Heparin Is a Major Activator of the Anticoagulant Serpin, Protein Z-dependent Protease Inhibitor*

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Protein Z-dependent protease inhibitor (ZPI) is a recently identified member of the serpin superfamily that functions as a cofactor-dependent regulator of blood coagulation factors Xa and XIa. Here we provide evidence that, in addition to the established cofactors, protein Z, lipid, and calcium, heparin is an important cofactor of ZPI anticoagulant function. Heparin produced 20–100-fold accelerations of ZPI reactions with factor Xa and factor XIa to yield second order rate constants approaching the physiologically significant diffusion limit (k_a = 10^6 to 10^7 M^{-1} s^{-1}). The dependence of heparin accelerating effects on heparin concentration was bell-shaped for ZPI reactions with both factors Xa and XIa, consistent with a template-bridging mechanism of heparin rate enhancement. Maximal accelerations of ZPI-factor Xa reactions required calcium, which augmented the heparin acceleration by relaying Glα domain inhibition as previously shown for heparin bridging of the antithrombin-factor Xa reaction. Heparin acceleration of both ZPI-protease reactions was optimal at heparin concentrations and heparin chain lengths comparable with those that produce physiologically significant rate enhancements of other serpin-protease reactions. Protein Z binding to ZPI minimally affected heparin rate enhancements, indicating that heparin binds to a distinct site on ZPI and activates ZPI in its physiologically relevant complex with protein Z. Taken together, these results suggest that whereas protein Z, lipid, and calcium cofactors promote ZPI inhibition of membrane-associated factor Xa, heparin activates ZPI to inhibit free factor Xa as well as factor XIa and therefore may play a physiologically and pharmacologically important role in ZPI anticoagulant function.

Protein Z-dependent protease inhibitor (ZPI)2 is one of the more recently characterized serpin superfamily proteins encoded by the human genome (1–3). This serpin circulates in blood plasma as a high affinity complex with its vitamin K-dependent cofactor protein, protein Z (4). As its name implies, ZPI is dependent on binding to protein Z as well as on two other cofactors, lipid and calcium, to promote the inhibition of its target protease, blood coagulation factor Xa (2). Calcium is needed to mediate the binding of the ZPI-protein Z complex and factor Xa to a lipid vesicle to allow rapid inhibition of the membrane-associated protease through a template-bridging mechanism (5–7). The recent x-ray structures of the ZPI-protein Z complex reported by our group and another group together with modeling of the ternary ZPI-protein Z-factor Xa complex on a membrane and complementary mutagenesis studies have demonstrated that exosite interactions between ZPI-protein Z complex and factor Xa are responsible for the specificity of ZPI for membrane-associated factor Xa (6, 8–10). ZPI is also a specific inhibitor of coagulation factor XIa, but rapid inhibition in this case does not require cofactors (5, 7).

The importance of ZPI and protein Z as anticoagulant regulators of factors Xa and XIa is suggested by the observations that deficiencies in either the serpin or its cofactor protein result in an increased risk of thrombosis and peripheral arterial diseases, especially when combined with other risk factors (11, 12). Moreover, complete deficiency of ZPI or protein Z in mice when combined with the factor V Leiden mutation results in thrombosis or embryonic lethality, respectively, supporting an important role in anticoagulant regulation of factor Xa and factor XIa (13, 14). Interestingly, the glycosaminoglycan, heparin, which is known to activate a number of serpins that circulate in blood, was found to enhance the rates of ZPI inhibition of factor Xa and factor XIa, but the enhancement was only a modest 2–3-fold (7). Heparin has been shown to bind ZPI and was originally used as an affinity ligand to purify ZPI from blood plasma (2).

In the present study, we have made a detailed kinetic investigation of the accelerating effects of heparin on ZPI reactions with factor Xa and factor XIa. Surprisingly, our studies reveal that heparin is a much more substantial activator of ZPI anticoagulant function than was previously appreciated. Heparin rate enhancements of ~100-fold for ZPI inhibition of fXa and ~20-fold for inhibition of fXIa were found, resulting in physiologically and pharmacologically significant association rate constants of 10^6 to 10^7 M^{-1} s^{-1}, approaching the diffusion limit. The former rate enhancement is dependent on calcium ions, in agreement with previous findings of a calcium requirement for maximal heparin acceleration of antithrombin inhibition of factor Xa (15). Rate enhancements of both reactions are optimal at heparin concentrations and heparin chain lengths comparable with those that activate other serpin-protease reactions of physiologic significance, and the bell-shaped dependence of
the rate enhancements on heparin concentration implies a template-bridging mechanism similar to that mediating other serpin-protease reaction rate enhancements (16, 17). Importantly, complexation of ZPI with its cofactor, protein Z, as occurs in blood plasma, minimally affects these rate enhancements. Together, these results suggest that whereas protein Z acts to target ZPI to a membrane to regulate membrane-associated factor Xa at a site of vascular injury, heparin may act as an additional physiologically important cofactor to allow ZPI to regulate free factor Xa as well as factor Xla and thereby play a significant role in ZPI anticoagulant function.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant human ZPI was expressed in baculovirus-infected insects cells and purified by successive SP-Sepharose, Mono S, and Sephacyr S200 chromatography steps, as in previous studies (5, 6). Recombinant protein Z was judged >95% pure by SDS-PAGE analysis. Molar concentrations of ZPI were determined from the absorbance at 280 nm using a molar extinction coefficient of 31,525 M⁻¹ cm⁻¹ calculated from the amino acid sequence (18). Human ZPI and human antithrombin were purified from blood plasma as reported previously (2, 19). Human factor Xa, human protein Z, and human factor Xla purified from blood plasma were purchased from Enzyme Research Laboratories (South Bend, IN). Protein Z preparations were judged fully functional based on the ability of stoichiometric levels to shift the electrophoretic mobility of ZPI on native PAGE and to maximally accelerate ZPI inhibition of factor Xa in the presence of lipid and calcium (5). Recombinant Glá domainless factor Xa was expressed and purified as described (15). Protease concentrations were determined by standard assays with chromogenic substrates that were calibrated with proteases of known active site concentration (20).

