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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 relieving Gla 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

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[2] The abbreviations used are: ZPI, protein Z-dependent protease inhibitor; SI, stoichiometry of inhibition; fXa and fXIa, factor Xa and XIa, respectively; HA, high affinity; LA, low affinity; GD-factor Xa, Gla domainless factor Xa.

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 XIa 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 Sephacryl S200 chromatography steps, as in previous studies (5, 6). Recombinant ZPI was judged >95% pure by SDS-PAGE analysis. Molar concentrations of ZPI were determined from the absorbance at 280 nm using a molar absorption 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 XIa 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 Gla 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 (Nadroparine 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 XIa 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 equimolar with the ZPI concentration, in the absence or presence of 5 mM $CaCl_2$ 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 fXa (American Diagnostica, Greenwich, CT) for factor Xa reactions or 100 μM S2366 (Diapharma, West Chester, OH) for factor XIa 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, Gla domainless factor Xa, or factor XIa 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 $CaCl_2$ for reaction times of 0.5–6 min. The effect of protein Z on reactions was assessed by including protein Z equimolar with ZPI in the reaction. The dependence of heparin-accelerated reaction rates on calcium concentration was determined at an optimal heparin concentration and varying $CaCl_2$ 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 Pefafluor fXa substrate (Centerchem, Norwalk, CT) for factor Xa reactions or ~30 μM Boc-Glu-(OBzl)-Ala-Arg-7-amido-4-methylcoumarin (Bachem) for factor XIa 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

nm. The observed pseudo-first order rate constant for reactions was measured from the equation,

$$k_{obs} = \ln((v_o - v_\infty)/(v_t - v_\infty))/t \quad \text{(Eq. 1)}$$

where v_t and v_∞ 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_o 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 ($k_{a,app}$) 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),

$$k_{obs} = k_d + k_{a,-H} \times [ZPI]_f + k_{a,T} \times [ZPI{\cdot}H] \times K_{Pr,H}/(K_{Pr,H} + [H]_f) + k_{a,B} \times [H]_f/(K_{Pr,H} + [H]_f) \quad \text{(Eq. 2)}$$

where

$$[ZPI{\cdot}H] = ([ZPI]_o + [H]_o + K_{ZPI,H} - (([ZPI]_o + [H]_o + K_{ZPI,H})^2 - 4[ZPI]_o[H]_o)^{1/2})/2 \quad \text{(Eq. 3)}$$

$$[ZPI]_f = [ZPI]_o - [ZPI{\cdot}H] \quad \text{(Eq. 4)}$$

$$[H]_f = [H]_o - [ZPI{\cdot}H] \quad \text{(Eq. 5)}$$

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, $[ZPI]_f$ and $[H]_f$ 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 to not 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 XIa in the presence of varying heparin concentration with or without 5 mM $CaCl_2$ were performed similar to ZPI reactions using 35 nM antithrombin, 0.3 nM factor XIa, and 10^{-10} to 10^{-5} 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 $CaCl_2$, and with or without 25 μM 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,

$$k_{obs} = k_d + k_a \times [ZPI]_o \quad \text{(Eq. 6)}$$

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

Binding of Proteins to Heparin-Agarose—Samples of 15–25 μg 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 $CaCl_2$. 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,

$$0.1 + 0.45 \times (V_e - V_o)/(V_f - V_o) \quad \text{(Eq. 7)}$$

where V_o, V_e, and V_f 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. 1*A*). 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-

