Heparin and Calcium Ions Dramatically Enhance Antithrombin Reactivity with Factor IXa by Generating New Interaction Exosites†

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ABSTRACT: Blood coagulation factor IXa has been presumed to be regulated by the serpin, antithrombin, and its polysaccharide activator, heparin, but it has not been clear whether factor IXa is inhibited by the serpin with a specificity comparable to that for thrombin and factor Xa or what determinants govern this specificity. Here we show that antithrombin is essentially unreactive with factor IXa in the absence of heparin ($k_{ass} \sim 10\ M^{-1}\ s^{-1}$) but undergoes a remarkable ~1 million-fold enhancement in reactivity with this proteinase to the physiologically relevant range ($k_{ass} \sim 10^7\ M^{-1}\ s^{-1}$) when activated by heparin in the presence of physiologic levels of calcium. This rate enhancement is shown to derive from three sources: (i) allosteric activation of antithrombin by a sequence-specific heparin pentasaccharide (300−500-fold), (ii) allosteric activation of factor IXa by calcium ions (4−8-fold), and (iii) heparin bridging of antithrombin and factor IXa augmented by calcium ions (130−1000-fold depending on heparin chain length). Mutagenesis of P6−P3′ reactive loop residues of antithrombin further reveals that the reactivity of the unactivated inhibitor is principally determined by the P1 Arg residue, whereas exosites outside the loop which are present on the activated serpin and on heparin are responsible for heparin enhancement of this reactivity. These results together with our previous findings demonstrate that exosites are responsible for the unusual specificity of antithrombin and heparin for three clotting proteases with quite distinct substrate specificities.

Antithrombin, a member of the serpin superfamily of proteins, is the principal regulator of blood clotting cascade proteinases in vertebrates (*1*, *2*). Inherited or acquired deficiencies of this serpin in humans are associated with an increased risk of thrombotic disease (*3*), and complete deficiency in mice leads to embryonic lethality due to a consumptive coagulopathy and fibrin deposition in the heart and liver (*4*). Antithrombin is thus essential for maintaining hemostasis and life. The serpin regulates the activity of clotting proteinases through a recently elucidated inhibition mechanism shared by other serpin family inhibitors of proteinases (*2*, *5*). In this mechanism, the proteinase initially binds to an exposed reactive loop of the inhibitor in a substrate-like manner and begins to cleave a reactive bond in the loop. However, the cleavage proceeds only to the acyl intermediate stage. At this point the severed N-terminal strand of the loop inserts into the center of the major β-sheet of the protein core, causing the acyl-linked proteinase to be translocated to the opposite pole of the serpin and be inactivated by deformation (*6*−*8*).

Efficient regulation of clotting enzymes by antithrombin requires the sulfated polysaccharides, heparin or heparan sulfate, as cofactors (*1*−*3*). A subset of these glycosaminoglycans contain a unique pentasaccharide sequence which binds and activates antithrombin (*9*−*13*), thereby producing a several thousand-fold acceleration of the inhibition of the main target enzymes, thrombin and factor Xa, to diffusion-limited rates. While conformational activation of antithrombin by the pentasaccharide principally accounts for accelerated inhibition of factor Xa, it is not sufficient to cause accelerated thrombin inhibition. The latter instead requires a heparin chain that is sufficiently long to bind thrombin alongside antithrombin to promote the encounter of the proteins in a ternary bridging complex (*13*−*15*). The X-ray structures of antithrombin, both free and in complex with the pentasaccharide, have shown that conformational activation of the serpin causes an increased level of exposure of a reactive proteinase binding loop on the protein surface (*16*, *17*). However, mutagenesis of the binding loop sequence indicates that this sequence determines the reactivity of the inhibitor with thrombin and factor Xa in the absence of heparin but minimally contributes to the heparin-enhanced reactivity (*18*, *19*). Heparin appears to increase antithrombin reactivity with these enzymes instead through exosite determinants outside the reactive loop sequence, which either become accessible on the serpin following conformational activation or are provided by the bound heparin molecule.

Factor IXa is known to be inhibited by antithrombin and its rate of inhibition accelerated by heparin (*20*−*22*). Addition of factor IXa to plasma with or without heparin or into the bloodstream of mice *in vivo* results in the complex-

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ation of the enzyme exclusively by antithrombin, suggesting the possibility that factor IXa is regulated *in vivo* by antithrombin (*23*, *24*). However, the wide variability in rate constants reported for antithrombin−factor IXa reactions in the absence and presence of heparin has made it unclear whether antithrombin exhibits a similar specificity for interacting with factor IXa as it does with thrombin and factor Xa. This raises the more perplexing question of how antithrombin can specifically inhibit three clotting proteinases whose macromolecular substrate specificities differ so greatly.

In this study, we show that antithrombin inhibits factor IXa at a diffusion-limited rate comparable with that for the reactions with thrombin and factor Xa when the inhibitor is activated by physiologic-type heparins and when physiologic levels of calcium are present. Remarkably, such rates are achieved by a ~1 million-fold heparin acceleration of an extremely slow rate of antithrombin inhibition of factor IXa. The unusually large heparin acceleration is shown to originate from similar contributions of conformational activation of antithrombin and of calcium-dependent bridging of antithrombin and factor IXa and also to require allosteric activation of factor IXa by calcium. Mutations in the antithrombin reactive loop sequence are shown to affect the reactivity of the inhibitor with factor IXa in the absence of heparin but not to affect the extent to which heparin enhances this reactivity. The latter findings indicate that heparin enhances antithrombin reactivity with factor IXa as it does with factor Xa and thrombin, by presenting new exosites on the serpin and on heparin with which the proteinase may interact. Exosite determinants thus can explain how heparin-activated antithrombin can be specific for three very different clotting proteinases.

EXPERIMENTAL PROCEDURES

Heparins. The synthetic antithrombin-binding heparin pentasaccharide was generously provided by M. Petitou of Sanofi-Synthelabo (Toulouse, France). Full-length heparins containing the pentasaccharide binding sequence and with narrow molecular weight distributions and average chain lengths of ~26, ~50, and ~70 saccharides were purified from commercial heparin by repeated gel exclusion chromatography and affinity chromatography on matrix-linked antithrombin, as described previously (*25*). Molar concentrations of the latter heparins were determined by dry weight, by a uronic acid assay, or by an Azure A dye binding assay standardized with heparins with known molar concentrations (*13*, *26*).

Antithrombin. Normal antithrombin was purified from human plasma by affinity chromatography on matrix-linked heparin, followed by successive ion-exchange and gel chromatographies, as described previously (*25*). A natural P1′[1] Ser → Leu variant, antithrombin Denver, was purified from the plasma of a patient with the mutant antithrombin gene (*27*). Recombinant wild-type and mutant antithrombins were prepared by expressing wild-type and mutant cDNAs in BHK cells, as previously described (*18*, *19*), and purifying the expressed proteins from serum-free medium by the procedure used for plasma antithrombin. Protein concentrations were determined by absorption measurements at 280 nm based on a molar absorption coefficient of 37 700 M^{-1} cm^{-1} (*28*), except for the recombinant P1 Trp antithrombin, in which case the absorption coefficient was corrected for the presence of an additional tryptophan (*19*).