Heparin—Full-length heparins with narrow molecular weight distributions and average chain lengths of ~26, ~50, and ~72 saccharides were purified from commercial heparin by repeated gel exclusion chromatography, as described previously (19). These heparins were additionally fractionated by antithrombin-agarose affinity chromatography, yielding chains possessing the pentasaccharide binding sequence for antithrombin (21). 26-Saccharide heparin chains lacking the pentasaccharide were obtained as the fraction not binding to the antithrombin affinity column after repeated chromatography to remove all high affinity binding species (22). Chromatography of ~100-µg samples of each heparin on a Superdex 200 HR 10/30 size exclusion column (GE Healthcare) that was equilibrated and run in 0.2 M NaCl at 0.5 ml/min confirmed that each heparin eluted in a narrow molecular weight range with an average size consistent with previous measurements on a larger column calibrated with standard heparins (19). Molar concentrations of heparins were determined from the weight concentration obtained by an Azure A dye binding assay and the molecular weight (19). A synthetic heparin pentasaccharide corresponding to the binding sequence for antithrombin (fondaparinux), unfractionated heparin (Heparin Na), and low molecular weight heparin (Nadroparina Na) were generously provided by Sanofi-Synthelabo Research (Toulouse, France). The fondaparinux concentration was determined by stoichiometric fluorescence titrations of antithrombin with the saccharide as described (19). Molar concentrations of unfractionated heparin and low molecular weight heparin were calculated based on reported average molecular weights of 15,000 and 4,500, respectively.

Experimental Conditions—All solution phase experiments were conducted in 50 mM Hepes buffer, pH 7.4, containing 0.1 M NaCl and 0.1% PEG 8000, at 25 °C unless specified otherwise.

Stoichiometries of ZPI Reactions with Proteases—Fixed concentrations of protease (~100 nm factor Xa or ~20 nm factor Xla active sites) were reacted with increasing concentrations of ZPI, ranging from an ~3- to 7-fold molar excess in the absence or presence of protein Z equivmolar with the ZPI concentration, in the absence or presence of 5 mM CaCl₂ and an optimal concentration of heparin in reaction volumes of 50 µl. Stoichiometries of ZPI reactions with factor Xa in the absence of heparin were determined at ~10-fold higher factor Xa concentrations. After allowing sufficient time to reach a reaction end point, based on measured second order association rate constants (~5–20 min), remaining proteolytic activity was measured by adding 1 ml of either 100 µM Spectrozyme Xa (American Diagnostica, Greenwichtown, CT) for factor Xa reactions or 100 µM S2366 (Diapharma, West Chester, OH) for factor Xla reactions, each containing 50–100 µg/ml Polybrene, and monitoring the rate of absorbance change at 405 nm. Aliquots of ZPI-factor Xa reactions in the absence of heparin were diluted into 1 ml of substrate for activity measurement. Initial rates of substrate hydrolysis were determined from computer fits of substrate hydrolysis progress curves by a second order polynomial function as described (5). The stoichiometry of inhibition (SI) was determined from the fitted abscissa intercept of a linear plot of residual protease activity against the molar ratio of inhibitor to protease (5).

Kinetics of ZPI-Protease Reactions—Heparin effects on the kinetics of ZPI-protease reactions were evaluated under pseudo-first order conditions (i.e. with a large molar excess of inhibitor over protease) by incubating reaction mixtures of 20–100 µl containing 20–70 nM ZPI, 0.1–1 nM factor Xa, Glá domainless factor Xa, or factor Xla and varying levels of heparin ranging from 10⁻⁹ to 10⁻⁴ M in standard pH 7.4 Hepes buffer with or without 5 or 1.5 mM CaCl₂ for reaction times of 0.5–6 min. The effect of protein Z on reactions was assessed by including protein Z equivmolar with ZPI in the reaction. The dependence of heparin-accelerated reaction rates on calcium concentration was determined at an optimal heparin concentration and varying CaCl₂ concentrations in the range 0.01–25 mM. Phospholipid effects on reactions were assessed by including 25 µM synthetic lipid vesicles consisting of 70% dioleoylphosphatidylcholine and 30% dioleoylphosphatidylserine (Avanti Polar Lipids, Alabaster, AL) that were prepared as described (5). Reactions were quenched by adding 1 ml of either 50 µM Pefafuor Xa substrate (Centerchem, Norwalk, CT) for factor Xa reactions or ~30 µM Boc-Glu-(OBzl)-Ala-Arg-7-amido-4-methylcoumarin (Bachem) for factor Xla reactions, each containing 50–100 µg/ml Polybrene and in some cases also 10 mM EDTA. The initial linear rate of substrate hydrolysis was monitored at an excitation wavelength of 380 nm and an emission wavelength of 440
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nm. The observed pseudo-first order rate constant for reactions was measured from the equation,

$$k_{obs} = \ln((v_o - v_a)/(v_t - v_a))/t$$

(Eq. 1)

where \(v_o\) and \(v_a\) are the initial velocities of protease hydrolysis of the substrate after reaction with ZPI for the fixed reaction time and after reaching the reaction end point, respectively, \(v_t\) is the velocity measured in control reactions without ZPI, and \(t\) is the fixed reaction time. Reaction end points were determined from full progress curves of protease inhibition measured in the absence and presence of optimal heparin concentrations. The nonzero end points obtained in full reaction time courses were due to both small amounts of degraded protease more resistant to inhibition (<5%) and the reaching of a steady-state end point for slow reactions that reflected a balance between the rates of complex formation and complex dissociation (5, 6). Apparent second order association rate constants \(k_{app}\) were calculated by dividing \(k_{obs}\) by the total ZPI concentration. The dependence of \(k_{obs}\) on heparin concentration was computer-fit by nonlinear least squares analysis by the ternary complex bridging model equation (22),

$$k_{obs} = k_d + k_{a-H} \times [ZPI]_o + k_{a-T} \times [ZPI-H] \times K_{Pr,H}(K_{Pr,H} + [H])$$  \(+ k_{a-B} \times [H]/(K_{Pr,H} + [H])$$

(Eq. 2)

where

$$[ZPI-H] = ([ZPI]_o + [H]_o + K_{zpi,h} - (([ZPI]_o + [H]_o + K_{zpi,h})^2 - 4[ZPI]_o[H]_o)^{1/2})/2$$

(Eq. 3)

$$[ZPI] = [ZPI]_o - [ZPI-H]$$  \(= [H]_o - [ZPI-H]$$

(Eq. 4)

