Mechanism of Catalysis of Inhibition of Factor IXa by Antithrombin in the Presence of Heparin or Pentasaccharide‡

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Running title: Heparin catalysis of factor IXa inhibition by antithrombin
Summary

Because of the homology between factor IXa and factor Xa (f.IXa and f.Xa, respectively), and the critical upstream position of f.IXa in the coagulation cascade, the contribution of the heparin-derived pentasaccharide to antithrombin-mediated inhibition of f.IXa was investigated. Pentasaccharide promotes inhibition of both f.IXa and f.Xa generated in recalcified plasma. This result demonstrates that antithrombin is the predominant inhibitor of f.IXa in plasma, and that antithrombin’s activity is promoted by pentasaccharide. Kinetic experiments reveal that pentasaccharide increases the rates of antithrombin-mediated inhibition of both f.IXa and f.Xa by 2-orders of magnitude. These findings indicate that pentasaccharide-induced conformational changes in antithrombin enhance its capacity to inhibit both f.IXa and f.Xa. In the presence of Ca\(^{2+}\), full-length heparin produces an additional ~10-fold increase in the rates of inhibition of both enzymes, consistent with a template role of heparin. Heparin binding to f.Xa was previously shown to be promoted in the presence of Ca\(^{2+}\). Binding studies with f.IXa reveal a 10-fold higher affinity for heparin in the presence of Ca\(^{2+}\) compared with its absence. Thus, Ca\(^{2+}\) promotes heparin-catalyzed inhibition of f.IXa and f.Xa by antithrombin by augmenting the template mechanism. These results indicate that heparin-mediated catalysis of f.IXa inhibition by antithrombin reflects both pentasaccharide-induced conformational changes and heparin-mediated bridging of antithrombin to f.IXa. Furthermore, our data suggest that the efficacy of pentasaccharide for prevention and treatment of thrombotic disorders may reflect its action at two sites in the coagulation system.
INTRODUCTION

Heparin and low molecular weight heparin (LMWH)\(^1\) act as anticoagulants by catalyzing antithrombin, a serpin that inactivates thrombin, factor (f.) IXa, and f.Xa (1). Binding of heparin and LMWH to antithrombin is mediated by a unique pentasaccharide sequence found on one-third of heparin chains and one-fifth of LMWH chains (2). Pentasaccharide-mediated binding of heparin or LMWH to antithrombin induces a conformational change in the reactive centre loop of antithrombin that facilitates its interaction with f.Xa (3-5). Consequently, both agents increase the rate at which antithrombin inhibits f.Xa by two or three orders of magnitude (6, 7).

Efficient catalysis of thrombin inhibition by antithrombin requires a specific pentasaccharide-containing heparin chain of sufficient length to bridge antithrombin to thrombin, thereby forming a ternary heparin-thrombin-antithrombin complex (8, 9). Only heparin chains that contain at least 13 saccharide units in addition to the pentasaccharide, or a minimum molecular mass of 5400, are long enough to bridge antithrombin to thrombin (10). With a mean molecular mass of 5000, the majority of LMWH chains are too short to provide this bridging activity. Consequently, LMWH has reduced ability to catalyze thrombin inhibition relative to heparin.

An analog of the antithrombin-binding pentasaccharide sequence in heparin or LMWH has recently been synthesized (11). This agent, known as fondaparinux, has been compared with LMWH for prevention and treatment of venous thromboembolism (12-15). When compared with LMWH for thromboprophylaxis in high risk orthopedic patients, fondaparinux produces a 55% reduction in the risk of postoperative venous thromboembolism. More recent studies have compared fondaparinux with LMWH for treatment of patients with deep vein thrombosis or with heparin for therapy of patients with pulmonary embolism. In these studies, the efficacy and safety of
F.IXa plays a critical role in the amplification of thrombin generation after clotting is triggered by tissue factor exposure (17, 18). Several studies have demonstrated that heparin increases the rate of f.IXa inhibition by antithrombin, the predominant inhibitor of f.IXa (19-25). Kinetic analyses suggest that, like the case with thrombin, heparin accomplishes this by serving as a template onto which f.IXa and antithrombin bind. These studies have been corroborated by the identification of the heparin-binding site on f.IXa, which has been localized to a region distinct from the active site of the enzyme (26). Although kinetic analyses suggest that heparin serves as a template to catalyze f.IXa inhibition by antithrombin, the effect of heparin chain length on this reaction has not been extensively evaluated.

In addition to heparin chain length, divalent cations also influence rates of protease inhibition. Ca\(^{2+}\) enhances the rate of f.Xa inhibition by antithrombin, presumably by inducing the exposure of a heparin-binding site on the protease, thereby allowing formation of a ternary heparin-antithrombin-f.Xa complex (27, 28). This concept is supported by surface plasmon resonance studies demonstrating that the binding of heparin to f.Xa is Ca\(^{2+}\)-dependent (29). In the same study, it also was determined that heparin binds to f.IXa with a \(K_d\) of 17.8 nM, but only in the presence of Ca\(^{2+}\). The Ca\(^{2+}\)-dependence of the heparin-f.IXa interaction may explain why, depending on the Ca\(^{2+}\) concentration, previous studies reported different rates of f.IXa inactivation by the heparin-antithrombin complex (21, 30).