Factor IXa. Human factor IXaβ was purchased from Enzyme Research (South Bend, IN) and dialyzed into a low-pH buffer, consisting of 5 mM Mes and 0.15 M NaCl (pH 5.5), to minimize autoproteolysis. Certain comparative experiments were done with preparations of the enzyme produced by activation of factor IX, including one used in previous work (*27*, *29*). Human factor IX, purified from plasma (*29*) or purchased from Enzyme Research, was activated at a concentration of 2−4 μM by incubation with 0.01−0.02 μM factor XIa (Enzyme Research) in 0.1 M Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.1% PEG 8000 (pH 7.4) for 30−60 min at 25 °C, after which the reaction was quenched with 6 mM EDTA. SDS−PAGE showed that the proenzyme was fully activated. The enzyme preparation used in previous studies was purified by chromatographies on an agarose-linked factor XI antibody and heparin-bound agarose (*29*). Freshly activated factor IXa prepared from commercial factor IX was analyzed without further purification.

Experimental Conditions. All experiments were carried out at 25 °C in buffers consisting of (i) 20 mM sodium phosphate, 0.1% PEG 8000, and 0.1 mM EDTA, (ii) 0.1 M Hepes, 0.1% PEG 8000, and 1 mM EDTA, or (iii) 0.1 M Hepes, 0.1% PEG 8000, and 5 mM $CaCl_2$. For all buffers, sodium chloride was added to produce an ionic strength of 0.15 and the pH adjusted to 7.4. However, for some antithrombin−heparin binding studies, the buffer ionic strength was adjusted to 0.05−0.1.

Functional Heparin Concentrations and Affinities of Heparin for Antithrombin. Concentrations of high-affinity pentasaccharide binding sites for antithrombin in heparin species were measured by stoichiometric titrations, monitored by the fluorescence increase induced by the binding, of 0.2−1 μM antithrombin with the saccharides in the absence of calcium at an ionic strength (I) of 0.05−0.15 (*25*). Values of K_D for the binding of the different heparin species to antithrombin were measured by similar titrations of 0.05−0.2 μM antithrombin in the absence or presence of calcium at an I of 0.15 (*25*). Both functional heparin concentrations (based on a 1:1 stoichiometry for binding of the saccharides to antithrombin), and K_D values were obtained by nonlinear regression analyses of the titration curves. Average values of these parameters were obtained from two to five titrations.

SDS−PAGE of Factor IXa and Antithrombin−Factor IXa Complexes. The purity of factor IXaβ was assessed by SDS−PAGE analysis under nonreducing and reducing conditions with 2−4 μg of the enzyme after inactivation with 1 mM 4-amidinophenylmethanesulfonyl fluoride (*30*). Most preparations of the enzyme were found to be >90% pure. The formation of SDS-stable complexes of factor IXa with antithrombin was investigated by incubating 2 μM proteinase with 5 μM antithrombin without or with 5 μM full-length heparin for ~10 s to 16 h at an I of 0.15 in the presence of calcium. The reaction times that were chosen were sufficient to fully (>98%) complex the enzymes with antithrombin, based on measured rate constants. Unreacted factor IXa was

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide electrophoresis; PEG, polyethylene glycol; P1′, P1, etc., residues in the antithrombin reactive loop numbered from the N-terminal side (P1, P2, etc.) and the C-terminal side (P1′, P2′, etc.) of the scissile bond.

inactivated with 2 mM Glu-Gly-Arg chloromethyl ketone (Bachem), and the samples were then analyzed by SDS−PAGE as described above.

Concentrations of Active Factor IXa. Active concentrations of factor IXa were determined by titrations with antithrombin in the presence of heparin. Samples of factor IXa at a fixed concentration of 0.1−0.25 μM were incubated for 2−20 h with antithrombin in molar ratios to the enzyme of 0−2.0 at an *I* of 0.15 in the presence of calcium. Pentasaccharide or full-length heparin was included in the titrations at concentrations 1−4-fold higher than those of the enzymes. Residual enzyme activity was measured by diluting the samples 10-fold into 300−500 μM methylsulfonyl-D-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide (Spectrozyme FIXa, American Diagnostica) in 0.1 M Hepes, 0.1 M NaCl, 10 mM $CaCl_2$, and 33% ethylene glycol (pH 8) (*31*). All substrate solutions also contained 50 μg/mL Polybrene to neutralize heparin. The absorbance increase at 405 nm, reflecting residual enzyme activity, was measured for several minutes. Concentrations of proteinase active in binding to antithrombin were obtained from the intercept with the abscissa axis of the linear segments of plots of residual enzyme activity versus antithrombin concentration. In the case of a few factor IXa preparations, the intercept with a non-zero activity end point (typically <5%, but ∼15% in one case) defined the active proteinase concentration.

Kinetics of Factor IXa Inactivation by Antithrombin and Antithrombin−Heparin Complexes. Kinetic analyses of antithrombin−factor IXa reactions were done under pseudo-first-order conditions, essentially as in previous work (*25*, *29*, *32*). In the analyses of free antithrombin, inhibitor concentrations ranged from 0.5 to 20 μM. In experiments with antithrombin−pentasaccharide complexes, the antithrombin concentration was fixed at 5 μM and saccharide concentrations were varied from 0.125 to 15 μM or antithrombin was present in a 1.2−2.5-fold molar excess over the saccharide. For experiments with antithrombin−full-length heparin complexes, the antithrombin concentration was fixed at 25 nM and heparin concentrations were varied over a wide range (from 2.5 nM to 10 μM) or the inhibitor concentration was set at 250 nM and the heparin concentration varied in the catalytic range (from 0.25 to 50 nM). The proteinase concentration was 5−100 nM except in some reactions of free antithrombin, in which 1−2 μM was used. In all cases, the inhibitor concentration was at least 5-fold and typically 10-fold higher than that of the enzyme, ensuring pseudo-first-order conditions.

Reactions were initiated by addition of the proteinase to antithrombin and pentasaccharide or full-length heparin when present in a total volume of 50−100 μL. After different amounts of time, reactions were terminated by addition of 900−950 μL of a solution of Spectrozyme FIXa (300−500 μM) or its fluorogenic analogue (50 μM), with 7-amido-4-methylcoumarin in place of *p*-nitroanilide (Pefafluor FIXa, Centerchem, Norwalk, CT), as indicated above for measurements of concentrations of active factor IXa. However, certain reactions with full-length heparins were carried out with a range of catalytic heparin concentrations for fixed reaction times. The residual activity was determined from the initial linear rate of absorbance increase at 405 nm or the fluorescence increase at 440 nm (with excitation at 380 nm). Observed pseudo-first-order rate constants (k_{obs}) were obtained in most cases by fitting the decrease in enzyme activity with time to a single-exponential function with a zero end point. In some cases, the data were fitted to a finite end point, based on the observation of a small fraction of enzyme that is less susceptible to inhibition and likely due to degraded, although still active, forms of the enzyme. Second-order rate constants for antithrombin−factor IXa reactions in the absence of heparin were obtained by dividing k_{obs} by the inhibitor concentration. Rate constants for reactions of antithrombin−heparin complexes with factor IXa measured as a function of time were determined by first subtracting the calculated contribution of the reaction of free antithrombin to k_{obs} and then dividing by the concentration of antithrombin−heparin complex calculated from the measured K_D. Rate constants determined at different antithrombin or antithrombin−heparin complex concentrations were averaged. Second-order rate constants for the reaction of antithrombin−heparin complexes with factor IXa measured with a series of catalytic heparin concentrations for a fixed time were obtained by fitting the decrease in enzyme activity as a function of the heparin concentration to the exponential function (*25*, *32*)

