 mM CaCl₂, and 12.5 μl of FXI-deficient plasma were added, and the clotting time was measured in an ST4 coagulometer (Stago). The base clot time without ZPI was ~100 s.

Surface Plasmon Resonance Binding Experiments—Monclonal antibody against FIX (14 μg/ml) was coupled through amine groups to a CM5 chip activated as described by Biacore, Inc. using a Biacore 3000 instrument at a flow rate of 5 μl/min until 2,100 response units were coupled. Unreacted sites on the chip were blocked with ethanolamine. FIXa (12 nM) was injected at a flow rate of 10 μl/min in filtered HBS-0.025% BSA-5 mM CaCl₂. After 2.5 min, buffer was injected, and FIXa (294 ± 4 response units) dissociated at a negligible rate. ZPI (4.5–36 nM) was injected in the same buffer at the same flow rate for 2.5 min, and binding was monitored. Another wash was used to follow the dissociation of ZPI from FIXa, and then the chip was regenerated with 0.1 M glycine, 0.05 M NaCl, pH 2.5. For some experiments, freshly prepared FVIIIa was injected after the FIXa followed by a wash and then ZPI.
FIGURE 1. Inhibition of FXa by ZPI. A, dose dependence of inhibition of FXase (2 nM FIXa, 25 μM phospholipids, 5 mM Ca2+, 0.2 μM FX) in the absence of FVIIIa by ZPI alone (●), by protein Z alone (■), or by ZPI and protein Z (▲). B, inhibition of FXase by ZPI/protein Z in the presence of 80 pM FVIIIa by ZPI alone (●) or ZPI with 2.5 μg/ml protein Z (▲). C, inhibition of FXase activity in 5 min in several experiments was well below its plasma level in several experiments (Fig. 2). D, inhibition of FXase activity in 5 min in several experiments was well below its plasma level in several experiments (Fig. 2).

RESULTS

Purified ZPI and rZPI—Plasma-derived ZPI was purified to >90% homogeneity as judged by SDS-PAGE; rZPI was purified to >95% homogeneity. rZPI comigrated with plasma-derived ZPI on SDS-PAGE. The blot region containing excess FX at a mobility of 65 kDa was removed; only FXa at 50 kDa is shown. C and D employed 12-well gels and 10-well gels, respectively, and 2 and 1 μg/ml ZPI, respectively, each with 2.5 μg/ml protein Z. Points or bars and error bars in all figures represent means ± S.E. of the mean.

Global fits of the series of sensorgrams were performed using Biavitation software to calculate k_on, k_off, and K_D = k_off/k_on.

Inhibition of Prothrombinase Activity by ZPI—Plasma-derived ZPI and rZPI inhibited the prothrombinase activity of FXa in a similar, strictly protein Z-dependent manner, as reported (3). The presence of both plasma-derived ZPI and rZPI was lost over time; therefore, small aliquots were frozen to minimize the number of freeze/thaw cycles.

Inhibition of Prothrombinase Activity by FXa—To determine whether ZPI might also inhibit FIXa, the FXa physical product of FXase mixtures was semiquantitatively using an immunoblotting assay. Aliquots were collected 1–2.5 min after the addition of FX and subjected to immunoblot. As shown in Fig. 1, C and D, aliquots of FXase mixtures that included ZPI/protein Z contained significantly less FXa than did control FXase mixtures without ZPI/protein Z. A quantitative scan of these blots through the center of each band revealed that 78% less FXa was generated by FXa in the presence of 2 μg/ml ZPI, and 42% less FXa was generated in the presence of 1 μg/ml ZPI.

Immunoblotting of FXase Inhibition by ZPI—To further test the notion that ZPI can inhibit FIXa, the FXa product of FXase mixtures was semiquantitated using an immunoblotting assay. Aliquots were collected 1–2.5 min after the addition of FX and subjected to immunoblotting. As shown in Fig. 1, C and D, aliquots of FXase mixtures that included ZPI/protein Z contained significantly less FXa than did control FXase mixtures without ZPI/protein Z. A quantitative scan of these blots through the center of each band revealed that 78% less FXa was generated by FXa in the presence of 2 μg/ml ZPI, and 42% less FXa was generated in the presence of 1 μg/ml ZPI.

Inhibition of FXase Activity by ZPI—To determine whether ZPI might also inhibit FIXa (a homologue of FXa), FXase assays containing FIXa, phospholipids, and Ca2+ were preincubated ± ZPI ± protein Z, before the addition of FIXa, and then the generation of FXa was monitored as described under "Experimental Procedures." As observed for prothrombinase assays, the level of ZPI required for 50% inhibition of FXase activity in 5 min was below its plasma level in several experiments (Fig. 1). In these initial experiments, the amidolytic activity of the FXa product was directly measured to monitor inhibition of FXase, but part of the inhibition observed could be correlated with the inhibition of the FXa product by ZPI/protein Z. However, the pattern of inhibition of FXase differed from that observed in prothrombinase assays in that ZPI inhibition of FXase was much less dependent on protein Z, especially in the presence of FVIIIa, as expected.