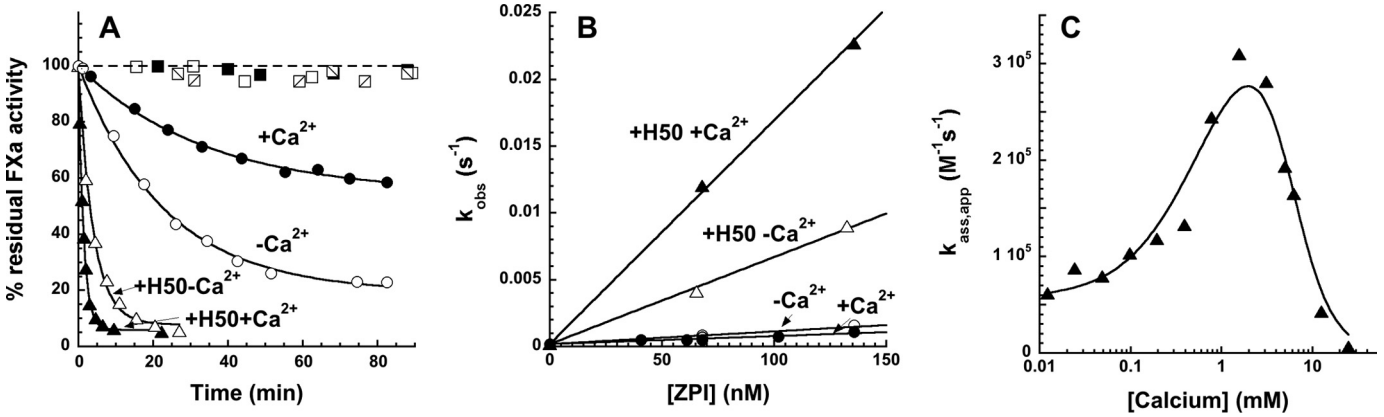

FIGURE 1. **Accelerating effects of heparin on ZPI inhibition of factor Xa in the absence and presence of calcium.** *A*, progress curves are shown for reactions of 68 nM ZPI with 0.15 nM factor Xa in the absence (*circles*) and presence of 1.4 μM 50-saccharide heparin (*H50*) (*triangles*) either with or without 5 mM Ca^{2+} (*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_{obs} 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 ($k_{a,app} = k_{obs}/[ZPI]_o$) 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

$k_{a,app}$ 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 $I \sim 0.15$, pH 7.4, buffer at 25 °C with or without 5 mM Ca^{2+}, protein Z (PZ) equimolar with ZPI, and ~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 $k_{a,app}$ and SI represents the corrected association rate constant for reaction through the inhibitory pathway.

ZPI ± heparin ± PZ	ZPI-fXa reactions without Ca^{2+}			ZPI-fXa reactions with Ca^{2+}		
	$k_{a,app}$	SI	$k_{a,app} \times$ SI	$k_{a,app}$	SI	$k_{a,app} \times$ SI
	M^{-1} s^{-1}	*mol I/mol E*	M^{-1} s^{-1}	M^{-1} s^{-1}	*mol I/mol E*	M^{-1} s^{-1}
ZPI	$9.5 \pm 0.6 \times 10^3$	3.6 ± 0.4	$3.4 \pm 0.6 \times 10^4$	$5.9 \pm 0.4 \times 10^3$	4.5 ± 0.2	$2.7 \pm 0.3 \times 10^4$
ZPI + PZ	$5.6 \pm 0.2 \times 10^3$	6.8 ± 0.1	$3.8 \pm 0.2 \times 10^4$	$8.7 \pm 0.9 \times 10^3$	5.3 ± 0.1	$4.6 \pm 0.6 \times 10^4$
ZPI + H26	ND	ND	ND	$3.2 \pm 0.2 \times 10^4$	4.3 ± 0.2	$1.4 \pm 0.2 \times 10^5$
ZPI + H50	$6.5 \pm 0.3 \times 10^4$	3.5 ± 0.1	$2.3 \pm 0.2 \times 10^5$	$1.7 \pm 0.1 \times 10^5$	4.0 ± 0.1	$6.8 \pm 0.6 \times 10^5$
ZPI + H50 + PZ	$2.9 \pm 0.1 \times 10^4$	4.0 ± 0.1	$1.2 \pm 0.1 \times 10^5$	$1.2 \pm 0.1 \times 10^5$	5.0 ± 0.1	$6.0 \pm 0.6 \times 10^5$
ZPI + H72	$1.1 \pm 0.1 \times 10^5$	2.7 ± 0.1	$3.0 \pm 0.4 \times 10^5$	$3.6 \pm 0.1 \times 10^5$	2.9 ± 0.1	$1.0 \pm 0.1 \times 10^6$
ZPI + H72 + PZ	$1.1 \pm 0.1 \times 10^5$	2.8 ± 0.1	$3.1 \pm 0.4 \times 10^5$	$3.6 \pm 0.3 \times 10^5$	3.2 ± 0.1	$1.2 \pm 0.1 \times 10^6$

gress 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. 1*B*), 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 ($k_{a,app}$) 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 $k_{a,app}$ 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. 1*C*). Optimal levels of calcium thus result in an overall ~60-fold heparin enhancement of $k_{a,app}$ 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 $k_{a,app}$ 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 $k_{a,app}$ 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. 2*A*). 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