The purpose of this study was to determine the mechanism of f.IXa inhibition by antithrombin and the role played by Ca\(^{2+}\). Preliminary experiments were performed in recalcified plasma to examine the effect of heparin or fondaparinux on antithrombin inhibition of f.IXa.
generated in situ. To establish the mechanism of heparin-catalysis of f.IXa inhibition by antithrombin, we compared the effects of fondaparinux or heparin on the rates of f.IXa inhibition in a buffer system in the absence and presence of Ca^{2+}. The influence of these glycosaminoglycans on the rates of f.Xa and thrombin inhibition by antithrombin also were determined for comparative purposes.
EXPERIMENTAL PROCEDURES

Materials

All reagents used were of analytical grade. Polybrene (hexadimethrine bromide), heparin (174 units/mg; from porcine intestinal mucosa), fluorescein isothiocyanate (FITC), and fluorogenic substrate B-9760 (Boc-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin hydrochloride) were purchased from Sigma-Aldrich Canada. Pefafuor 10148 (methoxysuccinyl-(D)-cyclohexylglycine-Gly-Arg-7-amino-4-methyl-coumarin-acetic acid) and Pefachrome FIXa (CH$_3$SO$_2$-D-cyclohexylglycine-Gly-Arg-p-nitroanalide-acetic acid), substrates for f.IXa, and Prionex were obtained from Centerchem Inc. (Stamford, CT). S-2765 (N-α-benzyloxy carbonyl-D-Arg-Gly-Arg-p-nitroanalide) was obtained from Chromogenix (Molndal, Sweden). Tosyl-Gly-Pro-Arg-p-nitroanalide (tGPR-pNA) was obtained from Roche Applied Sciences Canada. Fondaparinux was obtained from Sanofi Recherche (Markham, ON) / Organon (Scarborough, ON). Hypersulfated LMWH (HS-LMWH) was synthesized and labeled with FITC (fluorescein-HS-LMWH) as described (31). Human α-thrombin, and factor Xa were obtained from Enzyme Research Laboratories Inc. (South Bend, IN). Human f.IXa was obtained from Haematologic Technologies Inc (Essex Junction, VT). Human antithrombin was from Affinity Biologicals Inc. (Ancaster, ON).

Methods

Analysis of f.II, f.IX, and f.X activation and subsequent inhibition in activated plasma

Pooled platelet-poor plasma, collected from a minimum of ten healthy donors, was defibrinated by the addition of Arvin (Knoll Pharma Canada) to a concentration of 0.75 U/ml (1% v/v). After 20 min, the clot was wound around a plastic spatula and removed. The plasma was stored in aliquots at -70 °C. Equal volumes of plasma and Thrombosil (Beckman-Coulter Inc.,
Mississauga, ON) were mixed and incubated for 10 min at 37 °C. Subsequently, one-tenth volume of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TS) containing 0.01% polyethylene glycol (TSP), 6 µg/ml fondaparinux, or 6 µg/ml heparin was added to one of three tubes together with CaCl$_2$ to a final concentration of 16 mM. Immediately after CaCl$_2$ addition, and at timepoints thereafter, triplicate aliquots of 4 - 6 µl were removed into separate tubes containing 10 µl SDS gel sample buffer. Samples were placed in a boiling water bath for 3 min prior to electrophoresis on three 4-15% polyacrylamide gels (Criterion, BioRad, Mississauga ON). Standards containing zymogen, enzyme, and enzyme-antithrombin complex were included in each gel. After separating the proteins, the gels were blotted onto nitrocellulose membranes using a Transblot device, according to the manufacturer’s instructions (BioRad). Membranes were blocked by incubation in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.02% Tween-80 (TBS-Tw) containing 5% skim milk powder for 45 min at 23°C. After aspiration of the buffer, the membrane was incubated for 90 min with the antibody diluted in TBS containing 0.05% Tween-80. The membrane was then washed four times, for 5 min each, with TBS-Tw and then incubated with a detecting antibody. In all cases, chemiluminescence (ECL, Amersham-Pharmacia Biotech) was used to detect horseradish peroxidase (HRP)-linked second antibodies. Membranes were exposed to radiographic film for varying lengths of time and autoradiographs were scanned by densitometry (ImageMaster; Amersham-Pharmacia Biotech) and band densities were quantified using the instrument software. Membranes were stripped for reuse by washing for 30 min at 50°C in 62 mM Tris-HCl pH 6.7, 2% SDS, 70 mM mercaptoethanol.

Various antibodies were used for blotting; for f.IX, the first antibody was a mouse monoclonal antibody against human f.IX (Research Diagnostics Inc., Flanders, NJ), whereas the detecting antibody was a HRP-conjugated goat anti-mouse antibody (BioRad). For f.X, the first
antibody was a mouse monoclonal antibody against the light chain of human f.X (Sigma) while the second antibody was a HRP-conjugated goat anti-mouse antibody (BioRad). For f.II, the first antibody was a sheep polyclonal antibody against human thrombin (Research Diagnostics Inc.) and the second antibody was a HRP-conjugated donkey anti-sheep antibody (Sigma).

**Effect of glycosaminoglycans on the rates of f.IXa, f.Xa, and f.IIa inhibition by antithrombin**

Rates of f.IXa, f.Xa and thrombin inhibition by antithrombin were measured in a discontinuous assay under pseudo-first-order rate conditions at 23°C in TS buffer containing 0.5% Prionex (27, 32). Heparin and fondaparinux were tested at concentrations ranging from 0-12000 µg/ml. Enzyme concentrations ranged from 1 nM to 500 nM and antithrombin concentrations from 10 to 3500 nM with a minimum enzyme:antithrombin ratio of 1:10 in a 20-µl reaction volume. A solution containing antithrombin and glycosaminoglycan in TSP was aliquoted into wells of a 96-well plate. At various time intervals, aliquots of enzyme were added to individual wells. All reactions were stopped simultaneously by addition of 180 µl of a solution of polybrene (10 mg/ml) and 200 - 500 µM of the appropriate chromogenic or fluorogenic substrate. Chromogenic assays monitoring f.IXa contained 30% ethylene glycol (33). Residual enzyme activity was monitored in Spectra Max or Thermo Max Gemini plate readers (Molecular Devices Corporation, Sunnyvale, CA). The rate of substrate cleavage (V) was determined in each well over a 5 min period by the instrument software. The pseudo-first-order rate constant (k₁) was determined by plotting ln(V/V₀) versus time, where V₀ is the rate of substrate cleavage by the enzyme in the absence of antithrombin. The apparent second order inhibition rate constant (k₂) was determined by dividing k₁ by the antithrombin concentration.
Binding Experiments