In conclusion, a substantial amount of evidence has been presented in support of the hypothesis that ZPI is a key regulator of FXase in vivo. The results presented here provide a detailed understanding of the molecular basis for ZPI regulation of FXase activity and may have implications for the development of new therapeutic strategies for conditions associated with abnormal coagulation.
excess FVIIla. When FIXa was incubated with ZPI prior to FVIIla addition, the extent of inhibition of FIXa was not affected even when FVIIla was in molar excess (Fig. 3A). Interestingly, when ZPI was added after FIXa and FVIIla were allowed to form a complex, low concentrations of FVIIla appeared to stimulate ZPI inhibition of FIXa, whereas increasing FVIIla concentrations appeared protective of FIXa, and inhibition by ZPI was gradually diminished (Fig. 3B).

In other experiments not shown, FVa was added after or before the incubation of FIXa with ZPI/protein Z. When FIXa was incubated with ZPI prior to FVa addition, the extent of inhibition of FIXa by ZPI was not affected even when FVa was in molar excess. When ZPI was added after FIXa and FVa were allowed to form a complex, FVa was strongly protective, even when FIXa was in molar excess to FVa. Thus, FVa was more protective of FIXa than FVIIla was of FIXa.

Unusual Time Course for ZPI Inhibition of FIXa and FXa—ZPI inhibition of FIXa, like that of FXa, was rapid, reaching a maximum in \(<100\) s (40 s of preincubation plus 60 s of incubation before sampling) (Fig. 4); thus, maximum inhibition was reached in each of the experiments described above in which ZPI was incubated with FXase or prothrombinase components for 5 min. The maximum level of inhibition of FIXa was proportional to the concentration of ZPI. A plot of plateau activity versus concentration of ZPI from the data in Fig. 4 suggested that half-maximum inhibition of 1 nM FIXa occurred at 1 \(\mu g/ml\) ZPI (14 nM) (plot not shown). This concentration was found again to be below the plasma concentration of ZPI. When the same preparation of ZPI was used on the same day to inhibit prothrombinase with 20 pm FVa and FXase with 80 pm FVIIla, the potency of inhibition of each was similar (Fig. 4, dashed versus solid lines).

ZPI Inhibition of FX Activation Peptide Release—Since ZPI was reported not to inhibit FIXa (3) and since the activity assays shown in Figs. 2–4 might still be complicated by measuring a small amount of remaining ZPI inhibition of FXa product, several additional approaches were used to investigate ZPI inhibition of FIXa, as described below. FX was tritium-labeled to follow the release of \(^{3}\)H-labeled activation peptide during FX activation by FIXa. \(^{3}\)H-FX was 99.8% trichloroacetic acid-precipitable, whereas the released activation peptide was soluble in cold trichloroacetic acid; thus, the trichloroacetic acid-soluble fraction could be counted as a measure of FX activation. Using this method, 50%
inhibition of the FXase activity of FIXa was observed at ~1 μg/ml ZPI in the presence of 80 pm FVIIIa, and inhibition was essentially protein Z-independent (Fig. 5B). In the absence of FVIIIa, inhibition was modestly stimulated by protein Z (Fig. 5A). These data suggest that ZPI alone is an efficient inhibitor of FIXa but that either protein Z or low concentrations of FVIIIa can modestly enhance inhibition.

Inhibition of FIXa Amidolytic Activity by ZPI—The amidolytic activity of FIXa is not as physiologically relevant as the FXase activity of FIXa, but ZPI inhibition of FIXa does not involve possible inhibition of any FXa product. Therefore, a fluorogenic amidolytic substrate for FIXa was also used to measure the inhibition of FIXa by ZPI. Since this kind of assay requires higher concentrations of FIXa, 5 nM FIXa was preincubated with 7–20-fold molar excess of ZPI for 4 min in the presence of 5 mM Ca<sup>2+</sup> ± protein Z/phospholipids, and then amidolytic activity was measured. The data shown in Fig. 6 demonstrate that 50% inhibition of 5 nM FIXa activity was achieved with ~7.2 μg/ml (≈100 nM) ZPI. Protein Z and phospholipids were not required for ZPI inhibition. Protein Z did not inhibit FIXa and ZPI had no effect on the substrate in the absence of FIXa (data not shown). In other control experiments, ZPI did not inhibit the amidolytic activity of two other coagulation proteases, thrombin or activated protein C, in agreement with a previous study (1).