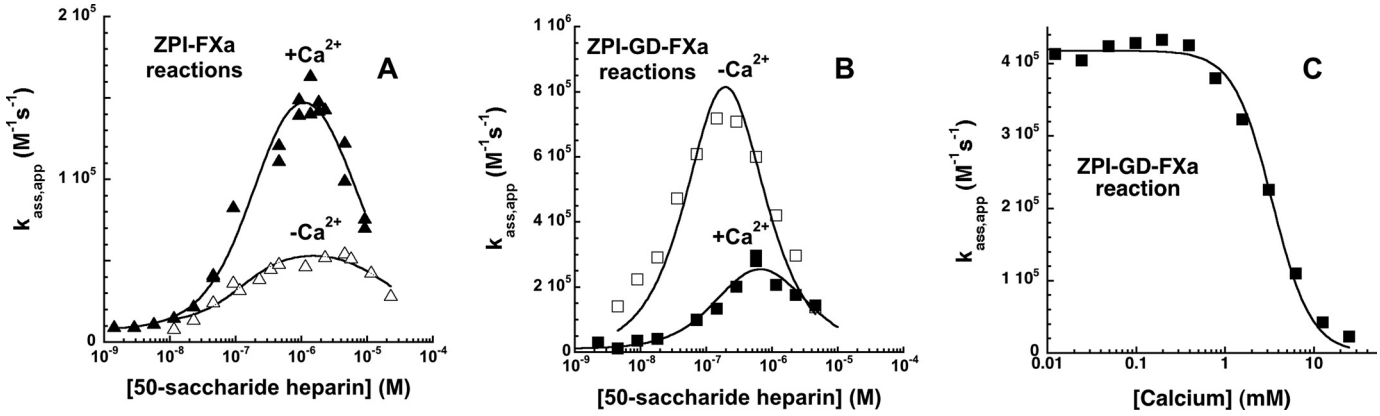

FIGURE 2. **Heparin concentration dependence of heparin accelerating effects on the inactivation of factor Xa and Gla domainless factor Xa by ZPI in the absence and presence of Ca^{2+}.** Dependence of $k_{a,app}$ for reactions of 68–70 nM ZPI with 0.1–0.2 nM factor Xa (*A*, *triangles*) or Gla domainless factor Xa (*B*, *squares*) in the absence (*open symbols*) and presence (*closed symbols*) of 5 mM Ca^{2+} on the concentration of 50-saccharide heparin. Reactions were allowed to proceed for fixed reaction times of 45–400 s, and k_{obs} was calculated from the measured residual protease activity assuming an exponential inactivation. Corrections were made for nonzero end points as described under "Experimental Procedures." *Solid lines*, fits of data by the equation for the ternary complex bridging model of heparin rate enhancement (Equation 2). *C*, the dependence of $k_{a,app}$ (k_{obs}/[ZPI]$_o$) for the reaction of 35 nM ZPI with 0.5 nM GD-factor Xa in the presence of 1.2 μM 50-saccharide heparin on calcium concentration. Reactions were allowed to proceed for a fixed time of 1 min and quenched with substrate, and residual GD-factor Xa activity was measured relative to an uninhibited control. k_{obs} was calculated assuming an exponential decay of GD-factor Xa activity. *Solid line*, a fit of the data assuming that a monophasic cooperative calcium interaction diminishes the heparin acceleration as in Fig. 1*C*.

studies showing that calcium augments the heparin-bridging rate enhancement of the reaction of the serpin, antithrombin, with factor Xa (15). This effect was shown to result from calcium disrupting an intramolecular interaction of the Gla domain of the protease with the heparin binding exosite in the catalytic domain that makes the heparin binding site accessible for bridging.

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. 2*A* 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 ~20 μM in the absence of calcium and ~6 μM 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 (~1 μM).

TABLE 2

Kinetic constants characterizing the bell-shaped dependence of heparin acceleration of ZPI-protease reactions on heparin concentration

Dissociation constants for ZPI interaction with heparin ($K_{ZPI,H}$) and for protease interaction with heparin ($K_{Pr,H}$) in binary complexes and association rate constants for reaction of ZPI with protease in a ternary bridging complex with heparin (k_T) were obtained from fits of the bell-shaped dependence of $k_{a,app}$ on heparin concentration by the template-bridging model equation (Equation 2). In cases where parameters were not well determined by the data, dissociation constant values were fixed at values determined for ZPI-factor Xa reactions done with the same heparin and calcium concentration.