$$A_H = A_o \times \exp\{-k_H[[AT]_o/(K_{AT,H} + [AT]_o)]t[H]_o\}$$

where A_H and A_o are the enzyme activities at the fixed time *t* in the presence and absence of heparin, respectively, k_H is the second-order rate constant for the reaction of the antithrombin−heparin complex with factor IXa, $[AT]_o$ and $[H]_o$ are the total antithrombin and heparin concentrations, respectively, and $K_{AT,H}$ is the dissociation constant for the antithrombin−heparin interaction. The fitted exponential constant in this case is equal to $k_H[[AT]_o/(K_{AT,H} + [AT]_o)]t$. Dividing the fitted constant by the fixed reaction time and by the factor $[AT]_o/(K_{AT,H} + [AT]_o)$, representing the fraction of heparin bound to antithrombin, thus yielded the second-order rate constant. Indistinguishable rate constants for inhibition by antithrombin were observed for all factor IXa preparations, including those freshly prepared by activation of the zymogen with factor XIa.

For analyses of k_{obs} for the 50-saccharide heparin-catalyzed antithrombin−factor IXa reaction over a wide range of heparin concentrations, k_{obs} was calculated as $[\ln(E_o - E_\infty)/(E_t - E_\infty)]/t$, where E_o, E_∞, and E_t represent the measured enzyme activities in the absence of inhibitor, after complete reaction (>10 half-lives), and after a fixed reaction time *t*, respectively. The dependence of k_{obs} on heparin concentration was fit by the equation for the ternary complex bridging model of heparin activation (*26*):

$$k_{obs} = (k_H[AT{\cdot}H]K_{IXa,H})/(K_{IXa,H} + [H]_{free})$$

where

$$[AT{\cdot}H] = [[AT]_o + [H]_o + K_{AT,H} - \sqrt{([AT]_o + [H]_o + K_{AT,H})^2 - 4[AT]_o[H]_o}]/2$$

and

$$[H]_{free} = [H]_o - [AT{\cdot}H]$$

In this equation, k_H is the second-order rate constant for inactivation of factor IXa by the antithrombin−heparin

Table 1: Association Rate Constants for Inhibition of Factor IXa by Human Antithrombin and Its Complexes with Different Heparins at 25 °C, pH 7.4, and *I* 0.15[a]

buffer	k_{uncat} (M^{-1} s^{-1})	k_{H5} (M^{-1} s^{-1})	k_{H26} (M^{-1} s^{-1})	k_{H50} (M^{-1} s^{-1})	k_{H70} (M^{-1} s^{-1})
NaP_i/EDTA	9.9 ± 1.3	$(4.0 \pm 0.3) \times 10^3$	$(7.9 \pm 0.2) \times 10^4$	$(2.1 \pm 0.1) \times 10^5$	$(6.5 \pm 0.1) \times 10^5$
Hepes/EDTA	$(1.5 \pm 0.1) \times 10$[b]	$(4.5 \pm 0.2) \times 10^3$	$(6.4 \pm 0.3) \times 10^4$ [$(1.4 \pm 0.1) \times 10^5$][b]	$(1.7 \pm 0.1) \times 10^5$ [$(9.4 \pm 0.6) \times 10^5$][b]	$(6.0 \pm 0.7) \times 10^5$ [$(1.0 \pm 0.1) \times 10^6$][b]
Hepes/Ca^{2+}	$(5.8 \pm 0.7) \times 10$	$(3.1 \pm 0.3) \times 10^4$	$(3.8 \pm 0.3) \times 10^6$ [$(4.8 \pm 0.4) \times 10^6$][b]	$(1.1 \pm 0.1) \times 10^7$ [$\geq 2 \times 10^7$][b,c]	$(3.1 \pm 0.3) \times 10^7$ [ND][d]

[a] Second-order association rate constants for reactions with factor IXa of antithrombin alone (k_{uncat}) and of complexes of the inhibitor with pentasaccharide (k_{H5}) and with full-length heparins of ~26 saccharides (k_{H26}), ~50 saccharides (k_{H50}), and ~70 saccharides (k_{H70}) were determined as described in Experimental Procedures. Rate constants for reactions with antithrombin−full-length heparin complexes were measured either with a series of catalytic heparin concentrations or with a 4−8-fold molar excess of heparin over antithrombin (values in brackets). Concentrations of antithrombin−heparin complexes were calculated on the basis of measured K_D values of 50 ± 6, 19 ± 3, and 20 ± 3 nM for pentasaccharide, 26-saccharide, and 50-saccharide heparins, respectively, in the absence of calcium and indistinguishable values in the presence of the cation. A value of 20 nM was assumed for the 70-saccharide heparin interaction. The buffers were 20 mM sodium phosphate and 100 μM EDTA (pH 7.4, $I = 0.15$) (NaP_i/EDTA), 0.1 M Hepes, and 1 mM EDTA (pH 7.4, $I = 0.15$) (Hepes/EDTA), and 0.1 M Hepes and 5 mM $CaCl_2$ (pH 7.4, $I = 0.15$) (Hepes/Ca^{2+}); all buffers also contained 0.1% polyethylene glycol 8000. Values are averages ± the standard error of the mean of at least three measurements, except where indicated. [b] Average ± range of two measurements. [c] Lower limit due to the first time point of full reaction progress curves representing ~95% completion of the reaction. [d] Not determined.

complex, [AT·H] is the concentration of the inhibitor−heparin complex, $[H]_{free}$ is the concentration of free heparin chains, $[AT]_o$ and $[H]_o$ are the total concentrations of antithrombin and heparin, respectively, and $K_{AT,H}$ and $K_{IXa,H}$ are dissociation constants for antithrombin−heparin and factor IXa−heparin binary complexes, respectively. The equation lacks terms for the uncatalyzed reaction and for the reaction between the antithrombin−heparin complex and factor IXa−heparin binary complexes, since the former made a negligible contribution and fitting of the latter indicated a rate constant indistinguishable from that of the uncatalyzed reaction.

The optimal calcium concentration for stimulating the reaction of the antithrombin−pentasaccharide complex with factor IXa was evaluated from full progress curves in Hepes buffer containing $CaCl_2$ concentrations of 0, 0.001, 0.01, 0.1, and 1 mM. Similar analyses of the calcium concentration dependence of the reaction of factor IXa with the antithrombin−50-saccharide heparin complex were carried out by fixed time assays at $CaCl_2$ concentrations of 0, 0.1, 0.5, 1, 2, 3, 5, and 10 mM.

Rate constants measured for antithrombin−factor IXa reactions by the synthetic substrate assay were validated by monitoring the rate of appearance of the antithrombin−factor IXa complex by SDS−PAGE. Antithrombin (10 μM) was allowed to react for varying times with factor IXa (2 μM) in the absence or presence of 1 μM pentasaccharide or 5 nM 50-saccharide heparin, and the reactions were then quenched by reducing the pH to ~2. After addition of SDS and subsequent boiling, the pH was increased to that of the electrophoresis running buffer, and the samples were analyzed by SDS−PAGE under nonreducing conditions. Antithrombin−factor IXa complex bands stained with Coomassie Blue R250 were quantified by scanning gels and imaging complex bands with Scion Image 4.0 software (Scion Corp., Frederick, MD). k_{obs} was obtained by fitting the time dependence of complex formation by a single-exponential function, and second-order rate constants were calculated as described above.