The affinity of fluorescein-HS-LMWH for f.IXa was measured as previously described and competition experiments were then performed to determine the affinities of heparin and fondaparinux for f.IXa (31). Briefly, the affinity of fluorescein-HS-LMWH for f.IXa was determined by monitoring fluorescence of a titration of fluorescein-HS-LMWH with f.IXa. The affinity of unlabeled heparin or fondaparinux was then measured in separate experiments where fluorescein-HS-LMWH was displaced from a fixed concentration of f.IXa by increasing concentrations of competing glycosaminoglycan.
RESULTS

Influence of fondaparinux and heparin on inhibition of thrombin, f.IXa, and f.Xa in activated plasma

Plasma experiments were performed to verify f.IXa inhibition by antithrombin and to determine the extent to which the reaction was promoted by fondaparinux or heparin. For comparative purposes, the inhibition of thrombin and f.Xa also was monitored. Defibrinogenated platelet-poor plasma was recalcified and activated at 23°C with Thrombosil, an agent that uses silica to activate the intrinsic pathway of coagulation, in the absence or presence of 0.6 µg/ml fondaparinux (0.33 µM) or heparin (0.04 µM). Aliquots were removed at various times and the extent of enzyme-inhibitor complex formation was assessed by SDS-PAGE followed by immunoblot analysis using antibodies that recognize the zymogen-, activated-, and antithrombin inhibited-forms of factors IX, X, or II (Fig. 1). Mobility and immunoreactivity of these forms were verified with markers prepared from purified zymogens, enzymes, and enzyme-antithrombin complexes. Membranes were scanned and the densities of individual bands were calculated as a percent of total (not shown).

In the absence of glycosaminoglycan, 80% of f.IX was activated over the 15 min incubation. By this time, about equal proportions of the initial f.IX were uninhibited as were in complex with antithrombin. These results confirm that antithrombin is the predominant inhibitor of f.IXa in plasma (24, 30). The time course of f.IX activation was attenuated slightly in the presence of heparin or fondaparinux. With both glycosaminoglycans, formation of f.IXa-antithrombin complexes was enhanced and accelerated, with heparin demonstrating slightly better efficacy than fondaparinux. These results indicate that both heparin and fondaparinux promote f.IXa inhibition
by antithrombin.

In the absence of glycosaminoglycan, f.X was activated more rapidly than f.IX, with about 80% f.X activation by 5 min. Free f.Xa accumulated to a lesser extent than f.IXa. However, much of f.Xa (70%) was rapidly inhibited by antithrombin in the absence of glycosaminoglycan. Consequently, f.Xa-antithrombin complexes formed earlier than f.IXa-antithrombin complexes and there was greater inhibition of f.Xa. In the presence of heparin, activation of f.X was attenuated, with only about 50% activation after 15 min. It is likely that retarded f.X activation reflects the inhibitory effect of heparin on upstream steps in the coagulation system, including inhibition of f.IXa. In comparison to heparin, fondaparinux had less effect on the time course of f.X activation. Like heparin however, fondaparinux reduced accumulation of free f.Xa and promoted formation of f.Xa-antithrombin complexes.

Prothrombin was activated more rapidly than f.IX or f.X in the absence of glycosaminoglycan, reaching full activation by 2 min. Levels of free thrombin rapidly reached 30%, and declined to about 5% after 15 min. Free f.Xa also showed a rapid increase with subsequent decline, whereas f.IXa demonstrated a gradual increase in concentration. Heparin delayed activation of prothrombin, as expected. Heparin was more effective at promoting thrombin inhibition by antithrombin than it was at enhancing f.Xa inhibition, a finding that is consistent with inhibition rate constants. Fondaparinux had little effect on prothrombin activation or thrombin inhibition in these experiments.

**Heparin-catalyzed rates of f.IXa inhibition**

To investigate the mechanism of heparin catalysis of f.IXa inhibition by antithrombin, rate constants of inhibition were determined under pseudo-first-order conditions in the absence or
presence of fondaparinux or heparin. Use of a f.IXa fluorogenic substrate permitted sensitive
determination of residual f.IXa activity in the inhibition assays (33). In the presence of 2 mM CaCl₂,
the plot of the second order rate constants of f.IXa inhibition by antithrombin versus heparin
concentration in 2 mM CaCl₂ is bell-shaped (Fig. 2A), characteristic of a template mechanism of
catalysis. The uncatalyzed rate of f.IXa inhibition by antithrombin, 1.34 x 10⁴ M⁻¹min⁻¹, was
increased up to 1210-fold by heparin to a maximum rate of inhibition of 1.62 x 10⁷ M⁻¹min⁻¹ (Table
I). Heparin caused a concentration-dependent increase in f.IXa inhibition in the presence of 2 mM
CaCl₂ until the rate reached a plateau at a heparin concentration of 2 µg/ml (0.13 µM), and heparin
concentrations above 200 µg/ml (13 µM) led to a decrease in k₂ values. These data support earlier
studies examining the effect of heparin on f.IXa inhibition by antithrombin which also suggested that
a template mechanism was important (20-22, 30). Second-order rate constants of 0.8 - 3 x 10⁴ and
0.3 - 10 x 10⁷ M⁻¹min⁻¹ for uncatalyzed and heparin-catalyzed rates of f.IXa inhibition by
antithrombin reported in these studies are in good agreement with the values determined in the
present study.

