Calcium Enhances Heparin Catalysis of the Antithrombin−Factor Xa Reaction by Promoting the Assembly of an Intermediate Heparin−Antithrombin−Factor Xa Bridging Complex. Demonstration by Rapid Kinetics Studies†

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ABSTRACT: Heparin catalyzes the inhibition of factor Xa by antithrombin mainly through an allosteric activation of the serpin inhibitor, but an alternative heparin bridging mechanism has been suggested to enhance the catalysis in the presence of physiologic calcium levels due to calcium interactions with the Gla domain exposing a heparin binding exosite in factor Xa. To provide direct evidence for this bridging mechanism, we studied the heparin-catalyzed reaction of antithrombin with factor Xa, Gla-domainless factor Xa (GDFXa), and a heparin binding exosite mutant of GDFXa in the absence and presence of calcium using rapid kinetic methods. The pseudo-first-order rate constant for factor Xa inhibition by antithrombin complexed with a long-chain ∼70-saccharide heparin showed a saturable dependence on inhibitor concentration in the presence but not in the absence of 2.5 mM Ca^{2+}, indicating the formation of an intermediate heparin−serpin−proteinase encounter complex with a dissociation constant of ∼90 nM prior to formation of the stable serpin−proteinase complex with a rate constant of ∼20 s^{-1}. Similar saturation kinetics were observed for the inhibition of GDFXa by the antithrombin−heparin complex, except that Ca^{2+} was not required for the effect. By contrast, no Ca^{2+}-dependent saturation of the inhibition rate constant was detectable over the same range of inhibitor concentrations for reactions of either a short-chain ∼26-saccharide high-affinity heparin−antithrombin complex with factor Xa or the long-chain heparin−antithrombin complex with the heparin binding exosite mutant, GDFXa R240A. These findings suggest that binding of full-length heparin chains to an exosite of factor Xa in the presence of Ca^{2+} produces a chain-length-dependent lowering of the dissociation constant for assembly of the intermediate heparin−antithrombin−factor Xa encounter complex, resulting in a several 100-fold rate enhancement by a heparin bridging mechanism.

Antithrombin is the primary serpin family inhibitor in plasma that regulates the activities of the serine proteinases of the blood coagulation pathway (*2*, *3*). Similar to other inhibitory serpins, antithrombin inactivates its target proteinases by binding to their active sites through an exposed reactive center loop and undergoing a conformational change which traps the enzymes in inactive, stable complexes (*4*). Antithrombin is unusual in inhibiting its target enzymes slowly but can be activated to inhibit these enzymes several 1000-fold faster by the polysaccharide cofactor, heparin (*2*−*4*). Heparin activates antithrombin by binding to the serpin through a specific pentasaccharide region (*5*−*7*) and inducing conformational changes in the serpin reactive center loop which enhance proteinase binding to the loop (*7*−*13*). However, such conformational changes appear to be specific for factor Xa (FXa)[1] since the heparin pentasaccharide effectively accelerates antithrombin inhibition of just factor Xa and not other target proteinases such as thrombin (*5*, *7*, *14*, *15*). Longer chain high-affinity heparins containing the pentasaccharide efficiently accelerate antithrombin inhibition of thrombin but by an alternative ternary complex bridging or template mechanism in which heparin binding to both the serpin and proteinase promotes the encounter between the two proteins (*1*, *14*−*20*). This bridging mechanism has been thought to minimally contribute to the acceleration of the antithrombin−factor Xa reaction by longer chain heparins since most of the acceleration by such heparins is accounted for by the pentasaccharide (*5*, *7*, *15*).

Previous rapid kinetic studies have shown that antithrombin inactivates factor Xa and thrombin by a two-step reaction mechanism in which an initial Michaelis-type enzyme−inhibitor complex, formed in the first reaction step, is

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[1] Abbreviations: AT, antithrombin; SI, stoichiometry of inhibition; FXa, active factor Xa; Gla, γ-carboxyglutamic acid; GDFXa, Gla-domainless factor Xa [factor Xa from which amino-terminal residues 1−45 [in the chymotrypsin numbering system of Bode et al. (*1*)] have been removed]; GDFXa R240A, Gla-domainless factor Xa derivative in which Arg^{240} has been substituted with an Ala; BSA, bovine serum albumin; PEG, poly(ethylene glycol).

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converted to an irreversible covalent complex in the second step of the reaction (*9*, *21*). The dissociation constant (K_D) for formation of the initial noncovalent serpin−proteinase complex is high (in the millimolar range) for the reactions of antithrombin with either factor Xa or thrombin in the absence of heparin. In the presence of a full-length heparin, the K_D for formation of the noncovalent complex decreases by ~10^4-fold at physiologic ionic strength and pH to ~100−200 nM in the case of the antithrombin−thrombin reaction due to the bridging effect of heparin (*17*, *21*). The K_D for noncovalent complex formation also decreases for the full-length heparin-catalyzed reaction of antithrombin with factor Xa, but this decrease (to ~200 μM) is considerably less than that observed for the reaction with thrombin and is primarily due to conformational activation of the serpin (*7*, *9*). Such K_Ds suggest that antithrombin bound to heparin or heparan sulfate chains in vivo would not effectively bind factor Xa and consequently would inhibit this enzyme much less efficiently than thrombin, in keeping with the ~40-fold slower bimolecular rate constant for antithrombin−heparin complex inhibition of factor Xa as compared to thrombin (*7*).