Effect of Calcium on Factor IXa Substrate Hydrolysis. The rate of factor IXa cleavage of the fluorogenic substrate was analyzed as a function of calcium chloride concentration with 20 nM enzyme and 45 μM fluorogenic substrate in Hepes buffer (pH 7.4) lacking EDTA and ethylene glycol. The initial linear hydrolysis rate was measured, and its dependence on calcium concentration was fit by the equation

$$v_{obs} = (v_0 K_{Ca})/(K_{Ca} + [Ca^{2+}]_o) + (v_{Ca}[Ca^{2+}]_o)/(K_{Ca} + [Ca^{2+}]_o)$$

where v_{obs}, v_o, and v_{Ca} are the rates of hydrolysis measured at a given calcium concentration, in the absence of calcium, and at a saturating calcium concentration, respectively, and K_{Ca} is the dissociation constant for calcium ion binding to the enzyme.

FPLC Analysis of Factor IXa−Heparin Interactions. Factor IXa (1−5 μg) was applied to a heparin−agarose column previously equilibrated in $I = 0.15$ Hepes buffer containing either 1 mM EDTA or 5 mM Ca^{2+}. The column was washed for 15 min, and a linear salt gradient to 1.5 M NaCl was then run over the next 45 min with a flow rate of 1 mL/min, followed by limit buffer for 10 min. Elution of factor IXa from the column was detected with a fluorescence monitor set at 280 nm for excitation and 340 nm for emission.

RESULTS

Kinetics of the Antithrombin−Factor IXa Reaction in the Absence of Heparin. Factor IXa formed SDS-stable complexes with antithrombin in a near-quantitative manner with minimal cleaved antithrombin appearing in the reaction either in the absence or in the presence of heparin, indicating no significant contribution of the substrate pathway in these reactions (*25*). Analysis of the kinetics of the antithrombin−factor IXa reaction with a sensitive chromogenic substrate assay (*31*) showed that antithrombin inhibited factor IXa with an extremely low second-order rate constant of ~10−15 M^{-1} s^{-1} at *I* 0.15, pH 7.4, and 25 °C (Table 1). A similar rate constant of 12 M^{-1} s^{-1} was obtained when the reaction kinetics were directly monitored by SDS−PAGE analysis of formation of the antithrombin−factor IXa complex (Figure 1), validating that the chromogenic substrate assay of factor IXa activity accurately reports the kinetics of factor IXa inhibition by antithrombin. Our own previous reports of higher rate constants for this reaction could be traced to

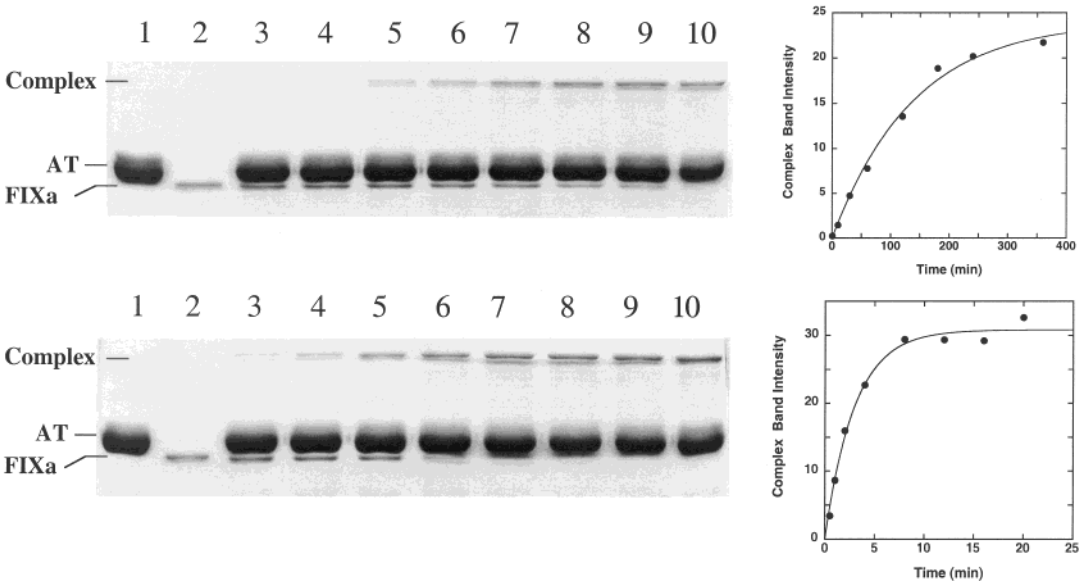

FIGURE 1: SDS−PAGE analysis of the kinetics of the antithrombin−factor IXa reaction. Top and bottom gels depict the reaction of antithrombin (10 μM) with factor IXa (2 μM) either in the absence of heparin after reaction times of 0.5, 10, 30, 60, 120, 180, 240, and 360 min (top gel, lanes 3−10, respectively) or in the presence of 1 μM heparin pentasaccharide for reaction times of 0.5, 1, 2, 4, 8, 12, 16, and 20 min (bottom gel, lanes 3−10, respectively). Unreacted antithrombin and factor IXa are shown in lanes 1 and 2 of the gels, respectively. The panels on the right show plots of the integrated complex band intensity as a function of time with nonlinear regression fits to a single-exponential function shown as solid lines.

heparin contamination of the enzyme preparation in one study (*29*) or the fluorogenic substrate used in another study measuring traces of factor XIa activity in the factor IXa preparation instead of factor IXa activity (*27*).

Effect of Calcium Ions on the Reaction. Addition of physiologic levels of calcium ions (5 mM) to the reaction significantly enhanced the second-order rate constant for antithrombin inhibition of factor IXa 4−6-fold to 58 ± 7 M^{-1} s^{-1}. Increasing the calcium ion concentration to 10 mM resulted in no further rate increase, indicating that 5 mM calcium was sufficient to saturate the effect. A similar rate-enhancing effect of calcium ions was observed on the initial rate of cleavage of the synthetic substrate by factor IXa. Calcium thus produced a saturable 43 ± 3% increase in the rate of chromogenic substrate hydrolysis by factor IXa (Figure 2), consistent with calcium acting as an allosteric effector of factor IXa enzymatic activity. The apparent dissociation constant for calcium binding to factor IXa of 15 ± 2 μM determined from these data is in keeping with the allosteric effector site on the enzyme being saturated at physiologic levels of the cation.