To quantify the contribution of pentasaccharide-induced conformational changes in
antithrombin to its interaction with f.IXa, the effect of fondaparinux on the rates of f.IXa inhibition
by antithrombin was monitored. The plot of k₂ versus fondaparinux concentration indicates that
fondaparinux enhances the rate of f.IXa inhibition (Fig. 2B). Whereas the plot of k₂ versus heparin
concentration is bell-shaped, a similar plot of fondaparinux-catalyzed f.IXa inhibition yields a
plateau, at a rate of approximately 1.44 x 10⁶ M⁻¹min⁻¹, with a fondaparinux concentration of 0.6
µg/ml (0.33 µM). To compare the effects of fondaparinux and heparin on the relative increases in
the rate of f.IXa inhibition by antithrombin, the maximum second-order rate constants of
fondaparinux- and heparin-catalyzed inhibition were divided by the uncatalyzed rates of inhibition. Fondaparinux produces a maximal 107-fold increase in the rate of inhibition of f.IXa by antithrombin compared with the rate measured in the absence of glycosaminoglycan (Fig. 3). With heparin, the rate is increased 1210-fold. Thus, heparin increases the rate of f.IXa inhibition by antithrombin 11-fold more than fondaparinux.

In contrast to the current results, a recent study failed to show a catalytic influence of fondaparinux on antithrombin inhibition of f.IXa (34). Methodological differences may account for these disparate results. We used a kinetic approach in the current study, whereas a single time point, measured in the absence of Ca$^{2+}$, was used to quantify inhibition in the work of Herault and colleagues. In addition, we directly quantified residual f.IXa activity by chromogenic assay, whereas an indirect tenase system was used in the previous publication. Another study, using methods similar to ours, demonstrates pentasaccharide-mediated stimulation comparable to that reported here (26).

**Rates of f.Xa and thrombin inhibition by antithrombin**

Because the mechanisms by which heparin catalyzes the inhibition of f.Xa and thrombin by antithrombin are distinct, inhibition studies of these two enzymes were performed for comparison purposes. In the presence of 2 mM CaCl$_2$, heparin produced a 1900-fold increase in the rate of f.Xa inhibition by antithrombin (from 1.83 x 10$^5$ M$^{-1}$min$^{-1}$ to 3.47 x 10$^8$ M$^{-1}$min$^{-1}$) (Fig. 2; Table I). The $k_2$ value continued to increase as a function of heparin concentration until a plateau was reached at a heparin concentration of approximately 6 µg/ml (0.4 µM). Heparin concentrations over 200 µg/ml (13 µM) caused dose-dependent decreases in the rate of f.IXa inhibition by antithrombin (Fig. 2A).

When the rate of f.Xa inhibition by antithrombin is plotted *versus* fondaparinux
concentration, there is a concentration-dependent increase until a plateau of $2.85 \times 10^7 \text{ M}^{-1}\text{min}^{-1}$ is reached with a fondaparinux concentration of 0.6 µg/ml (0.33 µM) (Fig. 2B). The effect of fondaparinux on the rate of f.Xa inhibition by antithrombin was similar to its effect on the rate of f.IXa inhibition (Fig. 3), with maximum stimulation of 156- and 107-fold, respectively. Like f.IXa, the rate of f.Xa inhibition by antithrombin is 11- to 12-fold higher with heparin than with fondaparinux (Fig. 3). These values are in agreement with earlier reports demonstrating that rates of f.Xa inhibition with heparin are higher than those with pentasaccharide (8, 27).

The plots of the second-order rate constants of f.IXa and f.Xa inhibition by antithrombin versus fondaparinux concentration both reach a plateau. However, the approximate 10-fold lower basal rate of inhibition of f.IXa versus f.Xa in the absence of a glycosaminoglycan is maintained in the presence of fondaparinux. The results presented here suggest that the mechanism of heparin-induced catalysis of f.IXa inhibition by antithrombin closely resembles that for f.Xa. In both cases, pentasaccharide-induced conformational changes in antithrombin enhance the serpin’s reactivity with the target protease. However, Ca$^{2+}$-dependent bridging by longer heparin chains produces a further enhancement in the rate of the inhibition reaction.

Heparin showed a typical template effect on the rates of thrombin inhibition by antithrombin, yielding a bell-shaped curve (Fig. 2A), as observed for f.IXa and f.Xa. The basal rate was $2.89 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$ and increased to a maximum of $7.14 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$ in the presence of heparin. The 2470-fold increase in the rate of thrombin inhibition produced by heparin was greater than the enhancement it produced in the rates of f.IXa or f.Xa inhibition (Fig. 3). The similar ascending portions of the f.IXa, f.Xa, and thrombin inhibition curves reflect the affinity of heparin for antithrombin (35). The peak and descending arms of the f.IXa and f.Xa plots occur at higher heparin
concentrations than the peak of the thrombin inhibition plot, suggesting that the affinity of heparin for f.IXa and f.Xa is lower than that for thrombin. The rates obtained in the present study are in agreement with published values; with an uncatalyzed second-order rate constant for the inhibition of thrombin by antithrombin of approximately $4.25 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$ that is increased 4000-fold to $1.7 \times 10^9 \text{ M}^{-1}\text{min}^{-1}$ in the presence of optimal concentrations of heparin (20).

Heparin pentasaccharide is known to have minimal effects on the rate of thrombin inhibition by antithrombin (8, 35, 36). In the present study, rates of thrombin inactivation by antithrombin were increased only 1.7-fold to $4.82 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$ by fondaparinux in the presence of Ca$^{2+}$ (Figs. 2B, 3).