In Equations 2–5, \(k_d\) is the first order rate constant for ZPI-protease complex dissociation, \(k_{a-H}\) is the second order rate constant for association of free ZPI and free protease, \(k_{a-T}\) is the second order rate constant for association of ZPI-heparin binary complex with free protease to form a ternary bridging complex, \(k_{a,B}\) is the second order rate constant for the association of ZPI-heparin and protease-heparin binary complexes, \([ZPI]_o\) and \([H]_o\) are total ZPI and heparin concentrations, \([ZPI-H]\) is the concentration of ZPI-heparin binary complex, \([ZPI]_o\) and \([H]_o\) are the free concentrations of ZPI and heparin, and \(K_{ZPI,H}\) and \(K_{Pr,H}\) are the dissociation constants for ZPI binding to heparin and for protease binding to heparin to form binary complexes, respectively. \(k_d\) was fixed at previously measured values because these were found not to be affected by heparin (5, 6). The association rate constant in the absence of heparin \(k_{a-H}\) was measured independently from full reaction progress curves. Because \(k_{a-B}\) was not well determined and could not be distinguished from the rate constant in the absence of heparin, it was fixed at the latter value. The fitted parameters were \(k_{a-T}\), \(K_{ZPI,H}\), and \(K_{Pr,H}\).

Reactions of antithrombin with factor Xla in the presence of varying heparin concentration with or without 5 mM CaCl2 were performed similar to ZPI reactions using 35 nm antithrombin, 0.3 nm factor Xla, and 10 \(^{-10}\) to 10 \(^{-7}\) M heparin and a fixed reaction time of 2.5 min. \(k_{obs}\) was calculated as for ZPI reactions, and the dependence of \(k_{obs}\) on heparin concentration was fit using the ternary complex bridging model equation above with antithrombin replacing ZPI except that \(k_{a,B}\) was fit as an additional parameter.

Full reaction progress curves were measured for ZPI-protease reactions in the absence and presence of optimal heparin concentrations at fixed 0.1–0.2 nM protease concentrations, as a function of the ZPI concentration in the range 5–200 nM, with or without protein Z equimolar with ZPI, with or without 5 mM CaCl2, and with or without 25 \(\mu\)M lipid. In this case, identical reaction mixtures were quenched at varying reaction times with substrate, and the time course of protease inactivation was measured from the decrease in initial rates of substrate hydrolysis. The first order rate of decrease in protease activity with time was computer-fit by a single exponential decay function with a nonzero end point and \(k_{obs}\) obtained from the fitted exponential decay constant. Second order association rate constants were obtained from the slopes of linear plots of \(k_{obs}\) versus the ZPI concentration according to the equation,

$$k_{obs} = k_d + k_a \times [ZPI]_o$$

(Eq. 6)

in which \(k_a\) was fixed at values determined independently in previous studies (5, 6).

Binding of Proteins to Heparin-Agarose—Samples of 15–25 \(\mu\)g of protein in 0.1 ml of equilibrating buffer were applied to a 1-ml Hi-Trap heparin column (GE Healthcare) equilibrated in 20 mM Hepes, 0.1 M NaCl, pH 7.4, with or without 5 mM CaCl2. After washing with several column volumes of equilibrating buffer, a 30-ml gradient from 0.1 to 0.55 M NaCl was applied at a flow rate of 1 ml/min, and the protein fluorescence of the eluate was continuously monitored. The salt concentration corresponding to the protein elution peak was determined from the relation,

$$0.1 + 0.45 \times (V_e - V_o)/(V_t - V_o)$$

(Eq. 7)

where \(V_o, V_e,\) and \(V_t\) are the volume at the start of the gradient, the volume corresponding to the elution peak of the protein, and the volume at the end of the gradient, respectively.

RESULTS

Heparin Accelerating Effects on the ZPI-Factor Xa Reaction—Previous studies have shown that heparin accelerates the inhibition of factor Xa by the serpin, ZPI, to a modest extent (~3-fold) as compared with the ~2000-fold acceleration produced by the cofactors, protein Z, phospholipid, and calcium ions (5, 7). To determine the potential physiologic relevance of the heparin accelerating effect, we studied its dependence on heparin concentration and heparin chain length as well as whether it was affected by calcium, protein Z, and lipid cofactors using recombinant ZPI. The kinetics of factor Xa inhibition by ZPI were measured in the absence and presence of an ~50-saccharide heparin and with or without 5 mM calcium ions under pseudo-first order conditions as a function of heparin concentration (Fig. 1A). Heparin significantly accelerated the rate of factor Xa inactivation by ZPI. The observed pseudo-first order rate constant \((k_{obs})\) obtained from the fitted exponential pro-
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FIGURE 1. Accelerating effects of heparin on ZPI inhibition of factor Xa in the absence and presence of calcium. A, progress curves are shown for reactions of 68 nM ZPI with 0.15 nM factor Xa in the absence (circles) and presence of 1.4 μM 50-saccharide heparin (H50) (triangles) either with or without 5 mM Ca2+ (closed and open symbols). Factor Xa activity controls without ZPI are shown in the absence of calcium with or without heparin (squares bisected by right or left diagonal lines) or in the presence of calcium with or without heparin (closed and open squares). Residual factor Xa activity was measured by quenching reactions at varying times by dilution into fluorogenic substrate and measuring the initial rate of substrate hydrolysis relative to a control without inhibitor, as described under “Experimental Procedures.” Solid lines, fits by a single exponential decay function with a nonzero end point. B, dependence of k_{app} for ZPI-factor Xa reactions measured as in A on the concentration of ZPI. Solid lines are linear regression fits with the intercept fixed at the independently measured rate constant for complex dissociation. C, dependence of the apparent second order rate constant (k_{app} = k_{obs}/[ZPI]) for the reaction of 66 nM ZPI with 1 nM factor Xa in the presence of 1.4 μM 50-saccharide heparin on calcium concentration. Reactions were allowed to proceed for a fixed time of 1.5 min and quenched with substrate, and residual factor Xa activity was measured relative to an uninhibited control as in A. k_{obs} was calculated, assuming an exponential decay of factor Xa activity. Solid line, a fit of the data assuming that a higher affinity noncooperative calcium interaction augments the heparin acceleration and a lower affinity cooperative calcium interaction diminishes the heparin acceleration.

