Calcium Enhances Heparin Catalysis of the Antithrombin-Factor Xa Reaction by a Template Mechanism

EVIDENCE THAT CALCIUM ALLEVIATES Gla DOMAIN ANTAGONISM OF HEPARIN BINDING TO FACTOR Xa*

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Alireza R. Rezaie‡

From the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

It is believed that heparin accelerates factor Xa (FXa) inactivation by antithrombin (AT) by conformationally activating the inhibitor rather than by bridging AT and FXa in a ternary complex (template effect). This is derived from kinetic studies done in the absence of Ca\(^{2+}\) or in the presence of EDTA. To test the possibility that the anionic Gla domain of FXa, when not neutralized by Ca\(^{2+}\) ions, prevents heparin binding to FXa, the heparin and pentasaccharide dependence of FXa inactivation by AT in both the absence (100 \(\mu\)M EDTA) and presence of Ca\(^{2+}\) (2.5 mM) was studied using wild-type FXa and a FXa derivative that lacks the Gla domain (GDFXa). AT inactivated both FXa derivatives similarly in both the absence and presence of Ca\(^{2+}\) \((k_2 = 1.7–2.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1})\). The active AT-binding pentasaccharide also accelerated the inactivation rates of both derivatives similarly in both the absence and presence of Ca\(^{2+}\) \((k_2 = 5.7–8.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\). However, in the presence of an optimum concentration of heparin \((\sim 50 \text{ nM})\) the inactivation rate constant of FXa in the presence of Ca\(^{2+}\) \((k_2 = 4.4 \times 10^7 \text{ M}^{-1} \text{s}^{-1})\) was 13-fold higher than the rate constant in the absence of Ca\(^{2+}\) \((k_2 = 3.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1})\). Heparin acceleration of GDFXa inactivation by AT was rapid and insensitive to the presence or absence of Ca\(^{2+}\) \((k_2 = 5.1–5.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\). The additional cofactor effect of heparin with all FXa derivatives was a bell-shaped curve, which disappeared if the ionic strength of the reaction was increased to \(-0.4\). These results suggest that although the major effect of heparin in acceleration of FXa inactivation is through a heparin-induced conformational change in the reactive site loop of AT, the template effect of heparin, nevertheless, contributes significantly to rapid FXa inactivation at physiological Ca\(^{2+}\).

The mechanism by which the therapeutic heparin catalyzes the inactivation of thrombin and factor Xa (FXa)\(^1\) by antithrombin (AT) has been extensively studied in the past (1–4). The consensus view is that heparin accelerates thrombin inactivation by AT by facilitating an initial interaction between the two proteins by a bridging mechanism (4–6). In FXa inactivation, however, it is believed that a template mechanism has a minimal role or no role at all in the acceleration and that a heparin-induced conformational change in the reactive site loop of AT accounts for all of the accelerating effect of heparin in the reaction (7–10). These distinct inactivation mechanisms are supported by the observation that a unique pentasaccharide fragment of heparin, which can bind and activate AT but is not long enough to bridge the inhibitor and enzyme, specifically promotes FXa but not thrombin inhibition (9, 10). For thrombin inhibition, heparin chains containing the pentasaccharide plus at least 13 additional saccharides are required to accelerate the reaction (2, 4).

All the previous heparin-catalyzed protease inactivation studies were conducted under experimental conditions that either lacked Ca\(^{2+}\) (11, 12) or contained EDTA in the reaction buffers (9, 13, 14). Under these conditions heparin-catalyzed thrombin inactivation by AT is at least 10–20-fold faster than the inactivation of FXa (9, 11–13). Unlike thrombin, however, FXa contains a highly anionic N-terminal domain, which is rich in \(\gamma\)-carboxyglutamic acid (Gla) residues that are involved in Ca\(^{2+}\)-dependent membrane binding. This domain of FXa contains 11 \(\gamma\)-carboxylated Glu residues, which are in a random disordered conformation in the absence of Ca\(^{2+}\) (15). To test the possibility that in the absence of Ca\(^{2+}\) the Gla domain of FXa antagonizes heparin binding to FXa and thereby prevents heparin from acting as a template in the FXa-AT reaction, the heparin and pentasaccharide dependence of FXa inactivation by AT was studied in both the absence and presence of Ca\(^{2+}\) using wild-type FXa and a recombinant FXa derivative, which lacks the Gla domain (GDFXa). It was found that in contrast to a similar \(-300\)-fold cofactor effect of the pentasaccharide in FXa inactivation in both the absence and presence of Ca\(^{2+}\), the cofactor effect of heparin was markedly higher if the reaction was carried out in the presence of Ca\(^{2+}\). The difference in the magnitude of the cofactor effect between heparin and the pentasaccharide was \(-5\) and \(-60\)-fold for FXa in the absence and presence of Ca\(^{2+}\), and \(-88\)–\(-74\)-fold for GDFXa in the absence and presence of Ca\(^{2+}\), respectively. These findings can explain why the anti-FXa activity of heparin exhibits a minimal chain length dependence in \(ex\ vivo\) and \(in\ vitro\) assays but significant chain length dependence in \(in\ vivo\) assays (16, 17).