**Heparin binding to f.IXa**

The template mechanism suggested by the heparin dose-response of f.IXa inhibition provides evidence for a heparin-f.IXa interaction. This concept is supported by experiments demonstrating that f.IXa binds to heparin-Sepharose (26, 37). Factor Xa and thrombin also bind to heparin-Sepharose; however, f.Xa only binds effectively in the presence of Ca$^{2+}$ (27). The Ca$^{2+}$-dependence of the heparin-f.Xa interaction is demonstrated by the fact that the heparin-catalyzed rate of f.Xa inhibition by antithrombin is increased 13-fold in the presence of Ca$^{2+}$ compared with that measured in its absence (27). Based on a previous study demonstrating that heparin-catalyzed inhibition of f.IXa by antithrombin was stimulated 6-fold by 5 mM Ca$^{2+}$ (21), we hypothesized that Ca$^{2+}$ may influence the affinity of f.IXa for heparin. Direct binding experiments were performed to quantify glycosaminoglycan-f.IXa interactions in the absence (2 mM EDTA) and presence of 2 mM CaCl$_2$. Measurements of intrinsic fluorescence of f.IXa or of fluorescein-FPR-f.IXa did not generate sufficient signal changes upon addition of heparin (data not shown). As an alternative, a competitive
binding assay was used in which the glycosaminoglycan of interest was employed to displace fluorescein-HS-LMWH bound to f.IXa (31). The fluorescence intensity (I) of fluorescein-HS-LMWH decreased in a concentration-dependent and saturable manner upon titration with f.IXa (not shown). Analysis of these data yielded a $K_d$ of 67.6 nM and a 35% decrease in fluorescence intensity in the presence of Ca$^{2+}$. For the displacement assay, initial addition of 80 nM f.IXa to fluorescein-HS-LMWH in the presence of Ca$^{2+}$ caused a 23% decrease in fluorescence intensity, consistent with binding (Fig. 4). Subsequent titration with heparin produced a concentration-dependent increase in the fluorescence signal of fluorescein-HS-LMWH to the intensity observed in the absence of f.IXa. This result is consistent with displacement of fluorescein-HS-LMWH from f.IXa by heparin. Analysis of these data revealed that the $K_d$ of heparin for f.IXa was 230 nM in the presence of Ca$^{2+}$. When fluorescein-HS-LMWH was titrated with f.IXa in the absence of Ca$^{2+}$, the decrease in fluorescence was 31% and a $K_d$ of 320 nM was obtained (not shown). Under the conditions of the competition experiment, addition of f.IXa to fluorescein-HS-LMWH in the absence of Ca$^{2+}$ caused a 10% decrease in fluorescence (Fig. 4). Titration of heparin restored the intensity to that of free fluorescein-HS-LMWH and analysis of the data yielded a $K_d$ of 2100 nM. Thus, the affinity of heparin for f.IXa is 9-fold higher in the presence of 2 mM CaCl$_2$ than in its absence. The affinity of heparin for f.IXa determined by plasmon resonance in the presence of Ca$^{2+}$ is 0.018 $\mu$M (29).

In comparison to the results with f.IXa, a previous study showed that the affinity of heparin for f.Xa is increased 126-fold in the presence of 4 mM CaCl$_2$ compared with its absence (from $K_d$ of 50.4 $\mu$M to 0.4 $\mu$M)$^2$. In contrast, the affinity of heparin for thrombin ($K_d = 1.1$ $\mu$M), is unaffected by Ca$^{2+}$ (35). Because heparin binds to f.IXa and f.Xa with similar affinities in the
presence of Ca\(^{2+}\), a similar mechanism of heparin binding is suggested for both proteases. This is consistent with the demonstration of similar heparin affinities of Gla-domainless forms of factors IXa and Xa (26).

**Effect of Ca\(^{2+}\) on glycosaminoglycan-catalyzed rates of f.IXa inhibition**

To directly address the role of Ca\(^{2+}\) in heparin catalysis of f.IXa inhibition by antithrombin, rate constants of f.IXa inhibition by antithrombin were determined in the absence (2 mM EDTA) and presence of 2 mM CaCl\(_2\). The uncatalyzed rate of inhibition of f.IXa by antithrombin was 7-fold higher in the presence of 2 mM CaCl\(_2\) than in its absence (1.19 x 10\(^4\) M\(^{-1}\)min\(^{-1}\) and 1.77 x 10\(^3\) M\(^{-1}\)min\(^{-1}\), respectively). For heparin-catalyzed inhibition, second-order rate constants in the presence of 2 mM CaCl\(_2\) were 2- to 10-fold higher than the corresponding values obtained in the presence of 2 mM EDTA over the range of heparin concentrations tested (Fig. 5A). These Ca\(^{2+}\)-dependent increases in the rates of heparin-catalyzed inhibition likely reflect a Ca\(^{2+}\)-dependent increase in the affinity of f.IXa for heparin. Similar results were obtained with f.Xa, where Ca\(^{2+}\) evoked up to a 9-fold increase in the rate of heparin-catalyzed inhibition over a range of heparin concentrations (Fig. 6A). Both f.IXa and f.Xa displayed bell-shaped dose response profiles in the presence of EDTA, suggesting that heparin plays a template role in the absence of Ca\(^{2+}\). As expected, Ca\(^{2+}\) had no effect on the heparin-catalyzed rates of thrombin inhibition (not shown).