Effects of the Heparin Pentasaccharide on the Reaction. The synthetic heparin pentasaccharide corresponding to the specific antithrombin binding sequence in heparin greatly enhanced the rate of antithrombin inhibition of factor IXa when the inhibition was monitored from the loss of enzyme activity in the chromogenic substrate assay. The observed pseudo-first-order rate constant (k_{obs}) for antithrombin inhibition of factor IXa in the absence of calcium increased in a saturable manner with increasing molar ratios of pentasaccharide to antithrombin (Figure 3), reaching a maximal value 400-fold greater than that determined in the absence of saccharide at a molar ratio of pentasaccharide to antithrombin of ~1 (Table 1). Analysis of the kinetics of the factor IXa reaction with the antithrombin−pentasaccharide complex using SDS−PAGE to monitor formation of the inhibitor−enzyme complex (Figure 1) confirmed the substantial

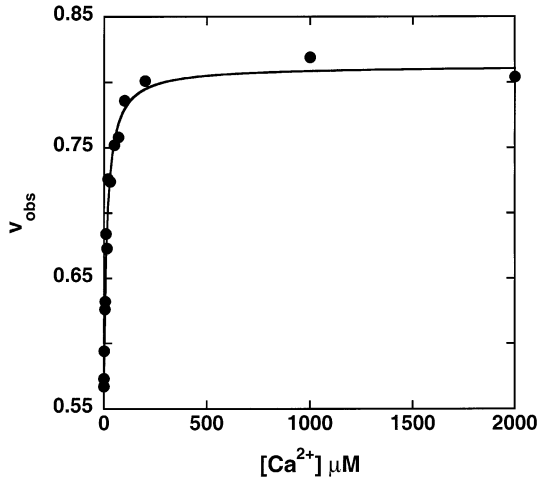

FIGURE 2: Calcium stimulation of factor IXa substrate hydrolysis. Initial rates of hydrolysis of 45 μM methylsulfonyl-D-cyclohexylglycyl-Gly-Arg-7-amido-4-methylcoumarin substrate by 20 nM factor IXa were measured as a function of calcium ion concentration as described in Experimental Procedures. The solid line shows a fit of the data by the binding equation given in the text.

activating effect of the pentasaccharide on the reaction and yielded a rate constant of 5500 M^{-1} s^{-1}, reasonably similar to that measured with the chromogenic assay. Saturating levels of the pentasaccharide added to antithrombin−factor IXa reaction mixtures containing 5 mM calcium also caused a similar ~500-fold increase in the rate constant over that measured in the absence of saccharide. The rate constant of 31 000 M^{-1} s^{-1} measured in this case was ~7-fold greater than that determined for the antithrombin−pentasaccharide complex reaction in the absence of calcium (Table 1). Calcium ions thus stimulated the rate constant for the antithrombin−pentasaccharide complex reaction with factor IXa to an extent similar to that for the uncomplexed inhibitor reaction. Approximately 50% of the calcium enhancement of this rate constant was achieved at 10 μM calcium ions,

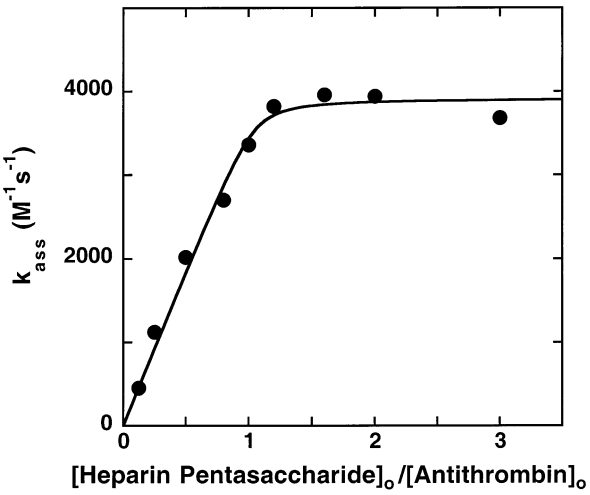

FIGURE 3: Heparin pentasaccharide acceleration of the antithrombin−factor IXa reaction. Apparent second-order association rate constants (k_{ass}) for reactions of 5 μM antithrombin with 100 nM factor IXa in Hepes buffer lacking calcium were determined as a function of added heparin pentasaccharide at the indicated molar ratios by dividing measured pseudo-first-order rate constants by the antithrombin concentration. Further details are given in Experimental Procedures. The solid line shows a nonlinear regression fit of data by the quadratic binding function for a 1:1 interaction.

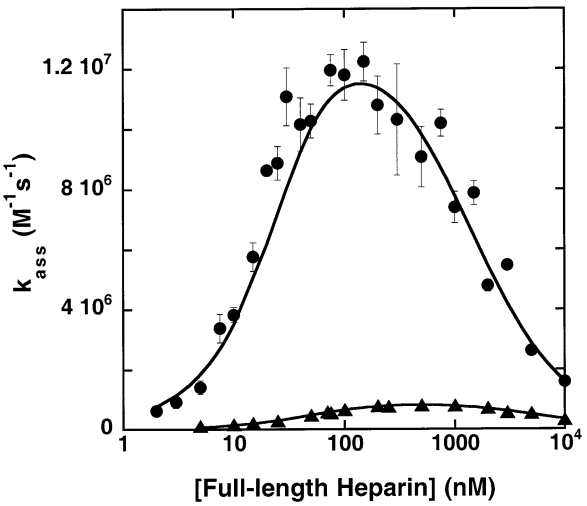

FIGURE 4: Accelerating effect of a full-length heparin on the antithrombin−factor IXa reaction in the absence or presence of calcium. k_{obs} for the reaction of 25 nM antithrombin with 5 nM factor IXa was measured as a function of added 50-saccharide heparin in Hepes buffer containing EDTA (▲) or 5 mM $CaCl_2$ (●) as described in Experimental Procedures. Reactions were allowed to proceed for 15 s in the presence and for 60 s in the absence of calcium before the residual factor IXa activity was measured. Apparent second-order rate constants were calculated by dividing k_{obs} by the antithrombin concentration. Each point represents an average of at least two determinations. Data were fit by the equation for the ternary complex bridging model for heparin acceleration of the reaction given in the text (solid lines).

and full enhancement was reached at 100 μM cation, suggesting that the calcium binding interaction which enhances inhibition of factor IXa by the antithrombin−pentasaccharide complex is also responsible for stimulating factor IXa chromogenic substrate hydrolysis.