TABLE 1

| ZPI ± heparin ± PZ | k_{app} | SI | k_{app} × SI |
|-------------------|--------|----|-------------|
|                   | M⁻¹ s⁻¹ | mol/mol E | M⁻¹ s⁻¹ |
| ZPI               | 9.5 ± 0.6 × 10⁸ | 3.6 ± 0.4 | 3.4 ± 0.6 × 10⁹ |
| ZPI + PZ          | 5.6 ± 0.2 × 10⁹ | 6.8 ± 0.1 | 3.8 ± 0.2 × 10⁹ |
| ZPI + H26         | ND     | ND   | ND         |
| ZPI + H50         | 6.3 ± 0.3 × 10⁹ | 3.5 ± 0.1 | 2.3 ± 0.2 × 10⁹ |
| ZPI + H50 + PZ    | 2.9 ± 0.1 × 10⁹ | 4.0 ± 0.1 | 1.2 ± 0.1 × 10⁹ |
| ZPI + H72         | 1.1 ± 0.1 × 10⁹ | 2.7 ± 0.1 | 3.0 ± 0.4 × 10⁹ |
| ZPI + H72 + PZ    | 1.1 ± 0.1 × 10⁹ | 2.8 ± 0.1 | 3.1 ± 0.4 × 10⁹ |

k_{app} and SI values for unaccelerated and heparin-accelerated reactions of ZPI with factor Xa in the absence and presence of 5 mM calcium ions and with or without protein Z.

Rate constants and stoichiometries for ZPI-factor Xa reactions in I = 0.15, pH 7.4, buffer at 25 °C with or without 5 mM Ca²⁺, protein Z (PZ) equimolar with ZPI, and I ≈ 1 μM 26-saccharide (H26), 50-saccharide (H50), and 72-saccharide (H72) heparins were measured as described under “Experimental Procedures.” Apparent second order rate constants were obtained from the slopes of the linear dependence of pseudo-first order rate constants on ZPI concentrations in the range 15–300 nM. The intercept of these plots was fixed at the independently measured value of 1.8 × 10⁻⁴ s⁻¹. The product of k_{app} and SI represents the corrected association rate constant for reaction through the inhibitory pathway.

progress curve for the reaction increased by ~7-fold when the glycosaminoglycan was present at an optimal concentration of ~1 μM. Notably, the effect of heparin on k_{obs} was markedly augmented in the presence of calcium, although calcium decreased the rate constant in the absence of heparin. The overall heparin rate enhancement thus increased under these conditions to a substantial ~30-fold. k_{obs} increased linearly with increasing ZPI concentration in the range of 60–140 nM when heparin was fixed at ~1 μM in the absence or presence of calcium (Fig. 1B), consistent with a bimolecular reaction between ZPI and factor Xa with no evidence for saturation of an intermediate Michaelis complex. Apparent bimolecular association rate constants (k_{app}) for these reactions were obtained from the slopes of the lines (Table 1). To determine the dependence of the calcium-augmented heparin rate enhancement on calcium concentration, k_{obs} for the ZPI-factor Xa reaction was measured at an optimal heparin concentration as a function of the calcium concentration. Calcium maximally augmented k_{app} at ~1.5 mM to an extent that was ~2-fold greater than the augmentation at 5 mM calcium, with higher concentrations reversing the augmentation (Fig. 1C). Optimal levels of calcium thus result in an overall ~60-fold heparin enhancement of k_{app} for the ZPI-factor Xa reaction. Indistinguishable calcium-dependent rate-enhancing effects of heparin on the ZPI-factor Xa reaction were found with ZPI purified from human plasma, despite ~2-fold lower absolute values of k_{app} for plasma than recombinant ZPI reactions both in the absence and presence of heparin and calcium (not shown). The inclusion of physiologic levels of magnesium ions (0.5 mM) had no effect on calcium-dependent heparin rate enhancements at either 1.5 or 5 mM calcium.

The dependence of k_{app} for the ZPI-factor Xa reaction on heparin concentration was bell-shaped both in the absence and presence of calcium ions with the maximal rate enhancement occurring at similar micromolar heparin concentrations (Fig. 2A). These findings were consistent with heparin acceleration being due to a template-bridging mechanism in which heparin binds both proteins and promotes their interaction in a ternary complex (22). Moreover, they were in keeping with previous
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The observation of second order reaction kinetics for the heparin-accelerated ZPI-factor Xa reaction at the nearly physiologic ZPI concentrations employed indicated that the ternary bridging complex is preferentially formed through a bimolecular reaction between the higher affinity protein-heparin binary complex and the other free protein (23). Fitting of the bell-shaped curves in Fig. 2A by the equation for the bridging mechanism provided the affinities of the binary protein-heparin complexes involved in ternary complex formation as well as the rate constants for reaction of ZPI and factor Xa within the ternary complex. Heparin bridging enhanced second order rate constants for the ZPI-factor Xa reaction 6- and 34-fold in the absence and presence of 5 mM calcium, respectively (Table 2). Because previous studies had established a $K_{D}$ for the factor Xa-heparin binary complex interaction in the micromolar range, the $K_{D}$ of 100–200 nM determined for the higher affinity binary protein-heparin complex that mediates ternary complex assembly in the ascending limb of the bell-shaped curves could be ascribed to the formation of a ZPI-heparin binary complex. The descending limbs thus reflect the antagonism of ternary complex formation by the increasing molar excess of free heparin chains over ZPI-bound chains that compete for the limiting factor Xa by forming factor Xa-heparin binary complexes (22). The fitted curves yielded a $K_{D}$ of $\sim$20 $\mu$M in the absence of calcium and $\sim$6 $\mu$M in the presence of calcium for this binary complex interaction. Because the $K_{D}$ values for the ZPI and factor Xa binary complex interactions are well separated in affinity, the optimal heparin rate enhancement is reached at a heparin concentration corresponding to saturation of the ZPI-heparin binary complex ($\sim$1 $\mu$M).