Ca\(^{2+}\) also increased the extent to which fondaparinux enhanced f.IXa inhibition by antithrombin (Fig. 5B). The maximum stimulation of the rate of fondaparinux-catalyzed f.IXa inhibition by antithrombin was about 110-fold in the absence or presence of 2 mM CaCl\(_2\). Over the range of fondaparinux concentrations tested, Ca\(^{2+}\) enhanced the rate of f.IXa inhibition from 6- to 20-fold. In contrast, Ca\(^{2+}\) had no effect on the fondaparinux dose-response with f.Xa (Fig. 6B).
DISCUSSION

When the intrinsic pathway of coagulation was activated in plasma, antithrombin was the predominant inhibitor of generated f.IXa. Inhibition by antithrombin is consistent with results of earlier studies where $^{125}\text{I}-\text{f.IXa}$ was added to plasma (24). To examine the role of glycosaminoglycans, the intrinsic pathway of coagulation also was activated in the presence of fondaparinux or heparin. Fondaparinux and heparin significantly promoted f.IXa inhibition by antithrombin to a similar extent. As expected, fondaparinux also enhanced f.Xa inhibition by antithrombin, but had no effect on thrombin inhibition. These studies verify that f.IXa inhibition by antithrombin is promoted by fondaparinux under conditions where f.IX is activated in plasma.

Because fondaparinux was as effective as heparin in this assay, detailed kinetic studies were performed in purified systems to quantify the relative effects of these two glycosaminoglycans on the rates of f.IXa inhibition by antithrombin. To permit mechanistic comparisons, their effects on the rates of f.Xa and thrombin inhibition by antithrombin were assayed in parallel experiments.

Because vulnerability to inhibition in the presence of the heparin pentasaccharide distinguishes f.Xa from thrombin and the template from conformational change models, quantification of fondaparinux contribution to the rate of f.IXa inhibition by antithrombin was determined. In the presence of $\text{Ca}^{2+}$, fondaparinux produced a 107-fold increase in the rate of f.IXa inhibition by antithrombin. With f.Xa, fondaparinux produced a 156-fold increase in the rate of inhibition, whereas it had only a 2-fold effect on the rate of thrombin inhibition. By comparison, heparin increased the rates of f.IXa and f.Xa inhibition by antithrombin 1210-fold and 1900-fold, respectively. These results demonstrate a similarity in the mechanism of glycosaminoglycan stimulation of inhibition of f.IXa and f.Xa by antithrombin. Thus, heparin enhances the rate of
f.IXa inhibition by antithrombin by about three orders of magnitude, whereas fondaparinux increases this rate by two orders of magnitude. These observations suggest that the 10-fold superiority of heparin over fondaparinux reflects the capacity of the longer heparin chains to bridge f.IXa to antithrombin.

Confirmation of this template mechanism comes from the observation that the heparin dose response for the inhibition of f.IXa by antithrombin is biphasic, with a bell-shaped profile similar to that for thrombin and f.Xa. F.Xa was only recently recognized to utilize this mechanism when it was determined that the enzyme binds heparin in the presence of Ca\(^{2+}\) (27). It was proposed that the Gla-domain of f.Xa obscures or binds to the heparin-binding site on f.Xa in the absence of Ca\(^{2+}\). In the presence of Ca\(^{2+}\), the heparin-binding site is exposed. Because f.IXa and f.Xa are homologous proteins with similar domain structures (38, 39), the potential role of Ca\(^{2+}\) in mediating interaction of heparin with f.IXa was investigated.

**Effect of Ca\(^{2+}\) on heparin-catalyzed rate of f.IXa inhibition by antithrombin**

Ca\(^{2+}\) enhances both the heparin- and the fondaparinux-catalyzed rates of f.IXa inhibition by antithrombin 2- to 9-fold. This finding is in agreement with the results of previous studies in buffer systems demonstrating that Ca\(^{2+}\) produces a 6-fold increase in the heparin-catalyzed rates of f.IXa inhibition by antithrombin (21). In plasma, Ca\(^{2+}\) is an absolute requirement for both heparin- and fondaparinux-stimulated neutralization of f.IXa activity because no inhibition of f.IXa was observed in the presence of EDTA (30). Results from the present study indicate that glycosaminoglycan-catalyzed inhibition of f.IXa by antithrombin occurs in the presence of EDTA in a purified system. The effect of EDTA on f.IXa inhibition in plasma was not examined because f.IXa-mediated activation of f.X is Ca\(^{2+}\)-dependent.
Explanations for increased inhibition in the presence of Ca\(^{+2}\) include a Ca\(^{+2}\)-mediated effect on the interaction between f.IXa and heparin or structural changes in f.IXa induced by Ca\(^{+2}\) binding.

a) Effect of Ca\(^{+2}\) on f.IXa-heparin interaction

F.IXa has a heparin binding site analogous to those of thrombin and f.Xa (26). Ca\(^{+2}\) increases the affinity of f.IXa for heparin by 9-fold. In the presence of Ca\(^{+2}\), the affinity of heparin for f.IXa is similar to that for f.Xa, with K\(_d\) values of 0.23 and 0.39 \(\mu\)M, respectively (40). It has been proposed that the enhanced heparin-induced catalysis of f.Xa inhibition by antithrombin observed in the presence of Ca\(^{+2}\) occurs because Ca\(^{+2}\) binding to the Gla-domain causes conformational changes that expose a heparin-binding site on f.Xa (27). A similar mechanism may be operative with f.IXa. Thus, Ca\(^{+2}\) may promote a template effect by increasing the affinity of f.IXa for heparin. However, the fact that the template effect is observed in the absence of Ca\(^{+2}\) is consistent with the ability of f.IXa to bind heparin in the absence of divalent cation. A similar mechanism occurs with f.Xa where it is considered that there is about a 10-fold bridging effect of heparin in the absence of Ca\(^{+2}\) (28).