Inhibition of FIXa in a Clotting Assay—In a FIXa-based clotting assay using FIX-deficient plasma, ZPI prolonged clotting times in a dose-dependent manner (Fig. 7). As observed above, protein Z had little effect in combination with ZPI, and the addition of protein Z antibodies to the plasma had no effect. These data suggested that direct protein Z-dependent FXa inhibition did not significantly affect the clot time prolongation.

Other experiments not shown compared plasma depleted of protein Z using anti-protein Z-Sepharose with the same plasma passed over a control column of non-immune horse IgG coupled to Sepharose. ZPI gave the same dose-dependent prolongation of FIXa-based clotting times in each plasma, suggesting that protein Z from the plasma was not necessary for the ZPI anticoagulant effect. Thus, inhibition of FIXa by ZPI at concentrations below plasma level was confirmed using both plasma and purified component assays.

Association and Dissociation of ZPI from FIXa—The time course in Fig. 4 suggests that an equilibrium is reached between ZPI and FIXa and that ZPI does not form a stable complex with FIXa in the manner of most serpins. Consistent with this notion, no complexes of ZPI with FIXa were observed on SDS-PAGE. Therefore, surface plasmon resonance binding studies were performed to determine whether ZPI could associate and dissociate from FIXa. A series of concentrations of ZPI was bound to antibody-captured FIXa in the presence and absence of FVIIIa, and sensorgrams showing association and dissociation are shown in Fig. 8. The $K_{D}$ for ZPI binding to FIXa was 12 nM in the absence of FVIIIa and 8.9 nM in the presence of 12 nM FVIIIa (TABLE ONE).

It is uncertain whether the difference in $K_{D}$ with and without FVIIIa is significant. However, less than saturating concentrations of FVIIIa may have modestly stimulated ZPI binding to FIXa. On the other hand, higher concentrations of FVIIIa (50 nM) interfered with binding of ZPI to FIXa (data not shown), similar to the findings in Fig. 3. Control studies showed that FVIIIa retained >80% of its activity over 10 min, whereas the binding experiments were of 8–9-min duration following
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FIGURE 8. Association and dissociation of ZPI from FIXa in surface plasmon resonance studies. FIXa was captured on a monoclonal anti-FIX-coupled channel in a Biacore instrument as described under “Experimental Procedures.” Free FIXa was washed away, and bound FIXa dissociated at a negligible rate (not shown). ZPI was then injected at increasing concentrations of ZPI of 9.0, 13.5, 18, 27, 32.6, and 36 nM, resulting in the increasing series of sensorgrams shown. In each case, response units (RU) in a blank channel treated the same way but without coupled antibody were subtracted from response units in the experimental channel. A, no FVIIIa was included. B, 12 nM FVIIIa was included between the FIXa-binding step and the ZPI-binding step as described under “Experimental Methods.” Individual sensorgrams were superimposed and subjected to global analysis, with the results shown in TABLE ONE.

TABLE ONE

| Condition          | \( k_{\text{on}} \) [M\(^{-1}\)s\(^{-1}\)] | \( k_{\text{off}} \) [s\(^{-1}\)] | \( K_{\text{off}} = k_{\text{off}}/k_{\text{on}} \) [M] |
|--------------------|---------------------------------|-------------------------------|---------------------------------|
| FVIIIa absent      | 5.4 ± 0.2 × 10^9                | 6.3 ± 0.2 × 10^3              | 12 × 10^{-9}                    |
| FVIIIa present     | 4.7 ± 0.2 × 10^9                | 4.2 ± 0.1 × 10^{-3}           | 8.9 × 10^{-9}                   |

FVIIIa activation. FVIIIa stability was probably improved after binding to FIXa, which is known to stabilize FVIIIa (27). Protein Z and phospholipids were not required for binding of ZPI to FIXa (Fig. 8). Although ZPI association and dissociation were superimposed on the dissociation of FIXa from the antibody, the \( k_{\text{off}} \) for FIXa from the antibody was only 4.2 × 10^{-7} s\(^{-1}\), which should have a negligible effect on the calculations for ZPI association and dissociation from FIXa.

**DISCUSSION**

Since at least half of adult deaths in the developed countries involve unwanted blood clots, regulation of blood coagulation is of high interest. Much can be learned from the study of how natural anticoagulant proteins such as ZPI work, and ZPI was shown to be a novel and potent inhibitor of FIXa. The studies presented here showed that FIXa is also efficiently and rapidly inhibited by ZPI. Since the product of FIXa in the FXase complex is FIXa, which can also be inhibited by FXa, this complication made it necessary to demonstrate inhibition of FIXa by several independent methods.