Recently, a heparin binding exosite was identified on the proteinase domain of factor Xa that is localized in the same three-dimensional region of the enzyme as the heparin binding exosite of thrombin (*22*, *23*). Kinetic data suggested that heparin can interact with this site of factor Xa to produce up to an ~300-fold greater acceleration of the reaction of antithrombin with factor Xa than the pentasaccharide only if the N-terminal negatively charged Gla domain of the proteinase is neutralized by binding Ca^{2+} ions (*22*, *24*, *25*). These results together with the observation that the dependence of heparin catalysis on heparin concentration is bell-shaped in the presence of calcium but shows simple saturation behavior in the absence of calcium suggested that the calcium enhancement of heparin catalysis was mediated by a bridging mechanism, similar to the mechanism by which heparin accelerates antithrombin inactivation of thrombin (*22*, *25*).

In the current study, we have made rapid kinetics measurements of the heparin-catalyzed reaction of antithrombin with factor Xa in both the absence and presence of calcium to determine (i) whether calcium enhances heparin catalysis by promoting the assembly of an intermediate heparin−antithrombin−factor Xa bridging complex and (ii) whether the extent of such promotion is consistent with ternary complex formation and rapid factor Xa inactivation occurring under physiologic conditions. Our results provide evidence for the saturation of such an intermediate ternary complex with a K_D of ~100 nM in the presence of physiologic levels of calcium and with a long-chain heparin but not in the absence of calcium or with pentasaccharide or short-chain heparins. Studies with Gla-domainless factor Xa and a heparin binding exosite mutant of the Gla-domainless enzyme implicate the Gla domain and the heparin binding exosite in these effects. Comparison with the heparin-catalyzed antithrombin−thrombin reaction using the long-chain heparin and in the presence of calcium revealed an intermediate ternary complex with a K_D of 20 ± 1 nM. Together, these results suggest that long-chain heparins similar to those found in vivo lower the K_D for formation of the noncovalent antithrombin−factor Xa complex in the presence of calcium by a bridging mechanism so as to result in ternary complex formation and an overall rate of factor Xa inhibition that is comparable to that of the heparin-catalyzed inhibition of thrombin by antithrombin at physiological levels of Ca^{2+}.

MATERIALS AND METHODS

Proteins and Other Reagents. Construction and expression of the Gla-domainless forms of wild type and the Arg^{240} to Ala mutant of factor X (GDFX and GDFX R240A, respectively) were previously described (*22*, *26*). Human plasma-derived factor X and its recombinant GDFX derivatives were activated by the factor X activating enzyme from Russell's viper venom (Haematologic Technologies Inc., Essex Junction, VT) and purified on SBTI−agarose as previously described (*27*). Preparations of plasma factor Xa were isolated predominantly in the α-form, whereas GDFXa derivatives were isolated mostly in the β-form due to the more facile autocatalytic cleavage of the Gla-domainless enzyme at the C-terminal Arg^{245}−Gly^{246} bond (*28*). Comparison of the α-form of plasma factor Xa with a preparation converted to the β-form (*27*) revealed no differences in their reactivities with antithrombin in the absence or presence of heparin or calcium, consistent with the C-terminal cleavage occurring outside of the heparin binding exosite of factor Xa (*22*, *23*). All reported experiments with plasma factor Xa were done with the α-form of the enzyme. Active site concentrations of plasma and recombinant factor Xa were determined by titrations with human antithrombin assuming a 1:1 stoichiometry (*27*). These concentrations were at least 70% of those determined from the absorbance at 280 nm using published absorption coefficients (*29*, *30*). Antithrombin was purified from human plasma, and its concentration was determined from the absorbance at 280 nm using an absorption coefficient of 37 700 M^{-1} cm^{-1} (*31*). Human plasma α-thrombin was a gift of Dr. John Fenton of the New York State Department of Health (Albany, NY), and its concentration was determined by active site titration as described (*32*). The active antithrombin binding pentasaccharide fragment of heparin was generously provided by Dr. Maurice Petitou (Sanofi Recherche, Toulouse, France). Full-length high-affinity heparins containing the pentasaccharide with a greatly reduced polydispersity and with an average molecular mass of ~8000 (~26 saccharides) or ~21 000 (~70 saccharides) were isolated from commercial heparin by size and antithrombin affinity fractionation as previously described (*33*). Concentrations of heparins were based on antithrombin binding sites and were determined by stoichiometric titrations of antithrombin with the polysaccharide, with monitoring of the interaction by changes in protein fluorescence (*7*, *8*, *33*). Comparison with heparin concentrations determined by dry weight or by carbazole assay indicated ~1.5 and ~2.8 antithrombin binding sites per heparin chain for ~26-saccharide and ~70-saccharide heparins, respectively (*7*, *33*). The presence of multiple sites on all or some of the chains in these preparations results from selecting for the highest affinity heparin species by overloading the immobilized antithrombin affinity column (*33*). Previous studies have shown that when heparin concentrations are expressed in terms of antithrombin binding sites, similar activities are found for chains of the same length but with multiple sites (*44*), indicating that the sites are

independent and noninteracting. The chromogenic substrates spectrozyme FXa (SpFXa) and PCa (SpPCa) were purchased from American Diagnostica (Greenwich, CT). Polybrene and the fluorogenic substrates *N-t*-Boc-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin (Boc-IEGR-amc) and *N-p*-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin (tosyl-GPR-amc) were purchased from Sigma (St. Louis, MO).