ZPI reaction components	$K_{ZPI,H}$	k_T	$K_{Pr,H}$
	μM	$M^{-1}\,s^{-1}$	μM
fXa, LA-H26 + 5 mM Ca^{2+}	0.7 ± 0.7	$2.6 \pm 0.6 \times 10^4$	Fix at 10
fXa, HA-H26 + 5 mM Ca^{2+}	0.30 ± 0.18	$5.6 \pm 0.7 \times 10^4$	Fix at 10
fXa, H50 - Ca^{2+}	0.09 ± 0.03	$5.6 \pm 0.4 \times 10^4$	23 ± 9
fXa, H50 + 5 mM Ca^{2+}	0.20 ± 0.06	$2.0 \pm 0.2 \times 10^5$	5.5 ± 1.4
fXa, H72 + 5 mM Ca^{2+}	0.10 ± 0.02	$6.3 \pm 0.5 \times 10^5$	2.0 ± 0.3
fXa, UFH + 1.5 mM Ca^{2+}	0.02 ± 0.01	$4.7 \pm 0.4 \times 10^5$	1.3 ± 0.3
fXa, LMWH+1.5 mM Ca^{2+}	1.4 ± 0.7	$3.7 \pm 0.6 \times 10^4$	Fix at 10
GD-fXa, H50 - Ca^{2+}	Fix at 0.09	$2.1 \pm 0.2 \times 10^6$	0.25 ± 0.06
GD-fXa, H50 + 5 mM Ca^{2+}	Fix at 0.2	$4.4 \pm 0.3 \times 10^5$	1.9 ± 0.4
fXIa, H50 - Ca^{2+}	Fix at 0.09	$8.3 \pm 0.6 \times 10^5$	0.7 ± 0.2
fXIa, H26 + 5 mM Ca^{2+}	Fix at 0.4[a]	$4.4 \pm 1.0 \times 10^5$	>10
fXIa, H50 + 5 mM Ca^{2+}	Fix at 0.2	$5.2 \pm 0.1 \times 10^5$	>10
fXIa, H72 + 5 mM Ca^{2+}	0.07 ± 0.02	$3.0 \pm 0.4 \times 10^6$	0.6 ± 0.2

[a] Value estimated from values for 50- and 72-saccharide heparins for corresponding ZPI-factor Xa reactions assuming a proportional relationship between $K_{ZPI,H}$ and the heparin chain length.

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 ~1 μM 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).

TABLE 3

Relative affinities of ZPI and proteases for heparin-Sepharose

~100-μg samples of protein in 0.1 ml of *I* 0.15, 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	NaCl concentration required to elute protein from heparin-Sepharose	
	Without Ca^{2+}	With 5 mM Ca^{2+}
	M	
ZPI	0.36	0.34
Factor Xa	0.16	0.34
GD-factor Xa	0.34	0.34
Factor XIa	0.33	0.32

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. 2*B*), 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. 2*B*). 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. 2*C*) that paralleled the inhibitory phase of the calcium dependence of heparin acceleration of the ZPI-full-length factor Xa reaction (Fig. 1*C*). 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 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. 3*A*). 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