b) Effect of Ca\(^{+2}\) on the f.IXa-antithrombin interaction

Because Ca\(^{+2}\) increases the fondaparinux-catalyzed rate of f.IXa inhibition by antithrombin, promotion of bridging interaction of antithrombin with f.IXa cannot be the only mechanism whereby Ca\(^{+2}\) enhances inhibition. Furthermore, Ca\(^{+2}\) promotes the rate of inhibition of f.IXa by antithrombin, even in the absence of glycosaminoglycan, an effect also observed with f.Xa (27, 41). The enhancement evoked by Ca\(^{+2}\) in the absence of glycosaminoglycan for f.IXa is about 10-fold, but for f.Xa it is less than 2-fold. A glycosaminoglycan-independent explanation for the
increased inhibition in the presence of Ca\(^{+2}\) could be that Ca\(^{+2}\) binding to the Gla-domain may indirectly affect the protease domain conformation, resulting in enhanced reactivity of f.IXa with antithrombin. In support of this concept, Ca\(^{+2}\) alters the binding of conformation-specific antibodies to the serine protease domain of f.IXa (42). Furthermore, incorporation of a tripeptide chloromethyl ketone into the f.IXa active site has been shown to be three times faster in the presence of Ca\(^{+2}\) than in its absence (43). Conformational changes in f.IXa also could result from direct interaction of Ca\(^{+2}\) with the Ca\(^{+2}\)-binding site in the protease domain (28, 43). Thus, occupation of the Ca\(^{+2}\)-binding site on the protease domain of f.Xa has been shown to influence active site function (41, 44, 45). Therefore, Ca\(^{+2}\) may augment f.IXa-antithrombin interaction by binding to f.IXa and modulating interactions in the vicinity of the active site. The effect of Ca\(^{+2}\) on the active site could be greater when antithrombin is activated by pentasaccharide.

Overall, the structural homology of f.IXa and f.Xa in the Ca\(^{+2}\)- and heparin-binding domains contributes to the similar mechanism by which heparin catalyzes inhibition by antithrombin. Thus, heparin acts, in part, as a template onto which f.IXa and antithrombin assemble. This is made possible by the ability of f.IXa and f.Xa to bind heparin. However, this template role augments the stimulation provided by the pentasaccharide moiety alone. The conformational change in antithrombin that results from pentasaccharide binding is proposed to generate a domain on antithrombin that is complementary to an exosite that resides near the active site of f.Xa (46). This promotes association of inhibitor and enzyme through a mechanism distinct from that provided by a heparin template. By extension, a similar exosite may exist on f.IXa that permits reactivity with pentasaccharide-activated antithrombin.
Physiological considerations and implications

The results of experiments in plasma demonstrate that f.IXa displays a greater resistance to inhibition than f.Xa, even in the presence of heparin or fondaparinux. This could result from the lower rate constant for inhibition or from protection of f.IXa from antithrombin by assembly into the intrinsic tenase complex. The latter phenomenon has been observed with f.Xa within the prothrombinase complex and has been attributed to the competitive effect of the substrate prothrombin on interaction of f.Xa with antithrombin (47, 48). Thus, f.IXa within its activation complex may be better protected than f.Xa in prothrombinase. Agents that disrupt these activation complexes may then permit greater access of antithrombin to the protease components. HS-LMWH fits this description as it disrupts both prothrombinase and intrinsic tenase complexes, likely by binding to the heparin-binding sites on f.Xa and f.IXa, respectively (31). Therefore, the heparin-binding site on f.IXa plays an important physiological role that may be exploited for therapeutic purposes.

f.IXa is a second physiological target of fondaparinux

Fondaparinux has been considered to specifically target f.Xa in an antithrombin-dependent fashion. Because early studies failed to identify an effect of fondaparinux on the inhibition of f.IXa, the anticoagulant mechanism of fondaparinux has only been considered from the perspective of f.Xa and thrombin. However, the striking effect of fondaparinux on the rate of inhibition of f.IXa suggests that anti-f.IXa action may contribute to the effectiveness of this drug. Although antithrombin inhibits f.IXa at lower rates than f.Xa, the position of f.IXa upstream of f.Xa in the coagulation cascade provides an opportunity for an intensified downstream effect.

Advancing our understanding of the anticoagulant mechanism of fondaparinux may help
to explain the clinical data obtained with this agent. By promoting f.IXa and f.Xa inhibition by antithrombin, fondaparinux attenuates thrombin generation. Even without enhancement in the rate of thrombin inhibition by antithrombin, the upstream effects of fondaparinux appear adequate to prevent thrombus growth as evidenced by its effectiveness in the prevention and treatment of venous thromboembolism (12-15). These findings raise the possibility that naturally occurring mechanisms are sufficient to regulate thrombin activity in this setting, provided that thrombin generation is attenuated (49).
References

Footnotes

1 The abbreviations used are: AT, antithrombin; f. factor; FITC, 5'-fluorescein isothiocyanate; LMWH, low molecular weight heparin; HS-LMWH, hypersulfated LMWH; HCII, heparin cofactor II; HRP, horse radish peroxidase; Gla, gamma carboxy glutamic acid; Z, zymogen; Za, active enzyme; Za-I, enzyme-inhibitor complex

2 O’Brien, Stafford, Fredenburgh, and Weitz, unpublished results
Table I. Second order rate constants ($k_2$) of inhibition of f.IXa, f.Xa, and f.IIa by antithrombin in the absence or presence of heparin or fondaparinux.