During our initial experiments, we found that FXa inhibition by ZPI could be reversed to an even greater extent than reported previously (3) by dilution into EDTA-containing buffer. This was used to advantage to monitor FXa activity generated by the FXase complex after allowing time for any FXa inhibition to be reversed. Then, using immunobLOTS, it was possible to show that the physical quantities of FXa generated in the presence of physiologic levels of ZPI/protein Z were substantially less than in their absence. Using labeled FX, it was possible to demonstrate that release of trichloroacetic acid-soluble activation peptide from FX by FIXa was significantly inhibited by ZPI/protein Z. The amidolytic activity of FIXa was also inhibited by ZPI. Finally, ZPI prolonged FXa-based plasma clotting times in a manner only modestly dependent on protein Z. Thus, inhibition of FIXa or FXa activity by ZPI was demonstrated by several different approaches.

In the time frame of FXase experiments such as in Fig. 2, B and C, about 1 nM FXa was produced in the absence of FVIIIa or ZPI, and about 10 nM was produced in the presence of FVIIIa and in the absence of ZPI. Thus, the amount of ZPI sequestered from FIXa due to interaction with the FXa product was negligible in the absence of FVIIIa but was significant in the presence of FVIIIa. Since 50% FXa inhibition was achieved at 1.3 μg/ml (18 nM) ZPI even in the face of competing interaction with FXa, the efficiency of FIXa inhibition by ZPI was suggested to be comparable with the efficiency of FXa inhibition. This is supported by data in Fig. 4 that compare inhibition of FXase with inhibition of prothrombinase.

Characteristics of ZPI inhibition of FXa or FIXa differed from those of typical serpins. Requirements for a protein cofactor and Ca\(^{2+}\) are very unusual, although plasminogen activator inhibitor-1 (PAI-1) is stabilized and localized to surfaces by vitronectin (28). Atypically, ZPI inhibition was rapid but plateaued at a level dependent on the concentration of FIXa. This behavior was quite unusual; when a serpin is used in excess to a target protease, activity loss is usually linear with time on a semilog plot (pseudo-first-order), and the slope of the line depends on the concentration of serpin inhibitor. These data suggested that ZPI can associate and dissociate from FIXa in an equilibrium interaction, and subsequent surface plasmon resonance experiments confirmed this. The \( K_{\text{on}} \) was 9–12 nM, similar to the I\(_{50}\) for inhibition of FXase. Consistent with the association/dissociation characteristics of ZPI interaction with FIXa, we were unable to detect covalent complexes of these molecules on gels. Nor were covalent complexes of ZPI with FIXa detected in previous studies (3), consistent with the reversible inhibition demonstrated here.

Reversibility of inhibition is also unusual for a serpin, although inhibition of kallikrein by protein C inhibitor and inhibition of human kallikrein-2 by PAI-1 (29) are accompanied by significant cleavage and inactivation of the respective inhibitors without covalent complex formation; thus, inhibition is potentially reversible in those cases. Significant cleavage of ZPI by FXa probably did not occur during the time frame of the experiments since activity was not regained in experiments similar to those in Fig. 4.

Even modest concentrations (20 pm) of FVa partly protected 0.5 nM FXa from ZPI/protein Z inhibition, and pre-exposure of FVa to a 3-fold molar excess of FVa completely protected FXa from ZPI inhibition, consistent with earlier studies. In contrast, 80 pm FVIIIa did not appear to protect 2 nM FIXa from ZPI. Furthermore, although inhibition of FXa is strictly protein Z-dependent, inhibition of FIXa in the presence of FVIIIa did not require protein Z, suggesting a different mechanism of inhibition. However, we cannot exclude that protein Z might have an effect on FIXa/FVIIIa inhibition under some particular set of physio-
logic conditions. Although it has been hypothesized that protein Z could enable ZPI to localize near the membrane surface (30), protein Z does not appear to fill this role in the case of FIXa or FXIa inhibition. From what is known about affinities of vitamin K-dependent factors for membrane surfaces (31), we would expect that most of 1–10 nM FXa or FIXa would be bound to the surface of 25 μM phospholipid vesicles. Thus, it is possible that ZPI has a greater affinity for the membrane-bound conformation of FIXa than for the membrane-bound conformation of FXa, in which no significant inhibition is observed unless protein Z is present.

The membrane-bound conformation of FIXa/FVIIIa complex might be yet more favorable for ZPI interaction than the membrane-bound conformation of FIXa alone. It is curious that in the absence of protein Z, ZPI inhibition of FIXa was pre-exposed to a large molar excess of FVIIIa, the efficiency of ZPI inhibition was decreased (Fig. 3). We speculate that FVIIIa promotes a conformation of FIXa that is favorable for ZPI interaction but could enable ZPI to localize near the membrane surface (30), protein Z is present.

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