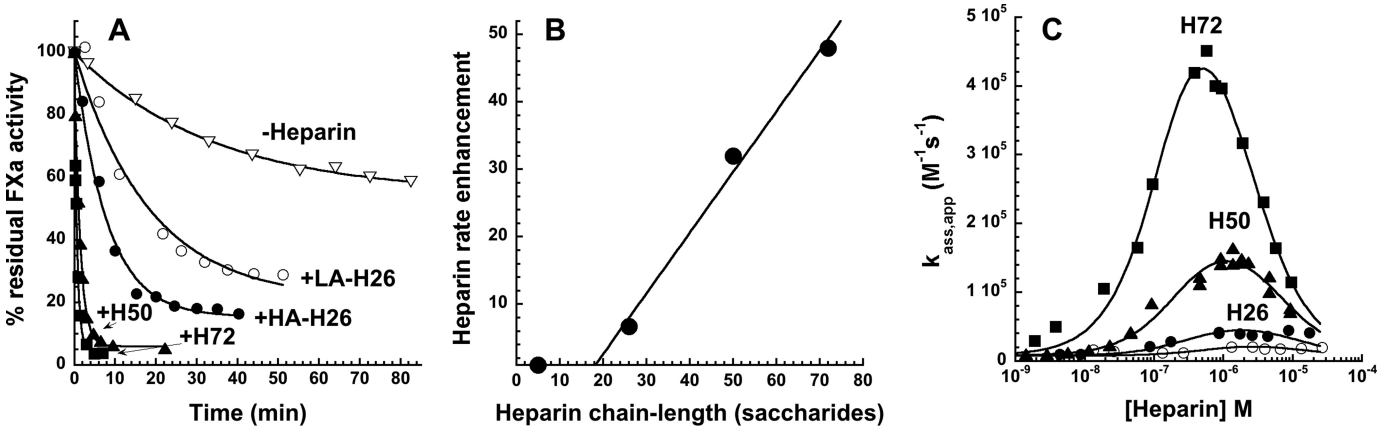

FIGURE 3. **Heparin chain length dependence of the accelerating effect of heparin on ZPI-factor Xa reactions in the presence of Ca^{2+}.** *A*, progress curves for reactions of 66–68 nM ZPI with 0.15 nM fXa (~0.1 nM) in the presence of 5 mM Ca^{2+} and in the absence (▽) or presence of optimal concentrations of heparins containing ~26 saccharides (1 μM *HA-H26* (●); 1 μM *LA-H26* (○)), ~50 saccharides (1.4 μM *H50* (▲)) or ~72 saccharides (1.2 μM *H72* (■)). All heparins contained a specific pentasaccharide binding sequence for antithrombin except LA-H26. *Solid lines*, fits of progress curves by a single exponential decay function with nonzero end point. *B*, the ratios of second order rate constants for ZPI-factor Xa reactions measured in the presence and absence of a pentasaccharide heparin and the heparins in *A* are plotted as a function of heparin chain length. Second order rate constants were obtained from the slopes of the linear dependence of k_{obs} for reactions at optimal heparin concentrations on ZPI concentration in Table 1. *C*, dependence of $k_{a,app}$ for heparin-accelerated reactions of 68 nM ZPI with 0.15 nM factor Xa in the presence of 5 mM Ca^{2+} on heparin concentration for the heparins in *A*. Data for the 50-saccharide heparin are reproduced from Fig. 2*A*. *Solid lines*, fits by the equation for the template bridging model for heparin rate enhancement (Equation 2).

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 $k_{a,app}$ 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. 3*B*), 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. 3*C*), indicating that the accelerations were all mediated by a similar ternary complex bridging mechanism. Moreover, k_{obs} 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 ~10^6 M^{-1} 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 ~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. 3*B*), 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 ~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 XIa—Factor XIa 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 XIa without heparin was

already quite fast. Fig. 5*A* shows that the 50-saccharide heparin increased k_{obs} for the ZPI-factor XIa reaction maximally ~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 XIa 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 XIa 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 XIa 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 XIa. The 50-saccharide heparin accelerated the antithrombin-factor XIa reaction with a bell-shaped dependence on heparin concentration in the absence or presence of calcium, consistent with heparin accelerating this reaction by a similar ternary complex bridging mechanism (Fig. 5*B*). 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 (~500-fold *versus* ~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 XIa reaction. It should be noted that the fitted limiting value of $k_{a,app}$ 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 XIa (28).

Heparin chain length affected the extent to which heparin accelerated the ZPI-factor XIa reaction, as was found for the ZPI-factor Xa reaction. Heparins of increasing chain length produced progressively greater heparin accelerations of the ZPI-factor XIa 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 XIa. All heparin-accelerated reactions showed bimolecular reaction kinetics based on the observed linear dependence of k_{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 XIa reaction, the binding parameters describing