The reaction of ZPI with factor Xa by the branched pathway suicide substrate mechanism of serpins is not completely efficient in that about three molecules of ZPI are required to inhibit one molecule of factor Xa (5). This is because about two ZPI molecules are cleaved by factor Xa through a competing substrate pathway for every molecule that inhibits factor Xa. To determine whether heparin and calcium rate-enhancing effects on the ZPI-factor Xa reaction involve a change in inhibition efficiency, we measured the SI in the absence and presence of $\sim$1 $\mu$M heparin and 5 mM calcium. Heparin produced a marginally significant reduction in the SI more evident in the presence than in the absence of calcium, indicating a modest increase in inhibition efficiency for the heparin-accelerated reaction (Table 1).
Mechanism of Calcium Enhancement of Heparin Accelerating Effects—To determine whether calcium augmented the heparin rate enhancement of the ZPI-factor Xa reaction by alleviating an inhibitory effect of the factor Xa Gla domain, we evaluated whether removing the Gla domain would augment the heparin rate enhancement in a manner similar to that of calcium. As expected, heparin produced a greater acceleration of the reaction of ZPI with Gla domainless factor Xa (GD-factor Xa) than with full-length factor Xa in the absence of calcium (Fig. 2B), confirming a large inhibitory effect of the Gla domain on heparin acceleration. However, removal of the Gla domain produced a far greater increase in heparin acceleration of the ZPI-factor Xa reaction than did the addition of calcium. The reason for this became clear when it was found that calcium inhibits the heparin acceleration of the ZPI-GD-factor Xa reaction, resulting in an acceleration similar to that of the ZPI-full-length factor Xa reaction in the presence of calcium (Fig. 2B). Calcium shifted the bell-shaped dependence of the heparin acceleration of the ZPI-GD-factor Xa reaction on heparin concentration to higher heparin concentrations and reduced the maximal heparin acceleration. This indicated that calcium inhibits the heparin acceleration by reducing the affinity of both ZPI and GD-factor Xa interactions for heparin and hence the ability of heparin to bridge ZPI and GD-factor Xa in a ternary complex (Table 2). Calcium inhibition of the heparin-accelerated ZPI-GD-factor Xa reaction showed a monophasic dependence on calcium concentration (Fig. 2C) that paralleled the inhibitory phase of the calcium dependence of heparin acceleration of the ZPI-full-length factor Xa reaction (Fig. 1C). Magnesium ions (0.5 mM) had no effect on this inhibition.

To provide direct evidence for the ability of calcium to both promote and inhibit heparin bridging of ZPI and factor Xa, we determined the relative affinities of the proteins for heparin by measuring the salt concentration required to elute the proteins from a heparin-agarose affinity column in the absence and presence of 5 mM calcium (Table 3). ZPI eluted from the heparin column at a much higher salt concentration than full-length factor Xa in the absence of calcium, indicating that ZPI bound heparin much tighter than full-length factor Xa under these conditions, in agreement with the affinities derived from the bell-shaped dependence of heparin acceleration on heparin concentration. Full-length factor Xa also bound to the heparin column much more weakly than GD-factor Xa in the absence of calcium, consistent with the Gla domain being responsible for the lower heparin affinity. Calcium greatly enhanced the binding of full-length factor Xa to the heparin column to an extent that resulted in identical salt concentrations for elution of full-length factor Xa and GD-factor Xa from the immobilized heparin. This was consistent with calcium relieving the inhibitory effect of the Gla domain on the factor Xa-heparin interaction. By contrast, calcium produced small or no reductions in the affinity of ZPI and GD-factor Xa for immobilized heparin, suggesting at most a marginal inhibitory effect of calcium on these protein-heparin interactions.

| Protein         | NaCl concentration required to elute protein from heparin-Sepharose | Without Ca$$^{2+}$$ | With 5 mM Ca$$^{2+}$$ |
|-----------------|-------------------------------------------------------------------|----------------------|-----------------------|
| ZPI             | 0.36                                                              | 0.34                 |                       |
| Factor Xa       | 0.16                                                              | 0.34                 |                       |
| GD-factor Xa    | 0.34                                                              | 0.34                 |                       |
| Factor Xa       | 0.33                                                              | 0.32                 |                       |

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Table 3: Relative affinities of ZPI and proteases for heparin-Sepharose

~100-μg samples of protein in 0.1 ml of 0.015 M, pH 7.4, equilibrating buffer were applied to a 1-ml Hi-Trap heparin column and eluted with a salt gradient from 0.1 to 0.55 M NaCl. Salt concentrations corresponding to the peak elution position for the protein based on continuous monitoring of the eluant by protein fluorescence were calculated from the elution volume. Details are provided under “Experimental Procedures.” Based on replicate runs, errors in salt concentrations were estimated to be ±0.01 M.

Protein Z and Lipid Effects on the Heparin-accelerated ZPI-Factor Xa Reaction—Because ZPI circulates in plasma as an equimolar complex with its cofactor, protein Z, we evaluated whether protein Z binding to ZPI affected the ability of heparin to accelerate the ZPI-factor Xa reaction. Protein Z binding was previously shown to reduce the apparent rate of ZPI inhibition of factor Xa in the absence of lipid and calcium cofactors solely because of a decreased efficiency of inhibition, as reflected by an increase in SI (5). Protein Z binding to ZPI produced a similar decrease in the apparent rate constant for the heparin-accelerated ZPI-factor Xa reaction in the presence of calcium that was accounted for by an increase in the SI (Table 1).

The effect of heparin on ZPI inhibition of membrane-associated factor Xa was evaluated in the absence of protein Z to determine whether membrane binding of factor Xa influenced the heparin rate enhancement. Heparin produced indistinguishable enhancements in $k_{obs}$ for the reaction of ZPI with factor Xa in the presence of calcium whether lipid was present or not (not shown), indicating that heparin is capable of bridging ZPI and factor Xa when factor Xa is membrane-bound. We next determined whether heparin affected the reaction of the ZPI-protein Z complex with factor Xa when both the serpin and protease were bound to a membrane through calcium-dependent interactions with the Gla domains of protein Z and factor Xa. Heparin at optimal levels (1 μM) decreased $k_{obs}$ for the reaction of 6 nm ZPI and equimolar protein Z with 0.2 nm factor Xa in the presence of calcium and lipid under bimolecular reaction conditions (i.e. below the $K_m$ for the membrane-dependent reaction) (5), from 0.0225 s$$^{-1}$$ to 0.0088 s$$^{-1}$$ (i.e. by ~3-fold), suggesting that heparin interactions with membrane-associated ZPI and factor Xa modestly reduced the rate of the reaction on the membrane.