Kinetic Methods. The rate of inactivation of factor Xa derivatives or thrombin by antithrombin in both the absence and presence of heparin was measured under pseudo-first-order conditions by a discontinuous assay method as previously described (*22*, *25*). In the absence of heparin, 1 nM factor Xa or thrombin was incubated with 250−1000 nM antithrombin in 0.1 M NaCl, 0.02 M Tris-HCl, pH = 7.4 (TBS buffer, ionic strength = 0.12), containing 1 mg/mL BSA, 0.1% PEG 8000, and either 2.5 mM $CaCl_2$ or 0.1 mM EDTA. In the presence of heparin, 0.2 nM factor Xa or thrombin was incubated with 100 nM antithrombin and catalytic levels of heparin (0.1−10 nM) or pentasaccharide (6.25−25 nM) in the same buffer system. All reactions were carried out in 50 μL volumes in 96-well vinyl plates at room temperature. After a period of time (10 s to 10 min depending on the rate of the reactions), 50 μL of the chromogenic substrate (500 μM SpFXa for factor Xa reactions or 500 μM SpPCa for thrombin reactions) in TBS buffer containing 0.1% PEG 8000 and 1 mg/mL polybrene was added to each well, and the remaining enzyme activity was measured with a V_{max} kinetics microplate reader (Molecular Devices, Menlo Park, CA). The observed pseudo-first-order rate constants (k_{obs}) were determined by fitting data to an exponential loss of activity with a zero end point. The second-order association rate constants for uncatalyzed and catalyzed reactions were obtained from the slopes of linear plots of k_{obs} vs the concentration of antithrombin or the antithrombin−heparin complex, respectively, in accordance with eq 1, as previously described (*33*).

$$k_{obs} = k_{uncat}[AT]_{free} + k_H[AT{-}heparin] \qquad (1)$$

In this equation k_{uncat} and k_H are the second-order rate constants for uncatalyzed and heparin-catalyzed reactions, respectively, and $[AT]_{free}$ and [AT−heparin] represent the free and antithrombin−heparin complex concentrations, which were calculated from the dissociation constant for the antithrombin−heparin interaction (K_D) and total concentrations of antithrombin ($[AT]_0$) and heparin ($[H]_0$) using the quadratic equation (*33*). K_D was fixed to values of 5 and 23 nM for the high-affinity heparin and pentasaccharide interactions, respectively, which were extrapolated from the ionic strength dependence of the interaction to the ionic strength of 0.12 used in the present studies (*7*). Since catalytic heparin levels were used in these experiments, $[AT]_{free}$ was closely approximated by the total antithrombin concentration in the presence of heparin, and thus plots of k_{obs} vs [AT−heparin] were linear with an intercept of $\sim k_{uncat}[AT]_0$. All values are the average of at least three independent measurements ± SE.

Rapid kinetic analysis was carried out to resolve the two-step reaction of the antithrombin−heparin complex with plasma and recombinant factor Xa and with thrombin in TBS buffer containing 0.1% PEG 8000 and either 2.5 mM Ca^{2+} or 0.1 mM EDTA at 25 °C. In this case the inactivation reaction was followed by a continuous assay in an Applied Photophysics SX-17MV stopped-flow instrument using the fluorogenic substrates, Boc-IEGR-amc or tosyl-GPR-amc, as reporters of factor Xa and thrombin inhibition, respectively, as previously described (*19*, *33*, *34*). The excitation wavelength was set to 380 nm and fluorescence detected with a 420 nm emission cutoff filter. For factor Xa reactions, antithrombin concentrations ranged from 100 to 5000 nM, high-affinity heparin concentrations from 12.5 to 1600 nM, and factor Xa from 2 to 300 nM, and Boc-IEGR-amc was present at 50 or 100 μM. For thrombin reactions, antithrombin concentrations ranged from 100 to 400 nM, high-affinity heparin from 1.6 to 200 nM, and thrombin from 0.25 to 65 nM, and substrate was present at 5 μM. Pseudo-first-order conditions were achieved by employing a >10-fold molar excess of the antithrombin−heparin complex over enzyme. To maintain a relatively constant fluorescence amplitude in these reactions, the ratio of heparin to enzyme was kept constant (*33*). k_{obs} was determined by computer fitting fluorescence progress curves to a single exponential function with a linear end-point rate (typically <1% of the initial rate). Corrections of k_{obs} for the reaction of excess free antithrombin were within the experimental error and were therefore neglected. The dependence of k_{obs} (the average of 7−12 progress curves) on the antithrombin−heparin complex concentration was computer fit by the hyperbolic equation (*33*, *34*):

$$k_{obs} = \frac{k[AT{-}heparin]}{K_{E,ATH}(1 + [S]_0/K_M) + [AT{-}heparin]} \qquad (2)$$

where k represents the limiting rate constant for conversion of the intermediate heparin−antithrombin−proteinase ternary encounter complex to a stable antithrombin−proteinase complex with release of heparin, [AT−heparin] is the concentration of antithrombin−heparin complex which was calculated using K_D values indicated above, $K_{E,ATH}$ is the dissociation constant for binding of proteinase to the antithrombin−heparin complex to form the ternary complex, $[S]_0$ is the concentration of the fluorogenic substrate, and K_M is the Michaelis constant for substrate hydrolysis by the enzyme. In these fits, K_M was fixed at measured values for factor Xa hydrolysis of Boc-IEGR-amc of 320 μM in TBS buffer containing 2.5 mM Ca^{2+} and 390 μM in TBS buffer containing 0.1 mM EDTA. Indistinguishable values were measured for GDFXa and GDFXa R240A. K_M for thrombin hydrolysis of tosyl-GPR-amc in the absence or presence of Ca^{2+} was fixed at the measured value of ~4 μM. Under conditions where $[AT{-}heparin] \ll K_{E,ATH}(1 + [S]_0/K_M)$, eq 2 reduces to the linear equation:

$$k_{obs} = \frac{k[AT{-}heparin]}{K_{E,ATH}(1 + [S]_0/K_M)} \qquad (3)$$

In those cases where the dependence of k_{obs} on [AT−heparin] was linear, the linear regression slope provided the overall second-order rate constant, $k/K_{E,ATH}$, after multiplying by the factor $1 + [S]_0/K_M$.