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<tr>
<td></td>
<td>$k_2$ (M$^{-1}$ min$^{-1}$)</td>
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<tr>
<td>f.IXa</td>
<td>$1.34 \pm 0.7 \times 10^4$</td>
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<td>f.Xa</td>
<td>$1.83 \pm 0.2 \times 10^5$</td>
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<td>f.IIa</td>
<td>$2.89 \pm 0.6 \times 10^5$</td>
<td>$7.14 \pm 1.1 \times 10^8$</td>
<td>$4.82 \pm 0.7 \times 10^5$</td>
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Second order rate constants were determined under pseudo first order conditions in the presence of 2 mM CaCl$_2$. Values in the presence of glycosaminoglycan (GAG) represent the maximum rate achieved over the range of concentrations tested. Values are the mean ± standard error for at least three determinations.
Figure legends

Fig. 1 Influence of fondaparinux and heparin on inhibition of f.IIa, f.IXa, and f.Xa in activated plasma. Defibrinated platelet-poor plasma was mixed with an equal volume of Thrombosil and warmed to 37 °C in the absence or presence of 0.6 µg/ml fondaparinux or heparin. Activation was initiated by the addition of CaCl$_2$ to 16 mM. Triplicate aliquots were removed at times up to 15 min and subjected to electrophoresis in 4-15% polyacrylamide gels. Purified zymogen (Z) and enzyme-antithrombin (Za-I) complexes were also subjected to electrophoresis for use as standards. Nitrocellulose blots of the gels were prepared and probed with antibodies that detect the zymogen, activated and antithrombin-inhibited forms of f.II, f.IX, and f.X, as well as heparin cofactor II-inhibited f.IIa. Panel A is the immunoblot generated with the antibody against f.IX, panel B was generated using the antibody against f.X, and panel C was generated with the antibody against f.II. Standards are indicated to the right of the gels. Times of sampling in minutes are noted below the lanes. The prepared f.IXa-AT complex standard underwent proteolysis upon storage (►). Nascent f.IXa-AT migrates with a slower mobility (☆), as determined in separate experiments.

Fig. 2. Influence of heparin and fondaparinux on the second order rate constants of inhibition of f.IXa, f.Xa, f.IIa by antithrombin. Second order rate constants ($k_2$) of inhibition by antithrombin in the presence of 2 mM CaCl$_2$ were determined under pseudo-first-order conditions at 23°C. Thrombin (●), f.IXa (■), or f.Xa (▲), in concentrations ranging from 1 - 500 nM, was incubated with a minimal 10-fold excess of antithrombin in the presence of varying concentrations of heparin (panel A) or fondaparinux (panel B). Reactions were stopped at various time intervals and residual enzyme activity was determined with a chromogenic or fluorogenic
substrate. Pseudo-first-order rate constants were divided by the antithrombin concentration to obtain second-order rate constants.

**Fig. 3. Comparison of the stimulatory effect of fondaparinux and heparin on catalysis of antithrombin-mediated inhibition of f.IIa, f.IXa, and f. Xa.** Second-order rate constants of inhibition ($k_2$) of f.IIa, f.IXa, or f.Xa by antithrombin, measured in the presence of heparin (open bars) or fondaparinux (closed bars), were divided by those determined in the absence of glycosaminoglycan (Fig. 2) and are plotted as fold increase over the uncatalyzed rate of inhibition.

**Fig. 4. Influence of Ca$^{2+}$ on heparin binding to f.IXa.** Binding of heparin to f.IXa in the presence of CaCl$_2$ or EDTA was monitored by displacement of f.IXa-bound fluorescein-HS-LMWH. The inset shows the fluorescence intensity values from the titrations. Initially, the fluorescence of 80 nM fluorescein-HS-LMWH in 2 mM CaCl$_2$ (circles) or EDTA (squares) was determined (white symbols). Subsequently, 50 nM f.IXa was added to each sample (gray symbols), resulting in 25 and 10% reductions in fluorescence intensity in the presence of 2 mM CaCl$_2$ or EDTA, respectively. The samples were then titrated with heparin and the fluorescence intensity after each addition was determined (black symbols). The inset shows fluorescence intensity values at each point in the titration ($I_{535}$). Fractions of maximal displacement were then calculated to normalize the data and plotted against the heparin concentration (main graph). K$_i$ values for displacement of fluorescein-HS-LMWH by heparin in the presence of Ca$^{2+}$ (●) or EDTA (■) were determined, taking into account the K$_d$ of fluorescein-HS-LMWH for f.IXa.

**Fig. 5. Influence of Ca$^{2+}$ on glycosaminoglycan-mediated stimulation of f.IXa inhibition by antithrombin.** Second-order rate constants of inhibition ($k_2$) of f.IXa by antithrombin were determined in the absence (2 mM EDTA, open symbols) or presence of Ca$^{2+}$ (2 mM CaCl$_2$, closed...
symbols) as described in the legend to Fig. 2. Experiments were performed in the presence of varying concentrations of heparin (panel A) or fondaparinux (panel B). Results are reported as mean of 2 to 4 experiments ± SEM.

**Fig. 6. Influence of Ca^{2+} on glycosaminoglycan-mediated stimulation of f.Xa inhibition by antithrombin.** Second-order rate constants of inhibition ($k_2$) of f.Xa by antithrombin were determined in the absence (2 mM EDTA, open symbols) or presence of Ca^{2+} (2 mM CaCl$_2$, closed symbols) as described in the legend to Fig. 2. Experiments were performed in the presence of varying concentrations of heparin (panel A) or fondaparinux (panel B). Results are reported as mean of 2 to 4 experiments ± SEM.
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Fig. 2 Wiebe et al

A

B

$\kappa_2 \text{(M}^{-1}\text{min}^{-1})$ vs. Heparin (M)

$\kappa_2 \text{(M}^{-1}\text{min}^{-1})$ vs. Fondaparinux (M)
Fig. 3 Wiebe et al
Fig. 4 Wiebe et al
Fig. 6 Wiebe et al

A

B

$[\text{Fondaparinux}] (\text{M})$

$[\text{Heparin}] (\text{M})$

$k_2 (\text{M}^{-1} \text{min}^{-1})$

$k_2 (\text{M}^{-1} \text{min}^{-1})$
Mechanism of catalysis of inhibition of factor IXa by antithrombin in the presence of heparin or pentasaccharide

Ericka M. Wiebe, Alan R. Stafford, James C. Fredenburgh and Jeffrey I. Weitz

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