Effects of Heparin Chain Length and Charge on Acceleration of the ZPI-Factor Xa Reaction—Heparin chain length has a significant effect on the ability of heparin to accelerate the antithrombin-factor Xa reaction as well as other serpin-protease reactions (15, 24). To determine whether chain length affected the extent of heparin enhancement of the ZPI-factor Xa reaction, we compared the effects of heparins ranging in length from 26 to 72 saccharides at a fixed concentration of ~1 μM in the presence of 5 mM calcium. The extent of heparin enhancement was found to increase as the heparin chain length increased with rate enhancements of 7-, 36-, and 77-fold for 26-, 50-, and 72-saccharide heparins, respectively (Fig. 3A). These rate enhancements were ~2-fold greater at 1.5 mM calcium. The SI for these reactions, although marginally reduced by the 50-saccharide heparin, was significantly decreased by the
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72-saccharide heparin (Table 1), indicating an improved efficiency of inhibition comparable with that induced by lipid and calcium cofactors (5). Plotting the rate enhancements after correction of \( k_{\text{app}} \) values for the SI as a function of chain length showed a proportional increase in rate enhancement with increasing chain length and a minimal extrapolated chain length of at least 18 saccharides in length for bridging ZPI and factor Xa (Fig. 3B), similar to the length requirements for heparin bridging antithrombin and factor Xa (15).

The heparin concentration dependence of the calcium-augmented heparin accelerations was bell-shaped for all heparins (Fig. 3C), indicating that the accelerations were all mediated by a similar ternary complex bridging mechanism. Moreover, \( k_{\text{app}} \) was proportional to the ZPI concentration when measured at optimal heparin concentrations over the range of 15–140 nM ZPI, indicating that all reactions were bimolecular under these conditions. Fitting of the bell-shaped curves indicated that the apparent \( K_D \) values for the binary protein-heparin complexes were significantly reduced as the chain-length increased, as is evident from the shift in the curves to lower heparin concentrations. This is in keeping with the increased number of binding sites on the longer chains for the proteins (25). The fits further showed that association rate constants for ZPI inhibition of factor Xa in the ternary bridging complex after correction for measured SIs were increased to \( \sim 10^{6} \text{ M}^{-1} \text{ s}^{-1} \) for the longer chain length heparins independent of protein Z binding to ZPI (Tables 1 and 2). Such rates imply that heparin effects on ZPI inhibition of factor Xa are physiologically significant.

The different chain length heparins examined all contained a specific pentasaccharide that binds the serpin, antithrombin, with high affinity (HA). To determine whether the pentasaccharide influences the accelerating effects of the different chain length heparins, we compared 26 saccharide heparins that contained (HA) or lacked (LA) this pentasaccharide for their ability to enhance the ZPI-factor Xa reaction rate. The pentasaccharide-containing HA-heparin produced a \( \sim 2 \)-fold greater rate-enhancing effect than the LA-heparin (Fig. 3, A and C). These modest differences are probably due to the higher sulfation of the pentasaccharide-containing chains than chains lacking this sequence (26), a reflection of the greater extent of processing of the pentasaccharide-containing chains along the biosynthetic pathway (27). That the pentasaccharide sequence itself was not important for heparin rate enhancement was suggested by the observations that (i) the pentasaccharide alone showed no ability to accelerate the ZPI-factor Xa reaction rate (Fig. 3B), and (ii) the pentasaccharide at levels equimolar with LA-heparin (1 μM) had no effect on the LA-heparin rate enhancement (not shown). These findings are consistent with heparin chains with greater sulfation having a higher affinity for ZPI and factor Xa due to their increased negative charge and consequently being more effective in bridging ZPI and factor Xa.

**Accelerating Effects of Unfractionated and Low Molecular Weight Heparins on the ZPI-Factor Xa Reaction**—To determine whether the rate-enhancing effects of heparin on the ZPI-factor Xa reaction were of pharmacologic significance with respect to heparins used clinically for anticoagulant therapy (20), we evaluated the effects of standard unfractionated heparin (UFH) and a low molecular weight heparin (LMWH) on the rate of the ZPI-factor Xa reaction in the presence of an optimal calcium concentration of 1.5 mM (Fig. 4). Unfractionated heparin produced a large \( \sim 100 \)-fold acceleration of the ZPI-factor Xa reaction at concentrations in the therapeutic range that was comparable with that observed with the longer chain length fractionated heparins. By contrast, LMWH produced a modest 8-fold accelerating effect that was similar to the shortest fractionated heparin chain examined (Table 2).

**Heparin Effects on ZPI Inhibition of Factor Xa**—Factor Xa is the only other well established target protease of ZPI (7). Heparin was found to also accelerate the inhibition of this protease by ZPI. However, the rate enhancement was much less than that observed for the reaction of ZPI with factor Xa, possibly because the ZPI reaction with factor Xa without heparin was...
already quite fast. Fig. 5A shows that the 50-saccharide heparin increased $k_{\text{obs}}$ for the ZPI-factor Xla reaction maximally $\sim$3-fold, with a bell-shaped dependence on heparin concentration suggestive of a template-bridging mechanism. Contrasting the agonist effects of calcium on the ZPI-factor Xa reaction, calcium antagonized the heparin acceleration of the ZPI-factor Xla reaction by shifting the bell-shaped curve to higher heparin concentrations. These results suggested that calcium diminishes the ability of heparin to bridge ZPI and factor Xla by reducing the affinity of the proteins for heparin in a manner similar to its effect on the ZPI-GD-factor Xa reaction. This would be in keeping with the absence of a membrane-binding Gla domain in factor Xla and thus with fully accessible heparin binding sites in the catalytic domain and apple domains of the protease (28). Fitting of the bell-shaped curves confirmed that calcium impaired bridging by weakening both binary and ternary protein-heparin complex interactions (Table 2).

To further demonstrate that calcium was a direct antagonist of protein-heparin bridging interactions, we examined the effects of calcium on the heparin-accelerated reaction of the serpin, antithrombin, with factor Xla. The 50-saccharide heparin accelerated the antithrombin-factor Xla reaction with a bell-shaped dependence on heparin concentration in the absence of calcium. Consistent with heparin accelerating this reaction by a similar ternary complex bridging mechanism (Fig. 5A). Notably, the heparin acceleration was more marked in the absence than in the presence of calcium, and the reduced acceleration in the presence of calcium resulted both from a lower maximal acceleration ($\sim$500-fold versus $\sim$2000-fold without calcium) and a shift of the bell-shaped curve to higher heparin concentrations. Fitting of the bell-shaped curves by the equation for the bridging mechanism confirmed that calcium impaired bridging by weakening protein-heparin binary and ternary complex interactions as with the ZPI-factor Xla reaction. It should be noted that the fitted limiting value of $k_{a,\text{app}}$ at high heparin concentrations represents a substantial residual rate enhancement that supports recent findings of an allosteric contribution to heparin rate enhancement involving heparin neutralization of repulsive interactions between antithrombin and factor Xla (28).