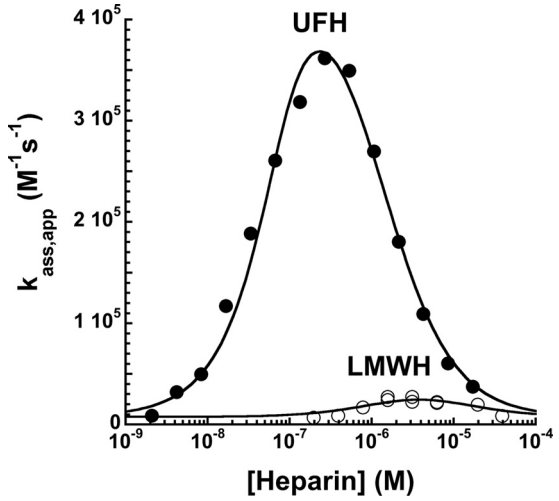

FIGURE 4. **Accelerating effects of pharmacologic heparins on ZPI-factor Xa reactions.** Dependence of $k_{a,app}$ for heparin-accelerated reactions of 66 nM ZPI with ~0.1 nM factor Xa in the presence of 1.5 mM Ca^{2+} on the concentration of unfractionated heparin (*UFH*, ●) or low molecular weight heparin (*LMWH*, ○). *Solid lines*, fits by the template bridging equation (Equation 2).

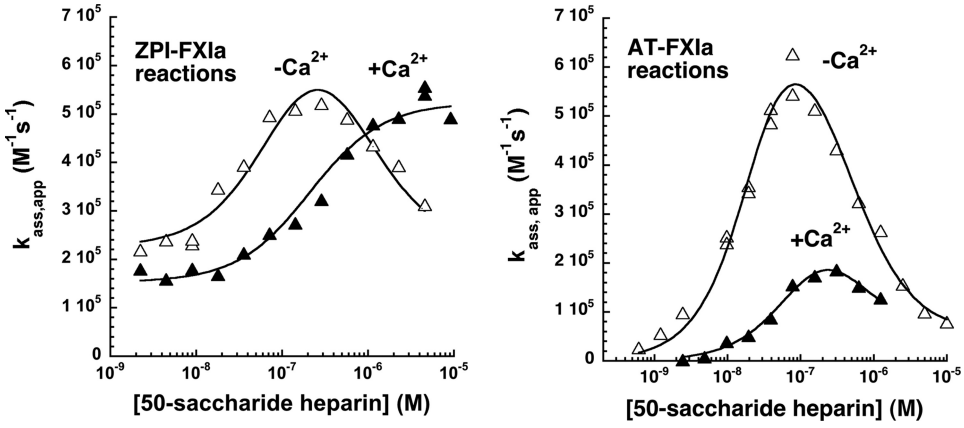

FIGURE 5. **Heparin accelerating effects on the inactivation of factor XIa by ZPI and antithrombin in the absence and presence of Ca^{2+}.** Dependence of $k_{a,app}$ for 50-saccharide heparin-accelerated reactions of 23 nM ZPI (*left*) or 35 nM antithrombin (*right*) with 0.15–0.3 nM factor XIa in the absence (△) and presence (▲) of 5 mM Ca^{2+} as a function of heparin concentration. Reactions were done for fixed times, and k_{obs} was calculated from the residual factor XIa activity assuming an exponential inactivation. *Solid lines*, fits by the template bridging equation (Equation 2).

the bell-shaped curves were not as well determined. Nevertheless, K_D values for ZPI binding to heparin from the ascending parts of the curves were in a similar range as those determined for heparin-accelerated ZPI-factor Xa reactions. Notably, the efficiency of factor XIa inhibition by ZPI was improved by heparin, as was evident from the decrease in SI from a value of 8 in the absence of heparin to values of 5–6 in the presence of the glycosaminoglycan (Table 4). Correction of apparent rate constants for the SI indicated that second order rate constants for 50- and 72-saccharide heparin-accelerated reactions reached diffusion-limited values ranging from 0.3 to 1 × 10^7 M^{-1} s^{-1} that were physiologically significant. Protein Z binding to ZPI reduced the apparent rate constant for heparin-accelerated factor XIa inhibition and increased the SI as with the heparin-accelerated ZPI-factor Xa reaction, resulting in corrected rate constants that were modestly reduced from those in the absence of protein Z but that still exceeded 10^6 M^{-1} s^{-1}. These findings affirm that protein Z binding to ZPI has at most minor effects on heparin-accelerated ZPI-protease reactions.