Inhibition Stoichiometries. Stoichiometries of inhibition of factor Xa, GDFXa, and thrombin by the antithrombin−~70-saccharide heparin complex were measured in TBS buffers with 2.5 mM Ca^{2+} or 0.1 mM EDTA by mixing fixed

Table 1: Second-Order Rate Constants for Uncatalyzed and Heparin-Catalyzed Reactions of Antithrombin with Factor Xa and GDFXa or with Thrombin in the Presence of Ca^{2+} or EDTA[a]

	k_{uncat} ($M^{-1}\,s^{-1}$)	k_{H5} ($M^{-1}\,s^{-1}$)	k_{H26} ($M^{-1}\,s^{-1}$)	k_{H70} ($M^{-1}\,s^{-1}$)
FXa (Ca^{2+})	$(2.6 \pm 0.1) \times 10^3$	$(0.82 \pm 0.07) \times 10^6$	$(6.3 \pm 0.3) \times 10^6$	$(140 \pm 10) \times 10^6$
FXa (EDTA)	$(1.8 \pm 0.1) \times 10^3$	$(0.75 \pm 0.06) \times 10^6$	$(1.9 \pm 0.1) \times 10^6$	$(10 \pm 1) \times 10^6$
GDFXa (Ca^{2+})	$(2.5 \pm 0.2) \times 10^3$	$(0.99 \pm 0.07) \times 10^6$	$(8.4 \pm 0.3) \times 10^6$	$(170 \pm 10) \times 10^6$
GDFXa (EDTA)	$(1.8 \pm 0.1) \times 10^3$	$(0.84 \pm 0.05) \times 10^6$	$(12 \pm 1) \times 10^6$	$(210 \pm 20) \times 10^6$
thrombin (Ca^{2+})	$(6.8 \pm 0.3) \times 10^3$	$(1.1 \pm 0.1) \times 10^4$	$(17 \pm 1) \times 10^6$	$(120 \pm 10) \times 10^6$
thrombin (EDTA)	$(6.4 \pm 0.3) \times 10^3$	$(1.1 \pm 0.1) \times 10^4$	$(21 \pm 1) \times 10^6$	$(230 \pm 30) \times 10^6$

[a] The uncatalyzed (k_{uncat}) second-order inactivation rate constants were determined from the residual chromogenic activities of factor Xa (1.0 nM) or thrombin (1.0 nM) after incubation at room temperature with antithrombin (100−1000 nM) for 5−10 min in TBS buffer containing 1 mg/mL BSA, 0.1% PEG 8000, and either 2.5 mM Ca^{2+} or 0.1 mM EDTA. Pentasaccharide (k_{H5}) or full-length heparin (k_{H26} and k_{H70}) catalyzed values were determined by the same procedures except that 0.2 nM enzyme was incubated with 6.25−25 nM pentasaccharide or 0.4−10 nM full-length high-affinity heparin and 100 nM antithrombin. The second-order inactivation rate constants were calculated from the slope of the linear plot of k_{obs} vs inhibitor or inhibitor−heparin complex concentration according to eq 1 as described under Materials and Methods.

levels of proteinase (100−200 nM of either factor Xa, GDFXa, or thrombin) with increasing molar ratios of antithrombin plus a fixed 2-fold molar excess of heparin over the highest concentration of the serpin. After incubation at room temperature for 1 h, residual enzyme activity was measured by addition of chromogenic substrate and measurement of the initial rate of substrate hydrolysis as in the discontinuous kinetic assays described above. The stoichiometry was obtained from the *x*-axis intercept of the linear regression fit of a plot of enzyme activity vs the inhibitor/enzyme molar ratio (*33*).

RESULTS

Table 1 shows the second-order association rate constants for the uncatalyzed (k_{uncat}), pentasaccharide-catalyzed (k_{H5}), and ~26-saccharide and ~70-saccharide high-affinity heparin-catalyzed (k_{H26} and k_{H70}) reactions of antithrombin with factor Xa and a Gla-domainless recombinant form of factor Xa (GDFXa) in both the absence and presence of physiologic levels of Ca^{2+} (2.5 mM), as measured by a discontinuous assay described under Materials and Methods. In agreement with previous results (*25*), the uncatalyzed and the pentasaccharide-catalyzed rates of inactivation of factor Xa derivatives by antithrombin were only slightly affected by Ca^{2+} (<1.5-fold). In contrast, the ~26-saccharide and ~70-saccharide heparin-catalyzed rates of factor Xa inactivation by the serpin were strongly dependent on the metal ion. As shown in Table 1, whereas k_{H26} for factor Xa inactivation was stimulated ~3-fold by physiologic levels of calcium, k_{H70} was ~14-fold higher in the presence than in the absence of Ca^{2+}. However, k_{H26} and k_{H70} for heparin catalysis of GDFXa inactivation by antithrombin were insensitive to Ca^{2+} but comparable in magnitude to the calcium-stimulated second-order rate constants for the reaction with intact factor Xa. These results suggested that factor Xa interacts effectively with the antithrombin−heparin complex only if Ca^{2+} is bound to the Gla domain or alternatively if the Gla domain is deleted. The small effect of Ca^{2+} on uncatalyzed and pentasaccharide-catalyzed rates is likely the result of Ca^{2+} binding to a site in the catalytic domain. Occupancy of this site by the metal ion is also known to slightly enhance the amidolytic activity of factor Xa toward cleavage of small synthetic substrates (*30*, *35*).