Effects of Full-Length Heparin on the Reaction. A full-length heparin of ~50 saccharides which contained the pentasaccharide binding sequence increased the rate constant for antithrombin inhibition of factor IXa to a much greater extent than the pentasaccharide. k_{obs} measured in the absence of calcium initially increased with increasing heparin concentration in parallel with the formation of an antithrombin−heparin binary complex, as judged from the agreement between the fitted K_D of 28 ± 5 nM for the initial saturable increase in k_{obs} and the K_D of 20 ± 3 nM measured directly by equilibrium binding titrations. This was followed by a gradual decline in k_{obs} at heparin concentrations of >1 μM (Figure 4). The bell-shaped dependence of k_{obs} on heparin concentration indicated that the additional rate enhancement produced by the full-length heparin arises from heparin bridging antithrombin and factor IXa in a ternary complex, similar to the mechanism by which full-length heparins accelerate antithrombin reactions with thrombin and factor Xa (*20*, *26*). Fitting of the kinetic data by the ternary complex bridging model showed that the full-length heparin produced a maximal rate enhancement of ~60000-fold, representing a rate enhancement ~200-fold greater than that produced by the pentasaccharide due to heparin bridging. A similar bell-shaped dependence of k_{obs} on the full-length heparin concentration was evident when reactions were conducted in the presence of 5 mM calcium (Figure 4). However, the maximal increase in k_{obs} was much greater in the presence of calcium than in its absence, and the subsequent decline in k_{obs} occurred at lower heparin concentrations (>0.1 μM) when calcium was present. Analysis of the data according to the ternary complex bridging model indicated a second-order rate constant for anithrombin−heparin complex inhibition of free factor IXa of $1-2 \times 10^7$ M^{-1} s^{-1},[2] corresponding to a >300000-fold overall rate enhancement due to heparin and an enhancement at least 600-fold greater than that produced by the pentasaccharide. The shift in the descending limb of the bell-shaped curve to lower concentrations of heparin in the presence of calcium ions suggested that factor IXa binds heparin with a higher affinity under these conditions (*26*). The fitted K_D for formation of the binary factor IXa−heparin complex in the descending limb in fact decreased from 5.9 ± 1.1 μM in the absence to 1.3 ± 0.2 μM in the presence of calcium. In support of this conclusion, factor IXa was eluted from a heparin−agarose column at a higher salt concentration in the presence (0.70 M) than in the absence (0.53 M) of calcium. Calcium thus promotes heparin bridging of antithrombin and factor IXa by enhancing the binding of factor IXa to antithrombin−heparin binary complexes. Calcium stimulation of the 50-saccharide heparin-catalyzed reaction was maximal at 2 mM calcium ions (not shown), indicating that higher calcium concentrations are required to promote heparin bridging of antithrombin and factor IXa compared to those required to activate factor IXa enzymatic activity. The physiological calcium concentration of 5 mM in fact reduced the rate constant ~25% from the optimum value measured at 2 mM calcium.

The dependence of the full-length heparin enhancement of the antithrombin−factor IXa reaction rate on heparin chain length was determined under conditions where heparin levels

[2] Full progress curves of 50-saccharide heparin-catalyzed antithrombin−factor IXa reactions in the presence of calcium measured at optimal heparin concentrations revealed that rate constants measured by the fixed time assay under these same conditions may be underestimated due to the high rate of the reaction (see Table 1).

Table 2: Association Rate Constants for Inhibition of Factor IXa by Human Antithrombin Reactive Loop Variants and Complexes of the Variants with Pentasaccharide and Full-Length Heparins at 25 °C, pH 7.4, *I* 0.15, and 5 mM $CaCl_2$[a]

antithrombin	k_{uncat} (M^{-1} s^{-1})	k_{H5} (M^{-1} s^{-1})	k_{H50} (M^{-1} s^{-1})	k_{H5}/k_{uncat}	k_{H50}/k_{H5}
wild-type	$(1.1 \pm 0.1) \times 10^2$	$(2.5 \pm 0.4) \times 10^4$	$(3.3 \pm 0.3) \times 10^6$	230 ± 60	130 ± 30
PN1 loop	$(1.5 \pm 0.1) \times 10$	$(5.1 \pm 0.2) \times 10^3$	$(1.1 \pm 0.1) \times 10^6$ [b]	340 ± 40	220 ± 30
P3F/P2P/P2′F	2.9 ± 0.5	$(1.9 \pm 0.2) \times 10^3$	$(3.8 \pm 0.7) \times 10^5$	660 ± 180	200 ± 60
P1W	0.007 ± 0.001[b]	1.5 ± 0.2	$(2.5 \pm 0.6) \times 10^2$ [c]	210 ± 60	170 ± 60
P1′L[d]	30 ± 9[e]	$(6.8 \pm 0.5) \times 10^3$	$(4.3 \pm 0.2) \times 10^6$	230 ± 90	630 ± 80

[a] Second-order association rate constants for reactions of factor IXa with recombinant and plasma antithrombins alone (k_{uncat}) or with complexes of the inhibitor variants with pentasaccharide (k_{H5}) or with ~50-saccharide full-length heparin (k_{H50}) were determined as described in Experimental Procedures in 0.1 M Hepes and 5 mM $CaCl_2$ (pH 7.4, $I = 0.15$). Reactive loop mutants were made on a wild-type background containing an Asn135 to Gln mutation and included the following: (i) P3 I → F, P2 A → P, P2′ L → F, a thrombin consensus sequence (P3F/P2P/P2′F), (ii) P6−P3′ VVIAGRSLN → AILIARSSP, the reactive loop sequence of protease nexin 1 (PN1 loop), and (iii) P1 Arg → Trp (P1W). A natural P1′ Ser → Leu mutant antithrombin (antithrombin Denver) was isolated from the plasma of a patient carrying the mutation (*27*). Values are averages ± the standard error of the mean of at least three measurements except where indicated. [b] Value ± the standard error from a single reaction of 15 μM inhibitor with 1.5 μM factor IXa followed for 17 days after correction for control losses in activity. [c] Average ± range of two measurements. [d] The control values for this variant are those given for normal plasma antithrombin in Table 1. [e] Value ± the standard error from a single reaction of 0.5 μM inhibitor with 10 nM factor IXa followed for 30 h after correction for control activity losses.

were catalytic and antithrombin was in large molar excess over the polysaccharide, both in the absence and in the presence of 5 mM calcium ions (Table 1). Surprisingly, heparin rate enhancements were smaller under these conditions than when measured with heparin in molar excess over antithrombin, as in the experiments whose results are depicted in Figure 4. Because the full-length heparins that were employed contained on average 1.3, 1.9, and 1.9 pentasaccharide sites per chain for 26-, 50-, and 70-saccharide heparins, respectively, as shown by stoichiometric titrations with antithrombin, these results imply that the observed rate enhancements were sensitive to the density of antithrombin molecules bound per heparin chain. Thus, lower rate enhancements appear to be associated with the higher binding densities achieved when a molar excess of antithrombin was used to saturate all sites on a heparin chain. Consistent with this conclusion, no difference was found in rate enhancements determined for the pentasaccharide when antithrombin or pentasaccharide was present in molar excess over the other. Regardless of the experimental design, the second-order inhibition rate constant was found to be quite sensitive to heparin chain length, its value, when measured at high antithrombin binding densities, increasing from ~7×10^4 to 6×10^5 M^{-1} s^{-1} in the absence of calcium and from 4×10^6 to 3×10^7 M^{-1} s^{-1} in the presence of calcium as the heparin chain length increased from ~26 to ~70 saccharides. The overall rate enhancements under these conditions thus ranged from 6000 to 60 000 without calcium and from 70 000 to 500 000 with calcium for 26- and 70-saccharide heparins, respectively. The effect of calcium on the full-length heparin-catalyzed reaction rate constants (50−60-fold increase) was significantly larger than on uncatalyzed and pentasaccharide-catalyzed reaction rate constants (4−7-fold increases), consistent with calcium acting both to activate factor IXa and to promote heparin bridging of antithrombin and factor IXa in a ternary complex. SDS−PAGE analysis confirmed the substantial enhancement of the full-length heparin-catalyzed antithrombin−factor IXa reaction rate in the presence of calcium (not shown).