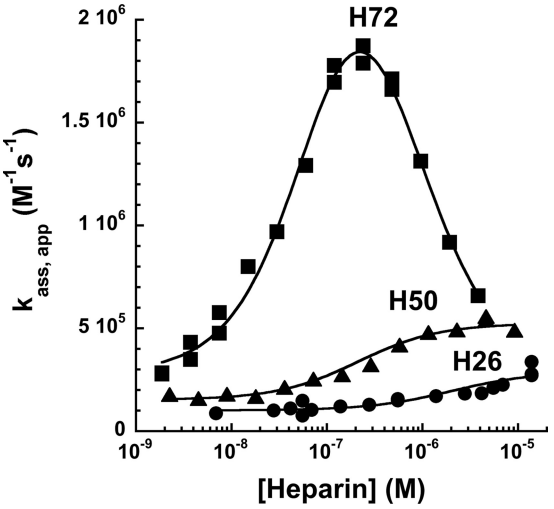

FIGURE 6. **Heparin chain length dependence of the accelerating effect of heparin on ZPI-factor XIa reactions.** Dependence of $k_{a,app}$ for 50-saccharide (*H50*) (▲) and 72-saccharide (*H72*) (■) heparin-accelerated reactions of 23 nM ZPI with 0.2–0.5 nM factor XIa and for 26-saccharide heparin (*H26*)-accelerated reactions of 41 nM ZPI with 0.2 nM factor XIa (●), all in the presence of 5 mM Ca^{2+} on the heparin concentration. Reactions were conducted for fixed reaction times, and k_{obs} was calculated from the residual factor XIa activity assuming an exponential inactivation. *Solid lines*, fits by the template bridging model equation (Equation 2).

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 XIa up to ~20-fold independent of calcium to yield association rate constants of ~10^6 to 10^7 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 effector of ZPI anticoagulant function based on findings of only modest ~2–3-fold heparin rate enhancements of ZPI reactions with factor Xa and factor XIa (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 complexed state with protein Z. Maximal apparent second order rate constants of 1–2 × 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 × 10^7 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-

TABLE 4

Apparent second order rate constants and stoichiometries of inhibition for unaccelerated and heparin-accelerated reactions of ZPI with factor XIa in the absence and presence of 5 mM calcium ions and with or without PZ

Rate constants and stoichiometries for ZPI-factor XIa reactions in I ~0.15, pH 7.4, buffer at 25 °C with or without 5 mM Ca^{2+}, protein Z (PZ) equimolar with ZPI, and ~1 μM 50-saccharide (H50) and 72-saccharide (H72) heparins were measured as described under "Experimental Procedures." $k_{a,app}$ was determined from linear plots of k_{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.

	ZPI-fXIa reactions without Ca^{2+}			ZPI-fXIa reactions with Ca^{2+}		
ZPI ± heparin ± PZ	$k_{a,app}$	SI	$k_{a,app}$ × SI	$k_{a,app}$	SI	$k_{a,app}$ × SI
	M^{-1} s^{-1}	*mol I/mol E*	M^{-1} s^{-1}	M^{-1} s^{-1}	*mol I/mol E*	M^{-1} s^{-1}
ZPI	1.4 ± 0.1 × 10^5	8.0 ± 0.3	1.1 ± 0.1 × 10^6	1.0 ± 0.1 × 10^5	7.9 ± 0.2	7.9 ± 1.0 × 10^5
ZPI + PZ	1.6 ± 0.1 × 10^5	14.7 ± 0.3	2.4 ± 0.2 × 10^6	7.6 ± 0.3 × 10^4	16.6 ± 0.3	1.3 ± 0.1 × 10^6
ZPI + H50	7.3 ± 0.3 × 10^5	5.3 ± 0.3	3.9 ± 0.4 × 10^6	5.4 ± 0.5 × 10^5	4.9 ± 0.2	2.6 ± 0.3 × 10^6
ZPI + H50 + PZ	3.4 ± 0.1 × 10^5	6.6 ± 0.2	2.2 ± 0.1 × 10^6	2.3 ± 0.3 × 10^5	5.9 ± 0.4	1.4 ± 0.3 × 10^6
ZPI + H72	4.1 ± 0.1 × 10^6	6.1 ± 0.3	2.5 ± 0.2 × 10^7	1.7 ± 0.2 × 10^6	6.1 ± 0.3	1.0 ± 0.2 × 10^7
ZPI + H72 + PZ	1.4 ± 0.1 × 10^6	10.0 ± 0.3	1.4 ± 0.1 × 10^7	7.7 ± 0.1 × 10^5	8.8 ± 0.4	6.8 ± 0.3 × 10^6

tor Xa activity at an injury site and thereby reinforce the role of these glycosaminoglycans as cofactors in antithrombin regulation of factor Xa (30). It should be noted in this regard that heparan sulfate glycosaminoglycans containing specific pentasaccharide binding sequences for antithrombin are limiting *in vivo* and that chains lacking these sequences may be the major physiologic activators of antithrombin (31–33). It is therefore of potential physiologic relevance that heparin glycosaminoglycans lacking antithrombin binding sequences accelerate ZPI and antithrombin reactions with factor Xa to comparable extents even at the 30-fold higher plasma concentrations of antithrombin over ZPI. This conclusion follows from our finding that ZPI has a ~100-fold higher affinity than antithrombin for such glycosaminoglycans (22).