Antithrombin inhibits factor Xa by a two-step reaction mechanism in which a noncovalent enzyme−inhibitor encounter complex is initially formed which is then converted to a stable covalent complex (*9*). Heparin accelerates this reaction at least partly by decreasing the K_D for forming the intermediate noncovalent complex. Rapid kinetic analysis was carried out to determine which reaction step Ca^{2+} affected to increase the reactivity of factor Xa with the antithrombin−heparin complex. The pseudo-first-order rate constant for factor Xa inhibition by the antithrombin−heparin complex (k_{obs}) was measured as a function of increasing concentrations of the inhibitor−heparin complex in both the absence and presence of physiological concentrations of Ca^{2+} and for pentasaccharide, ~26-saccharide, and ~70-saccharide heparin-catalyzed reactions. A saturable dependence of k_{obs} on the antithrombin−heparin complex concentration was observed in the presence but not in the absence of Ca^{2+} when a full-length ~70-saccharide heparin was the catalyst (Figure 1), indicating the saturation of an intermediate heparin−antithrombin−factor Xa encounter complex prior to formation of the stable antithrombin−factor Xa complex. Nonlinear regression analysis of data for the calcium-stimulated reaction by the hyperbolic equation (eq 2) yielded a value of 90 ± 10 nM for the ternary complex dissociation constant ($K_{E,ATH}$)

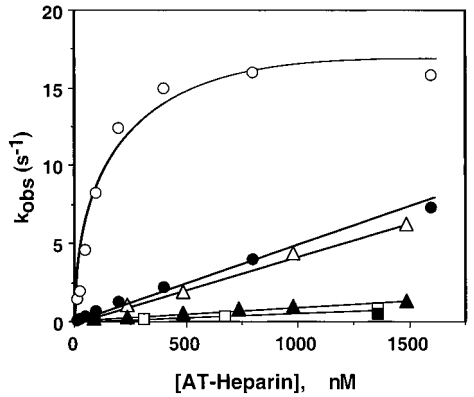


FIGURE 1: Dependence of k_{obs} for reactions of the antithrombin−heparin complex with factor Xa on the inhibitor−heparin complex concentration. k_{obs} was determined from the exponential decrease in the rate of hydrolysis of the fluorogenic substrate, Boc-IEGR-amc, by factor Xa due to antithrombin inhibition of the enzyme in the presence (open symbols) or absence (filled symbols) of Ca^{2+} for ~70-saccharide heparin (circles), ~26-saccharide heparin (triangles), or heparin pentasaccharide (squares) catalyzed reactions, as described under Materials and Methods. All reactions used a molar excess of antithrombin over heparin to saturate the polysaccharide, with exact concentrations of the antithrombin−heparin complex calculated on the basis of measured K_D values for the interaction. The solid lines are least squares computer fits of data by the linear equation (eq 1) (open and filled squares or triangles and filled circles) or hyperbolic equation (eq 2) (open circles).

Table 2: Kinetic Constants for the Heparin-Catalyzed Reactions of Antithrombin with Factor Xa, GDFXa, R240A GDFXa, and Thrombin in the Presence of Ca^{2+} or EDTA As Measured by Rapid Kinetic Methods[a]

	heparin	$K_{E,ATH}$ (10^{-6} M)	k (s^{-1})	k_2 (10^6 M^{-1} s^{-1})
FXa (Ca^{2+})	H_{70}	90 ± 10	18 ± 1	200 ± 20
FXa (EDTA)	H_{70}	ND	ND	8.6 ± 0.6
FXa (Ca^{2+})	H_{26}	ND	ND	4.8 ± 0.1
FXa (EDTA)	H_{26}	ND	ND	1.0 ± 0.1
FXa (Ca^{2+})	H_5	ND	ND	0.8 ± 0.6
FXa (EDTA)	H_5	ND	ND	0.6 ± 0.5
GDFXa (Ca^{2+})	H_{70}	150 ± 10	20 ± 1	130 ± 15
GDFXa (EDTA)	H_{70}	140 ± 15	15 ± 1	110 ± 20
GDFXa R240A	H_{70}	ND	ND	10 ± 1
thrombin (Ca^{2+})	H_{70}	20 ± 1	3.2 ± 0.1	160 ± 10
thrombin (EDTA)	H_{70}	8 ± 1	4.0 ± 0.1	500 ± 80

[a] The kinetic constants were determined by rapid kinetic analyses of the observed pseudo-first-order rate constant (k_{obs}) for factor Xa inactivation by the antithrombin−heparin complex as a function of the antithrombin−heparin complex concentration in TBS buffer containing either 2.5 mM Ca^{2+} or 0.1 mM EDTA. Antithrombin−heparin complexes were formed by mixing full-length (H_{26} or H_{70}) or pentasaccharide heparin (H_5) with a molar excess of antithrombin sufficient to saturate the polysaccharide. Correction of k_{obs} values for the free antithrombin reaction were <1% and were therefore neglected. $K_{E,ATH}$ and k values were obtained from computer fits of the saturable dependence of k_{obs} on the antithrombin−heparin complex concentration according to eq 2 after correction for the competitive effect of the reporter substrate as described under Materials and Methods. Overall second-order rate constants (k_2) were calculated from the ratio of $k/K_{E,ATH}$ or were determined from the slope of the linear regression line fitted to plots of k_{obs} vs inhibitor−heparin complex concentration according to eq 3 after correction for substrate competition. ND, not determinable.