Contribution of Reactive Loop Residues to Antithrombin Reactivity with Factor IXa. To determine whether the specificity of antithrombin for factor IXa is encoded in the serpin reactive loop, we examined the effect of reactive loop mutations previously shown to alter antithrombin specificity on the reactivity of antithrombin with factor IXa in the absence and presence of pentasaccharide and full-length heparins (*18*, *19*). The mutants consisted of (i) a triple P3 Ala → Phe, P2 Gly → Pro, and P2′ Leu → Phe mutant in which the altered residues correspond to consensus residues found in natural protein substrates of thrombin, (ii) a replacement of antithrombin P6−P3′ residues, VVIAGRSLN, except for P1−P1′, with those of the serpin, protease nexin-1, AILIARSSP, the latter a specific inhibitor of thrombin, (iii) a single P1 Arg → Trp mutation which converts antithrombin from an inhibitor of trypsin and trypsin-like proteases to an inhibitor of chymotrypsin (*19*), and (iv) a single P1′ Ser → Leu mutation which greatly reduces thrombin reactivity (*27*). The first three of these mutants were engineered recombinant antithrombins, whereas the last was a natural antithrombin variant. The reactive loop mutations significantly decreased the reactivity of antithrombin with factor IXa measured in the presence of calcium (Table 2), with second-order rate constants reduced 38-, 7-, 16000-, and 2-fold for the triple mutant, the protease nexin-1 loop mutant, the P1 Trp mutant, and the P1′ Leu mutant, respectively. It should be noted that the wild-type recombinant antithrombin had a 2-fold higher reactivity with factor IXa than plasma-derived antithrombin, similar to the difference in reactivity of recombinant and plasma antithrombins with factor Xa reported previously (*32*). This difference appears to be due to a small activating effect of the Asn135 → Gln mutation in all recombinant antithrombins due to the elimination of carbohydrate at this position and its interference with conformational activation of the serpin (*32*). Pentasaccharide or full-length heparins greatly enhanced the reactivity of the natural or recombinant mutant antithrombins with factor IXa. Interestingly, the extents of rate enhancement were very similar to those observed with the recombinant wild-type or plasma inhibitor reactions (Figure 5 and Table 2). These results indicate that the reactive loop residues, most importantly the P1 Arg residue, are critical determinants of the reactivity of antithrombin with factor IXa in the absence of heparin, but do not influence the heparin enhancement of this reactivity.

DISCUSSION

The purpose of this study was to establish the physiologic relevance and mechanism of heparin catalysis of the antithrombin−factor IXa reaction by examining the effects of pentasaccharide and full-length heparin chains of defined

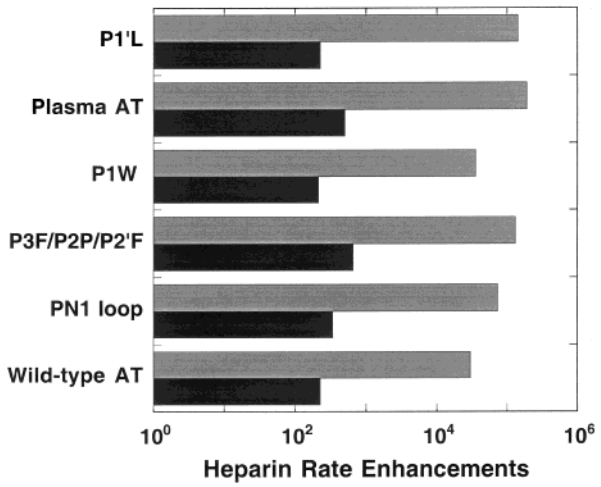

FIGURE 5: Heparin rate enhancements of reactions of factor IXa with antithrombin reactive loop variants. The relative increases in reactivity of normal and variant antithrombins with factor IXa produced by pentasaccharide (black bars) or 50-saccharide heparins (gray bars) in the presence of 5 mM calcium were calculated as the ratio of second-order association rate constants measured in the presence and absence of heparin (Table 2).

length and with high affinity for antithrombin as well as the influence of calcium ions on the reaction. An additional goal was to use mutagenesis to elucidate the reactive loop determinants of antithrombin's reactivity with factor IXa in the absence and presence of heparin for comparison with those previously shown to govern the inhibitor's reactivity with thrombin and factor Xa. Our findings have shown that antithrombin is an extremely unreactive inhibitor of factor IXa in the absence of heparin, whereas it becomes a remarkably efficient inhibitor of the enzyme in the presence of the polysaccharide activator when physiologic levels of calcium ion are present. The basal rate constant of $\sim$10 M^{-1} s^{-1} that we measured for antithrombin inhibition of factor IXa in the absence of heparin is considerably lower (200−900-fold) than basal rate constants for antithrombin inhibition of factor Xa and thrombin (*13*, *32*). This rate constant is also much lower than several previously reported values (*20*−*22*) which may be due to traces of heparin present in inhibitor or enzyme preparations purified by heparin−agarose chromatography. It agrees, however, with previous estimates of antithrombin reactivity with factor IXa in plasma (*23*, *24*, *33*). The unusually low reactivity is not surprising in view of the poor reactivity of factor IXa toward synthetic substrates (*31*) and in particular the k_{cat}/K_M of 15 M^{-1} s^{-1} measured for the substrate Z-Ala-Gly-Arg-*p*-nitroanilide, which mimics the antithrombin reactive loop sequence (*35*). The low reactivity of the enzyme appears to be due to a distortion of the S1 pocket and steric hindrance of active site-bound substrates by the 99 loop (*36*−*38*).

The rate constant for antithrombin inhibition of factor IXa was found to be dramatically stimulated $\sim 10^6$-fold by full-length heparins to the physiologically significant diffusion-limited range ($\sim 10^7$ M^{-1} s^{-1}), to approach rate constants for thrombin and factor Xa inhibition by antithrombin−heparin complexes under these conditions (*13*, *34*). This stimulatory effect, attained with well-defined high-affinity heparins in our study, is considerably higher than the 4000−10000-fold heparin rate enhancements indicated by earlier work, the majority of which were measured with unfractionated heparin (*20*−*22*, *33*). The values reported by Jordan et al. (*20*) and Mauray et al. (*22*) which were ostensibly measured in the absence of calcium are particularly anomolous in that they are closer to values we measured in the presence of calcium. A possible explanation is that sufficient calcium was adventitiously introduced by adding high concentrations of bovine serum albumin, a calcium binding protein, to reaction mixtures in such studies.

The large heparin rate enhancement of the antithrombin−factor IXa reaction was found to originate from three sources: (i) allosteric activation of factor IXa by calcium, (ii) conformational activation of antithrombin by the heparin pentasaccharide and (iii) bridging of antithrombin and factor IXa by full-length heparins, augmented by calcium. The allosteric effect of calcium ions on factor IXa reactivity was indicated from the similar stimulating effects of calcium on the basal rate of antithrombin inhibition of factor IXa and on the rate of factor IXa cleavage of a synthetic substrate. A comparable stimulation by calcium of the rate of factor IXa activation of factor X in the absence of the cofactors, phospholipid, and factor VIIIa has also been reported (*39*). These effects of calcium appear to arise from a high-affinity calcium interaction with the enzyme ($K_D \sim 15$ μM). The effects presumably involve the specific calcium binding loop in the catalytic domain of factor IXa corresponding to residues 70−80 (chymotrypsin numbering), which is known to mediate calcium-dependent cofactor binding in factor IXa as well as in factor Xa and factor VIIa (*40*−*43*). The affinity of calcium for the factor IXa effector site found in this study is thus similar to that reported for the 70−80 loop in trypsin ($K_D \sim 10$ μM) (*44*).