Heparins used clinically for anticoagulant therapy were also found to activate ZPI in the presence of physiologic calcium concentrations, suggesting that ZPI inhibition of factor Xa and factor XIa may contribute to the pharmacologic action of heparin. This is particularly true for unfractionated heparin, which produced activating effects comparable with those of the longer chain fractionated heparins on ZPI reactions with factor Xa and factor XIa. However, the low molecular weight heparin produced considerably smaller activating effects on ZPI-protease reactions that are not likely to be important for the anticoagulant effects of such heparins. Comparison with the activating effects of these heparins on antithrombin inhibition of factor Xa and factor XIa suggests that antithrombin is the more important serpin responsible for their anti-factor Xa activity because of the substantial fraction of chains containing pentasaccharide binding sequences for antithrombin (about one-third), whereas ZPI may contribute to their anti-factor XIa activity (20, 34).

The minimal effects of protein Z binding to ZPI on heparin acceleration of ZPI-protease reactions suggest that protein Z and heparin bind to distinct sites on ZPI with little or no interaction between these sites. This finding is not surprising in view of the recent x-ray structures of the ZPI-protein Z complex reported by us and another group, which show that the ZPI site for binding protein Z involves the negatively charged helix G/helix A region, which would not be expected to bind the like charged heparin polysaccharide (6, 8). Interestingly, our findings suggest that the affinity of ZPI for heparin is reasonably high and comparable with or greater than that of other heparin binding serpins (16). The high affinity binding of ZPI to heparin and considerable bridging activation of ZPI reactions with proteases as a result of this binding suggest that the heparin binding site of ZPI has functional significance. The location of the heparin binding site is presently unclear, but our recent x-ray structure of the ZPI-protein Z complex suggests one candidate highly basic region that is distinct from the helix D site used by many other serpins. Future mutagenesis studies will be necessary to localize this binding site.

Our studies have provided insight into the mechanism of heparin acceleration of ZPI-protease reactions. In all cases, the heparin concentration dependence of the heparin acceleration is bell-shaped, indicative of a template-bridging mechanism of rate enhancement in which heparin binds both ZPI and protease and promotes their interaction in a ternary bridging complex (22, 35). Evidence was obtained that the ternary bridging complex is formed through a bimolecular reaction between a ZPI-heparin binary complex and free protease with a second order rate constant determined by the K_m for formation of the ternary Michaelis complex and the rate constant for ZPI inhibition of protease within the complex (24).

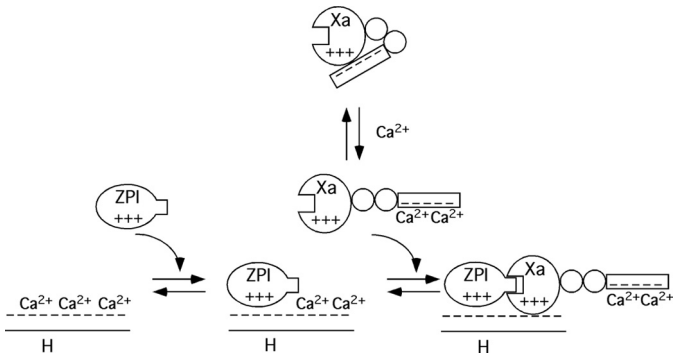

FIGURE 7. **Calcium effects on heparin bridging of ZPI and factor Xa.** The *schematic diagram* depicts the stimulatory and inhibitory effects of calcium on heparin bridging of ZPI and factor Xa. In the absence of calcium, factor Xa exists in a compact conformation in which the negatively charged Gla 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 Gla domain from the terminal protease domain are depicted as *circles*. Calcium ions disrupt the factor Xa domain-domain interaction by binding to the Gla 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 Gla 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_m 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 XIa and antithrombin-factor XIa reactions, although such effects measured

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 iduronate 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 $k_{a,\mathrm{app}}$ 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 XIa 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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