Heparin chain length affected the extent to which heparin accelerated the ZPI-factor Xla reaction, as was found for the ZPI-factor Xa reaction. Heparins of increasing chain length produced progressively greater heparin accelerations of the ZPI-factor Xla reaction in the presence of calcium and shifted the bell-shaped dependence of the acceleration to lower heparin concentrations (Fig. 6). This behavior paralleled that observed with the ZPI-factor Xa reaction in the presence of calcium and can be accounted for by the increased number of binding sites and consequent increased affinity of the longer heparin chains for ZPI and factor Xla. All heparin-accelerated reactions showed bimolecular reaction kinetics based on the observed linear dependence of $k_{\text{obs}}$ on ZPI concentration in the range of 5–50 nM at the optimal concentrations of these heparins. The second order rate constants determined from this dependence indicated maximal accelerations for 26-, 50-, and 72-saccharide heparins of 4-, 5-, and 17-fold, respectively (Table 4). Because heparin rate enhancements were smaller for the ZPI-factor Xla reaction, the binding parameters describing
versus ZPI concentration in the range 5–50 nM as in Table 1 except that the intercept was fixed at zero because of the negligible value for complex dissociation.

TABLE 4

| ZPI ± heparin ± PZ | $k_{\text{app}}$ | SI | $k_{\text{app}} \times SI$ | $k_{\text{app}}$ | SI | $k_{\text{app}} \times SI$ |
|------------------|----------------|-----|-----------------|----------------|-----|-----------------|
| ZPI              | 1.0 ± 0.1 $\times 10^5$ | 80 ± 0.3 | 1.1 ± $\times 10^6$ | 0.2 ± 0.1 $\times 10^4$ | 1.7 ± 0.2 | 9.9 ± 0.3 |
| ZPI + PZ         | 1.0 ± 0.1 $\times 10^5$ | 80 ± 0.3 | 1.1 ± $\times 10^6$ | 0.2 ± 0.1 $\times 10^4$ | 1.7 ± 0.2 | 9.9 ± 0.3 |
| ZPI + H50        | 1.0 ± 0.1 $\times 10^5$ | 80 ± 0.3 | 1.1 ± $\times 10^6$ | 0.2 ± 0.1 $\times 10^4$ | 1.7 ± 0.2 | 9.9 ± 0.3 |
| ZPI + H50 + PZ   | 1.0 ± 0.1 $\times 10^5$ | 80 ± 0.3 | 1.1 ± $\times 10^6$ | 0.2 ± 0.1 $\times 10^4$ | 1.7 ± 0.2 | 9.9 ± 0.3 |

FIGURE 6. Heparin chain length dependence of the accelerating effect of heparin on ZPI-factor Xa reactions. Dependence of $k_{\text{app}}$ for 50-saccharide (H50) (A) and 72-saccharide (H72) (B) heparin-accelerated reactions of 23 nM ZPI with 0.2–0.5 nM factor Xa and for 26-saccharide heparin (H26) accelerated reactions of 41 nM ZPI with 0.2 nM factor Xa (C), all in the presence of 5 mM Ca++. The accelerating effect on ZPI-factor Xa reactions with Ca++ was calculated from the residual factor Xa activity $k_{\text{app}}$ was calculated from linear plots of $k_{\text{obs}}$ versus ZPI concentration in the range 5–50 nM as in Table 1 except that the intercept was fixed at zero because of the negligible value for complex dissociation.

DISCUSSION

The results of this study provide compelling evidence that heparin is a potentially significant physiologic and pharmacologic activator of the serpin, ZPI, and thus suggest that heparin and heparin-like glycosaminoglycans may act as important cofactors of ZPI anticoagulant function. Heparin was found to accelerate the reactions of ZPI with factor Xa up to ~100-fold in a calcium-dependent manner and to accelerate the reaction with factor Xa up to ~20-fold independent of calcium to yield association rate constants of ~$10^5$ to $10^6$ M$^{-1}$ s$^{-1}$ that are in the diffusion-limited, physiologically relevant range. The maximal rate enhancements occur at heparin concentrations comparable with those that mediate physiologically significant accelerations of other serpin reactions, such as those of heparin cofactor II (22, 29), and at physiologic calcium concentrations. Prior studies did not consider heparin to be a significant effect of ZPI anticoagulant function based on findings of only modest ~2–3-fold heparin rate enhancements of ZPI reactions with factor Xa and factor Xa (7). Detailed investigations of the heparin effect, however, were not performed, and optimal concentrations of heparin and calcium were not used in studies of factor Xa inhibition.

In view of the fact that ZPI circulates in plasma as a tight complex with the cofactor, protein Z (4), it was important to find that protein Z binding to ZPI has little effect on heparin accelerations of ZPI-protease reactions. Heparin is thus a significant activator of ZPI in its physiologically relevant complex state with protein Z. Maximal apparent second order rate constants of 1–2 $\times 10^6$ M$^{-1}$ s$^{-1}$ were found for the reaction of heparin-activated ZPI-protein Z complex with factor Xa at optimal physiologic levels of calcium in the absence of a membrane, which contrast with values of 1–2 $\times 10^6$ M$^{-1}$ s$^{-1}$ for the reaction of ZPI-protein Z complex with factor Xa on a membrane surface (5, 6). Although the membrane reaction is clearly preferred, we found that the membrane reaction was slowed ~3-fold by heparin, presumably due to the ability of heparin to interact with its bridging sites on ZPI and factor Xa when the proteins are membrane-bound and to thereby affect the ZPI-factor Xa reaction. These observations suggest a potential means of shuttling of factor Xa and ZPI between membrane and glycosaminoglycan sites at a site of vascular injury that could ensure that ZPI inactivates factor Xa even if the proteins dissociate from a membrane. Matrix or cell surface heparin-like glycosaminoglycans may thus contribute to ZPI regulation of fac-
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**FIGURE 7.** Calcium effects on heparin bridging of ZPI and factor Xa. The schematic diagram depicts the stimulatory and inhibitory effects of calcium on heparin bridging of ZPI and factor Xa. In the absence of calcium, factor Xa exists in a compact conformation in which the negatively charged Glα domain of factor Xa (rectangle) makes an intramolecular interaction with the positively charged heparin binding site in the protease domain. The two EGF domains that separate the Glα domain from the terminal protease domain are depicted as circles. Calcium ions disrupt the factor Xa domain-domain interaction by binding to the Glα domain, resulting in an extended conformation that exposes the heparin binding site. Calcium ions also interact with negatively charged groups on heparin and must be displaced by an ion exchange process to allow the binding of the positively charged sites on ZPI and factor Xa to form the ternary bridging complex. Divalent calcium ions compete more effectively than monovalent cations for the binding of proteins to heparin and therefore reduce the affinity of the protein-heparin interactions relative to those formed in the presence of only monovalent cations. Another possible mechanism of calcium inhibition of protein-heparin bridging interactions may involve bound calcium promoting nonproductive protein-heparin interactions (see “Discussion”) (not shown).