It was surprising to find that the heparin pentasaccharide, which conformationally activates antithrombin but is incapable of bridging the inhibitor and proteinase in a ternary complex, caused such a large enhancement of the antithrombin−factor IXa reaction rate. The pentasaccharide has been thought to specifically activate antithrombin to react with factor Xa, since the saccharide accelerates the antithrombin−factor Xa reaction $\sim$300-fold so that it approaches the diffusion-limited range and minimally or only modestly affects the serpin's reactivity with thrombin and other enzymes (*13*). A previous report of Pieters et al. (*21*) in fact suggested that the pentasaccharide did not influence antithrombin reactivity with factor IXa. However, a later report of this group suggested that the pentasaccharide significantly stimulated the antithrombin−factor IXa reaction rate in recalcified plasma, although the extent of the stimulation was unclear (*33*). Our findings clearly show a substantial $\sim$300−500-fold pentasaccharide acceleration of the antithrombin−factor IXa reaction, comparable to the accelerating effect on the antithrombin−factor Xa reaction. These findings contrast with early and more recent studies of the chain length dependence of heparin's accelerating effect on the antithrombin−factor IXa reaction which suggested that factor IXa resembles thrombin in showing a marked dependence of the acceleration on heparin chain length with little reactivity observed for chains of fewer than 17−18 saccharides (*45*, *46*). However, it was not appreciated in these studies how poorly reactive antithrombin is with factor IXa in the absence of heparin and that the observed low reactivity with small pentasaccharide-containing heparin fragments represents a significant enhancement over a much lower basal reactivity.

The activating effect of the pentasaccharide, while substantial, is thus still not sufficient to produce a physiologically significant rate of factor IXa inhibition by antithrombin.

The additional rate-enhancing effect of full-length heparins on the antithrombin−factor IXa reaction and the bell-shaped heparin concentration dependence of the effect indicate that such heparins are capable of promoting the reaction by bridging antithrombin and factor IXa in a ternary complex (*20*, *26*). The rate enhancement due to heparin bridging ranges from 14- to 130-fold for ~26- to ~70-saccharide full-length heparins in the absence of calcium but increases to 130−1000-fold for these heparins when physiologic levels of calcium are present. Calcium ions thus augment the rate enhancement due to heparin bridging by ~8−9-fold in a manner independent of heparin chain length. This effect of calcium is distinct from the allosteric effect of the divalent cation, as judged from its requirement for higher calcium concentrations, and was associated with calcium ions enhancing the affinity of factor IXa for heparin in the ternary bridging complex. The effect presumably results from calcium ions binding to the acidic γ-carboxyglutamic acid (Gla) domain of factor IXa and disrupting an intramolecular interaction of this domain with the basic heparin binding exosite in the catalytic domain of the protease (*47*). This follows from the similar effect of calcium ions in promoting heparin bridging of antithrombin and factor Xa and the demonstration that this effect involves calcium interactions with the Gla domain which make the heparin binding exosite of factor Xa accessible to heparin (*34*, *48*). However, in contrast to the antithrombin−factor Xa reaction in which an insignificant heparin bridging effect is observed without calcium, the antithrombin−factor IXa reaction shows a significant heparin bridging effect when calcium is absent. This implies that the intramolecular interaction between the Gla domain and the heparin binding exosite may be more easily disrupted by heparin in factor IXa than in factor Xa, possibly because of the higher affinity of heparin for factor IXa than factor Xa (*20*). The bridging effect was found to be 8−15-fold greater with an ~70-saccharide heparin than with an ~26-saccharide heparin either in the absence or in the presence of calcium. This observation suggests a pronounced chain length dependence of the bridging effect similar to that previously found for heparin bridging antithrombin and factor Xa and to a lesser extent also for heparin bridging antithrombin and thrombin (*34*). Such a dependence is a characteristic feature of heparin bridging and is thought to reflect the larger target area of longer heparin chains which enhances the capture of a protease by nonspecific binding and promotes one-dimensional diffusion of the enzyme to specifically bound antithrombin (*49*). The bridging effect also appears to be diminished when full-length heparins possessing multiple pentasaccharide binding sites for antithrombin have all sites saturated with the serpin, as compared to when only a single site is occupied. This may result from the effective length of such chains being reduced when multiple antithrombins are bound.

Our finding that conformational activation of antithrombin by the heparin pentasaccharide enhances the serpin's reactivity with factor IXa as much as it does with factor Xa implies that the serpin conformational change must promote the interaction of the antithrombin reactive loop with the active sites of both enzymes despite their markedly different substrate specificities. To determine whether this arises from a more optimal presentation of the reactive center loop sequence of the serpin to the enzyme, we used several reactive loop mutants previously designed to assess the reactive loop contribution to antithrombin's specificity for factor Xa and thrombin. These mutants were found to decrease the reactivity of antithrombin with factor IXa from 2- to 16000-fold in the absence of heparin but to have little or no effect on the enhancement of antithrombin reactivity with factor IXa either with pentasaccharide or with full-length heparins. Such findings indicate that the reactive loop sequence, and most importantly the P1 Arg residue, are principal determinants of the reactivity of unactivated antithrombin with factor IXa, but this sequence does not contribute to the heparin enhancement of antithrombin reactivity. Similar findings were made when the reactivity of these and other antithrombin reactive loop mutants was analyzed with factor Xa and thrombin (*18*, *19*). We thus conclude that the antithrombin reactive loop sequence determines the reactivity of the unactivated serpin with factor IXa, factor Xa, and thrombin, whereas heparin enhances antithrombin reactivity with all enzymes through exosite determinants outside the reactive loop P6−P3′ sequence which are present both on the serpin and on heparin. The serpin exosites are made available upon conformational activation of antithrombin, whereas the heparin exosites are the bridging sites present in longer heparin chains next to the pentasaccharide site.

Exosites are thus key mediators of the reactivity of heparin-activated antithrombin with three different clotting proteinases and can explain why the antithrombin reactive loop sequence need not be optimal for recognition by any of these target enzymes. Such a design for the reactive loop sequence may reflect the need for antithrombin to inhibit only procoagulant proteinases and not the anticoagulant proteinase, activated protein C (*50*). Indeed, the extremely low reactivity of heparin-activated antithrombin with activated protein C [$\sim 10^7$-fold lower than with thrombin, factor Xa, or factor IXa (*51*)] appears to be due to the reactive loop sequence having evolved to prevent binding to the active site of this proteinase. The location of the proposed exosites on antithrombin which enhance recognition of factor Xa and IXa remains to be determined, but an alignment of the 13 available antithrombin sequences, which include representatives from all five vertebrate families (*52*), reveals a number of surface residues surrounding the reactive proteinase binding loop which are highly conserved and thus are prime candidates for the exosite (*53*). Mutagenesis of Gln61 of the 60 loop and Arg150 of the autolysis loop of factor Xa was recently shown to abolish a large part of the pentasaccharide enhancement of antithrombin reactivity with factor Xa, suggesting that these enzyme residues may interact with the proposed antithrombin exosite (*54*, *55*). Future mutagenesis of candidate antithrombin residues will be required to prove the existence of such exosites.

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