and 18 ± 1 s^{-1} for the rate constant for stable complex formation (Table 2). The calculated ratio of k to $K_{E,ATH}$, corresponding to the overall second-order rate constant, agreed reasonably well with the directly determined value from Table 1 and was ∼20-fold greater than the value determined for the reaction in the absence of calcium from the slope of the linear dependence of k_{obs} on antithrombin−heparin complex concentration (Figure 1). Contrasting these findings, k_{obs} for the pentasaccharide and ∼26-saccharide heparin-catalyzed reactions increased linearly throughout the same range of antithrombin−heparin complex concentration in the absence or presence of calcium. Pentasaccharide-catalyzed reactions showed indistinguishable slopes with and without calcium, whereas the ∼26-saccharide heparin-catalyzed reactions showed a 4−5-fold greater slope in the presence than in the absence of calcium, with both slopes exceeding those for the pentasaccharide-catalyzed reactions. All second-order rate constants determined from these slopes agreed reasonably well with values measured by the discontinuous assay (Table 2). These results indicated that the stimulating effect of calcium on the heparin-catalyzed antithrombin−factor Xa reaction is largely due to calcium lowering the dissociation constant for formation of the initial noncovalent ternary encounter complex and that such lowering of the ternary complex K_D depends on heparin chain length.

k_{obs} for the inactivation of GDFXa by antithrombin complexed with the longer chain heparin showed a similar saturable dependence on the antithrombin−heparin complex concentration, but this was observed in both the absence and presence of Ca^{2+} (Figure 2). K_Ds for ternary complex

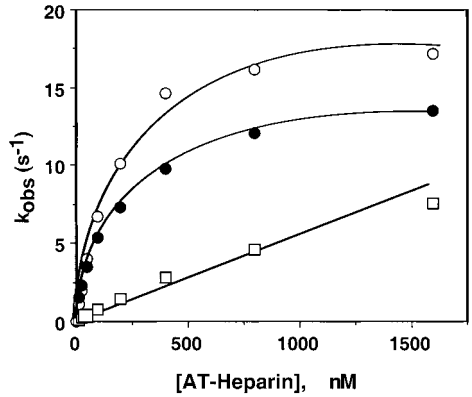


FIGURE 2: Dependence of k_{obs} for the reactions of the antithrombin−heparin complex with wild type and the Arg^{240} to Ala mutant GDFXa. k_{obs} for GDFXa inhibition in the presence (open circles) or absence (filled circles) of Ca^{2+} or for GDFXa R240A inhibition only in the absence of Ca^{2+} (squares) was determined for ∼70-saccharide heparin-catalyzed reactions as in Figure 1. The solid lines are least squares computer fits of data to the linear equation (eq 3) for the GDFXa R240A reaction or hyperbolic equation (eq 2) for GDFXa reactions.

formation of 140−150 nM and rate constants of 15−20 s^{-1} for stable complex formation were found for the reactions with and without calcium by nonlinear regression analysis of these data by eq 2 (Table 2), similar to the values found for the calcium-stimulated reaction of the antithrombin−heparin complex with intact factor Xa. The saturable dependence of k_{obs} on the antithrombin−heparin complex concentration was changed to a linear dependence when GDFXa was replaced with an Arg^{240} to Ala mutant of GDFXa (R240A), which was previously shown to be defective in binding heparin due to the mutation being localized in a heparin binding exosite of the enzyme (Figure 2) (*22*). These results suggest that a functional heparin binding exosite of factor Xa is required to lower the dissociation constant for formation of the noncovalent heparin−serpin−proteinase ternary complex. The results further show that deletion of the factor Xa Gla domain eliminates the requirement for calcium ions to lower the ternary complex dissociation constant.

To evaluate the relative efficacy of the high molecular weight heparin to accelerate thrombin and factor Xa inactivation by antithrombin in the absence and presence of calcium, rapid kinetic analysis of the ∼70-saccharide heparin-catalyzed antithrombin−thrombin reaction was carried out under experimental conditions identical to those described above for factor Xa, except that the thrombin fluorogenic substrate, tosyl-GPR-amc, was used to report enzyme inhibition (Figure 3). k_{obs} for thrombin inhibition by the antithrombin−∼70-saccharide heparin complex showed a saturable dependence on the inhibitor−heparin complex concentration in both the absence and presence of calcium. In the presence of calcium, nonlinear regression analysis of data according to eq 2 yielded a value of 20 ± 1 nM for the ternary complex dissociation constant ($K_{E,ATH}$) and 3.2 ± 0.1 s^{-1} for the rate constant for stable complex formation (k). Somewhat improved values of $K_{E,ATH}$ = 8 ± 1 nM and k = 4.0 ± 0.1 s^{-1} were observed for the interaction of thrombin with the antithrombin−heparin complex in the presence of EDTA. A lower affinity of thrombin for heparin in calcium (*43*) may account for this observation.

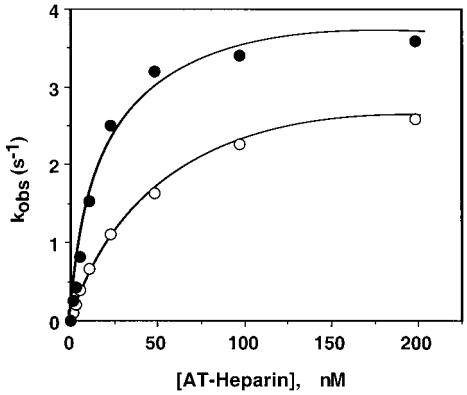


FIGURE 3: Dependence of k_{obs} for reactions of the antithrombin−heparin complex with thrombin on the inhibitor−heparin complex concentration. k_{obs} values were determined for ~70-saccharide heparin-catalyzed reactions in the presence (open circles) or absence (filled circles) of calcium as in Figure 1 except that tosyl-GPR-amc was the reporter thrombin substrate as detailed in Materials and Methods. Solid lines are least squares computer fits by the hyperbolic equation (eq 2).