Calcium was found to greatly augment the heparin acceleration of the ZPI-factor Xa reaction. Studies with GD-factor Xa showed that this was a result of calcium relieving Glα domain antagonism of heparin bridging of factor Xa and ZPI, similar to the mechanism by which calcium promotes heparin bridging of antithrombin and factor Xa (15) (Fig. 7). However, the studies with GD-factor Xa also revealed that calcium inhibits heparin bridging of ZPI and factor Xa, an effect also evident from published data on calcium bridging of antithrombin and GD-factor Xa (15). The inhibition results from calcium reducing the affinity of ZPI and GD-factor Xa for heparin in binary and ternary complexes as indicated by both a shift of the bell-shaped heparin concentration dependence of heparin acceleration to higher heparin concentrations and a reduction in the maximal acceleration. The latter probably reflects the decreased affinity of the protease for heparin in the ternary complex and consequent reduced $K_{\text{m,ter}}$ for ternary complex formation. Calcium inhibition of heparin bridging was also evident from the biphasic dependence of calcium’s augmenting effect on the heparin-accelerated ZPI-factor Xa reaction, which showed that the augmentation reached an optimum at 1.5 mM calcium and declined at higher calcium concentrations. The inhibitory effects of calcium on heparin bridging of ZPI-protease interactions were directly observable in the case of the ZPI-factor Xla and antithrombin-factor Xla reactions, although such effects measured...
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at 5 mM calcium are expected to be reduced at physiologic calcium concentrations.

Calcium inhibition of heparin bridging interactions with proteins is understandable in terms of the ion exchange mechanism by which these interactions are formed. According to this mechanism, the binding of positively charged residues of a protein to negatively charged groups on the heparin chain requires the displacement of small cations tightly associated with the polysaccharide (35). Because calcium ions are divalent, they associate more strongly with heparin than monovalent sodium ions and therefore are exchanged less readily with positively charged sites of a protein and as a result could produce an inhibitory effect on protein-heparin bridging interactions (Fig. 7). Another possible inhibition mechanism is suggested by the report that ~1 calcium ion specifically associates with idurionate carboxylate groups within each heparin tetrasaccharide at physiologically relevant calcium concentrations (36). Such specific calcium-heparin interactions could inhibit productive protein-heparin bridging interactions by allowing nonproductive interactions of bound calcium with negatively charged regions of proteins. This promotion of an alternative type of protein-heparin interaction could explain why calcium was observed to minimally inhibit protein interactions with immobilized heparin. The specificity of calcium-heparin interactions could also explain why physiologic magnesium levels were not found to inhibit protein-heparin bridging interactions.

Most importantly, our findings show that the stimulatory effects of calcium on the heparin-accelerated ZPI-factor Xa reaction are dominant at physiologic calcium concentrations and outweigh the inhibitory effects. Moreover, calcium stimulation was found to be enhanced by increases in heparin chain length. This was evident from the progressive shifts of the bell-shaped heparin concentration dependence of heparin-accelerated ZPI-protease reactions to lower heparin concentrations and from the increases in maximal heparin acceleration as heparin chain length was increased. These effects suggest that ZPI and protease interactions with heparin in binary and ternary complexes are enhanced by increasing heparin chain length. This finding is in agreement with previous studies showing that the strength of nonspecific electrostatic interactions of proteins with heparin is a function of an intrinsic heparin binding site dissociation constant and the number of overlapping binding sites on the heparin chain (25). The number of binding sites increases in proportion with chain length beyond a minimal size and accounts for the increase in apparent binding affinity. We observed such a proportional relation between $k_{a,app}$ for reaction of ZPI with protease in a ternary bridging complex and heparin chain length that suggested a minimal chain length of ~18 saccharides for heparin rate enhancement of the ZPI-factor Xa reaction. This agrees well with the heparin chain length dependence and minimal chain lengths required for heparin bridging of other serpin-protease reactions (15, 37). Evidence that heparins with higher sulfation are more effective at bridging was also obtained, consistent with increases in heparin charge density enhancing the intrinsic site dissociation constant for protein-heparin bridging interactions. Notably, the combined activating effects of calcium and heparin chain length on heparin bridging of ZPI and factor Xa are comparable with those reported for heparin bridging of antithrombin and factor Xa (15).

Together, our findings thus support a role for heparin and heparin-like glycosaminoglycans in activating ZPI to inhibit factor Xa and factor Xla at rates approaching the diffusion limit. We have shown that the physiologic form of ZPI, a high affinity complex with protein Z, is activated by heparin to an extent similar to that of free ZPI to inhibit both target proteases. Heparin binds ZPI with an affinity comparable with or better than that of other serpins that are thought to be physiologically activated by heparin. In the presence of a procoagulant membrane surface, protein Z localizes ZPI to the membrane, where template bridging of ZPI and factor Xa is the dominant mode of regulation of membrane-associated factor Xa. However, the ZPI-protein Z complex may also interact with heparin or heparin-like glycosaminoglycans at an injury site and be activated to inhibit factor Xa that escapes from a membrane site. Heparin-like glycosaminoglycans on the blood vessel wall may additionally serve as reservoirs for serpins, such as ZPI and antithrombin, to enable them to rapidly inhibit any free factor Xa formed in the absence of injury so as to maintain blood fluidity and hemostasis. The pivotal roles of factor Xa in activating blood coagulation and inflammation pathways suggest that a fine-tuned regulatory mechanism is necessary to keep factor Xa activity in check (38). Our findings expand the potential multiple roles of ZPI in this regulation.

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