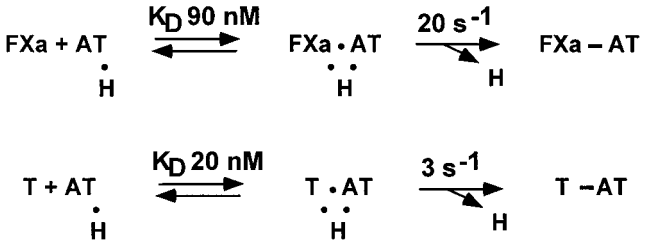
In addition to accelerating the inhibition of thrombin and factor Xa by antithrombin, heparin promotes a substrate reaction of antithrombin with both proteinases (*7*, *14*, *36*, *37*). In view of the findings that Ca^{2+} dramatically enhances the high molecular weight heparin-catalyzed rate of factor Xa inactivation by antithrombin, we determined whether Ca^{2+} influences the substrate reaction of the serpin with factor Xa and thrombin in the presence of the ~70-saccharide heparin. Inhibition stoichiometries of 1.4−1.5 for the heparin-catalyzed antithrombin−factor Xa reaction were observed in both the absence and presence of Ca^{2+}, indicating that the substrate pathway of the reaction is not affected by the metal ion. A somewhat lower stoichiometry of 1.2−1.3 was found for the heparin-catalyzed antithrombin−GDFXa reaction, but again this stoichiometry was unaffected by Ca^{2+}. Corresponding values for the high molecular weight heparin-catalyzed reactions of antithrombin with thrombin were 1.4−1.5 in the absence of calcium and 1.1−1.2 in the presence of the metal ion, indicating that the substrate pathway is reduced by calcium in the reaction with thrombin. Thus the lower heparin accelerating effect on the antithrombin−thrombin reaction in the presence of calcium (Table 1 and Figure 3) is not due to calcium enhancing the substrate pathway.

DISCUSSION

A heparin binding exosite on the proteinase domain of factor Xa was recently identified in a region homologous to heparin binding exosite 2 of thrombin, and it was noted that heparin binding to this site is greatly enhanced when the Gla domain of the proteinase is neutralized by physiological concentrations of Ca^{2+} (*22*, *23*, *25*). It was further shown that, in addition to the ~300-fold allosteric acceleration of the antithrombin−factor Xa reaction by heparin pentasaccharide, full-length heparin accelerated the reaction several 100-fold more when Ca^{2+} was present (*22*). This increased acceleration appeared to be mediated by a heparin bridging mechanism based on the bell-shaped dependence of the additional reaction rate enhancement on heparin concentration. Resolution of the two-step reaction of factor Xa with antithrombin in complex with a long-chain ~70-saccharide heparin in this study supports a mechanism of calcium enhancement of this reaction involving heparin bridging of an intermediate antithrombin−factor Xa encounter complex. Thus, as anticipated for such a mechanism, calcium greatly enhances formation of the intermediate serpin−proteinase encounter complex. Calcium was not required to observe the saturation of a similar encounter complex with Gla-domainless factor Xa, consistent with calcium binding to the Gla domain being responsible for the promotion of encounter complex formation and with deletion of the Gla domain mimicking this promoting effect without the need for calcium. These results support the previous proposal that the heparin binding exosite of factor Xa has limited accessibility to heparin if the Gla residues are not neutralized by calcium (*22*, *25*). Consistent with calcium or deletion of the Gla domain promoting ternary complex formation by enhancing a heparin−factor Xa bridging interaction, reaction of the antithrombin−heparin complex with the heparin binding exosite mutant Arg 240→Ala GDFXa that is defective in binding heparin (*22*) showed no promotion of ternary complex formation. Mutations in the heparin binding exosite of thrombin similarly abrogate heparin promotion of antithrombin−thrombin encounter complex formation by reducing the ability of heparin to bridge the encounter complex (*18*−*20*).

The promoting effect of calcium on formation of the heparin−antithrombin−factor Xa ternary complex was dependent on heparin chain length. Calcium enhanced the ternary complex interaction by at least 50-fold with a long-chain heparin, as indicated from the K_D of ~100 nM observed for the ternary complex in the presence of calcium and lower limit K_D of ~5 μM that can be estimated for this complex in the absence of calcium from the lack of ternary complex saturation over the range of inhibitor concentrations examined (Figure 1). By contrast, calcium did not result in any detectable saturation of a ternary complex in the short-chain heparin-catalyzed reaction up to ~1 μM antithrombin−heparin complex, even though calcium enhanced the accelerating effect of this heparin 3−5-fold. Considering the weak ternary complex K_D of ~200 μM previously measured for the short-chain heparin-catalyzed reaction in the absence of calcium (*9*), our results are consistent with calcium lowering the ternary complex K_D for the short-chain heparin but with this K_D still being much greater than 1 μM. It has been previously shown that calcium stimulation of the heparin−antithrombin−factor Xa reaction reaches a maximal value for heparin chains ranging from ~35 to 64 saccharides (*25*), implying that maximal calcium promotion of ternary complex formation is likely to be achieved with a heparin half the size of the ~70-saccharide heparin used in the present study. Such a chain-length requirement would be satisfied by the lengths of anticoagulantly active glycosaminoglycan chains encountered in vivo (*38*) and the ~50−60-saccharide average length of clinically administered heparin (*39*). This chain-length requirement contrasts with the shorter ~26-saccharide heparin chain length which is sufficient to promote a substantial ternary complex bridging effect for the thrombin reaction, although the maximum bridging effect in this case is reached with the same range of chain lengths as for the factor Xa reaction (*14*, *17*). The longer minimal heparin chain-length requirement for effective bridging in the factor Xa reaction may reflect the need for a longer chain to achieve

Scheme 1



a high enough affinity for the exosite of factor Xa or be due to additional interactions of long-chain heparins outside the exosite of factor Xa, possibly with the growth factor domains, which could stabilize factor Xa in an "open" conformation in which the heparin binding exosite is accessible.

The finding that calcium increased the overall accelerating effect of the long-chain heparin on the antithrombin−factor Xa reaction ~20-fold whereas it lowered the ternary complex K_D at least 50-fold can be accounted for by calcium reducing the rate of the covalent reaction between antithrombin and factor Xa within the ternary bridging complex. Thus, the rate constant of 18 s^{-1} observed for the covalent reaction step in the presence of calcium is significantly slower than the minimum estimate of ~30 s^{-1} for this rate constant in the absence of calcium (Figure 1) and the value of 140 s^{-1} previously determined for this rate constant with the short-chain heparin in the absence of calcium (*9*). The diminished rate of covalent complex formation associated with calcium promotion of a heparin bridging complex is in keeping with previous rapid kinetic studies of heparin-catalyzed antithrombin−proteinase reactions in which heparin bridging was also associated with reduced rates of covalent complex formation (*21*, *34*, *40*). This can be rationalized by the reduced conformational freedom of the serpin and proteinase in the ternary bridging complex, which could reasonably increase the activation energy for the covalent reaction step.

Comparison of the overall second-order rate constants for the heparin-accelerated reactions of antithrombin with factor Xa and thrombin in the presence of physiologic levels of calcium revealed that both reactions occur at close to diffusion-limited rates of $(1-2) \times 10^8$ M^{-1} s^{-1} (Table 1). Resolution of the two steps of these reactions (Scheme 1) shows that the relative contributions of assembly of the ternary bridging complex and formation of the covalent complex to the attainment of such rates differ for the two reactions.

Thus, the K_D value for ternary complex formation is ~4−5-fold higher for the factor Xa reaction than for the thrombin reaction, but this is more than compensated for by an ~6-fold higher rate constant for covalent complex formation with factor Xa than with thrombin. It should be emphasized, however, that heparin bridging accounts for nearly all of the ~10 000-fold accelerating effect of heparin on the antithrombin−thrombin reaction, whereas both conformational activation of the serpin and heparin bridging contribute to the acceleration of the antithrombin−factor Xa reaction. On the basis of the pentasaccharide accelerating effect on the latter reaction, the relative contributions of the two mechanisms to the acceleration of factor Xa inactivation at physiologic levels of Ca^{2+} can be calculated to be ~300-fold for the conformational activation mechanism and 200−300-fold for the bridging mechanism, with ~10-fold of the bridging effect being expressed in the absence of calcium (*7*, *14*) and the remainder in the presence of calcium (Tables 1 and 2). The lesser contribution of bridging to heparin acceleration of the antithrombin reaction with factor Xa than with thrombin appears to be correlated with a more specific recognition of the antithrombin reactive center loop sequence by factor Xa than by thrombin (*34*), suggesting that heparin bridging may be more effective when the requirements for serpin−proteinase recognition in the bridging complex are relaxed. The comparable near-diffusion-limited rates at which long-chain high-affinity heparins, resembling the anticoagulantly active glycosaminoglycans found in vivo (*38*), catalyze antithrombin inhibition of factor Xa and thrombin in the presence of physiologic levels of calcium underscore the fact that these two clotting proteinases are primary targets of antithrombin and that factor Xa is likely to be inactivated by antithrombin at a much faster physiologic rate than was previously thought due to the failure to consider the calcium effect on this reaction. It should be noted, however, that factor Xa in the prothrombinase complex is expected to be inhibited at a slower rate because factor Va binds to the heparin binding exosite of factor Xa and thus prevents heparin bridging of the enzyme and antithrombin in a ternary complex (*22*), an observation in keeping with the reported protection of factor Xa from antithrombin−heparin inactivation when bound in the prothrombinase complex (*41*, *42*).

Calcium differentially affected a substrate reaction of antithrombin with factor Xa and thrombin in the presence of heparin in which uncomplexed antithrombin cleaved at the reactive bond is formed rather than a stable inhibited complex (*7*, *14*, *36*, *37*). The substrate pathway effectively competes with the inhibitory pathway in the presence of heparin, with about one out of three antithrombin molecules being cleaved instead of forming a covalent complex in the reactions with both thrombin and factor Xa (*7*, *14*). Conformational activation of antithrombin appears to be responsible for the substrate pathway competition in the case of factor Xa, whereas heparin bridging appears to be responsible for this competition in the case of thrombin (*7*, *14*). While calcium was found to decrease the flux through the substrate pathway in the reaction with thrombin in parallel with a decrease in the heparin bridging effect, calcium had no effect on the substrate pathway for the factor Xa reaction despite calcium enhancing the heparin bridging effect. These findings indicate that heparin bridging interactions with thrombin and factor Xa exosites differentially affect the competition between substrate and inhibitor pathways and imply that factor Xa is most responsible for generating the antiangiogenic cleaved antithrombin species under physiologic conditions (*45*).

In summary, our results have shown that calcium stimulates the heparin-catalyzed antithrombin−factor Xa reaction by promoting the encounter between antithrombin and factor Xa in a ternary bridging complex with heparin. This promotion results from calcium interactions with the factor Xa Gla domain increasing the accessibility of a heparin binding exosite of factor Xa, thereby enabling heparin to effectively bridge the serpin−proteinase encounter complex. Effective bridging appears to require longer heparin chains with factor Xa than with thrombin, possibly due to interactions of such longer chains outside the heparin binding exosite, which act to stabilize factor Xa in a conformation

competent for bridging. These effects of physiologic calcium may ensure that factor Xa is inhibited by antithrombin at a diffusion-limited rate comparable to that of thrombin when the serpin is bound to anticoagulantly active heparin or heparan sulfate chains in vivo.

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