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents—Human plasma FXa, GDFXa (18), and recombinant human thrombin (19) were prepared as described previously. Human plasma AT was purchased from Hematologic Technologies Inc., VT. Unfractionated heparin from porcine intestinal mucosa, sodium salt (169.2 USP units/mg), and Polybrene were purchased from...
Sigma. Spectrozymes FXa (SpFXa) and PCa (SpPCa) were purchased from American Diagnostica, Greenwich, CT. The oligosaccharides, ranging in size from 6 to 18 saccharide units, were generous gifts from Dr. Ingemar Björk (Swedish University of Agricultural Sciences, Uppsala, Sweden), and high affinity heparin fragments with 22–64 saccharide units were generous gifts from Dr. Steven Olson (University of Illinois, Chicago).

Kinetic Methods—The rate of inactivation of FXa, GDFXa, and thrombin in the absence or presence of heparin and the pentasaccharide fragment of heparin (1 μM) were measured under pseudo-first order rate conditions by a discontinuous assay as described previously (19, 20). The heparin concentration dependence of the inactivation reactions was determined by incubating 0.2 μM FXa or GDFXa with 3–20 nM AT and varying concentrations of heparin (0–50 μM) in Tris-HCl (pH 7.5), 0.1 mM NaCl (TBS buffer, I = 0.12), 1 mg/ml BSA, and 0.1% PEG 8000 containing either 2.5 mM CaCl2 or 100 μM EDTA in 50-μl reactions. After 10 s to 1 min of incubation at room temperature (−25 °C), 50 μl of SpFXa in TBS buffer containing 1 mg/ml Polybrene (to block heparin function immediately) was added to give a final concentration of 0.4 mM. The remaining activities and the second-order inhibition rate constants (k2) for heparin-catalyzed inactivation of FXa derivatives were determined as described previously (19, 20). The bell-shaped dependence of k2 on heparin concentration was computer fit to the following equation as described previously (6),

\[
k_2 = k_{2,max} + K_{AT,H}^{-1} + S - \left(k_{2,max} + K_{FXa,H}^{-1} + S\right) \\
 \times \frac{K_{FXa,H}}{K_{FXa,H} + [H]} - 4[A] \times (H/S)^{1/2} \times (A + 4[A] \times K_{FXa,H}/K_{FXa,H} + [H] + K_{penta})
\]

(Eq. 1)

where [AT], is total antithrombin concentration, [H], is total heparin concentration, S is the stoichiometry of heparin-AT interaction (this value was 1.7 for the unfractionated heparin used in this study as determined by fluorescence measurements), KAT,H is the dissociation constant for heparin-AT interaction, k2,max is the maximum second-order inhibition rate constant, k2,penta represents the pentasaccharide-accelerated second-order rate constant, and KFXa,H is the dissociation constant for the FXa-heparin interaction. This equation neglects the uncatalyzed rate constant because its contribution was found to be negligible within experimental error. Fitting of the data by this equation revealed that k2,penta was not well determined. Its value was therefore fixed at the independently determined value.

RESULTS

Fig. 1 shows the heparin concentration dependence of FXa inactivation by AT in the presence of either 100 μM EDTA (open circles) or 2.5 mM Ca2+ (closed circles). The heparin concentration dependence of the second-order rate constant (k2) for FXa inactivation in the presence of EDTA reaches saturation at ~50 nM heparin (Fig. 1), and further increasing the concentration of heparin does not result in a decline in k2. The maximal rate constant achieved under these conditions was k2 = 3.5 ± 0.7 × 106 M−1 s−1 (Table I). In the presence 2.5 mM Ca2+, the accelerating effect of heparin was significantly increased over that measured in EDTA, and the dependence of k2 on heparin concentration was bell-shaped, with an initial increase in k2 followed by a decrease. The maximal k2 of 4.4 ± 0.4 × 106 M−1 s−1, reached at ~50–500 nM heparin, was 13-fold higher than that measured in the absence of Ca2+ (Fig. 1 and Table I). At higher heparin concentrations, k2 decreased such that at ~20 μM heparin there was essentially no difference between the inactivation rates in the absence or presence of Ca2+ (Fig. 1). Thus, only the additional 13-fold cofactor effect of heparin in the presence of Ca2+ was abolished at high heparin concentration, suggesting that a ternary complex by bridging or template mechanism was responsible for this portion of the cofactor effect of heparin in FXa inactivation.

FXa has at least three known Ca2+-binding domains that can influence the magnitude of the heparin cofactor effect in the FXa-AT reaction: the N-terminal Gla domain, the epidermal growth factor-like domain-1 (EGF-1), or the C-terminal catalytic domain (15, 21). To determine which one of these domains is responsible for the Ca2+-dependent cofactor effect of heparin in the reaction, the inactivation studies were carried out with two FXa derivatives, one lacks the Gla domain (GDFXa) and the other lacks both the Gla and EGF-1 domains (E2FXa). It was previously demonstrated that except for the membrane binding properties, all other enzymatic properties of wild-type FXa are preserved in these two mutants (18). Unlike the cofactor effect of heparin in FXa inactivation, which was a bell-shaped curve only in the presence of Ca2+, the accelerating effect of heparin in GDFXa inactivation exhibited a bell-shaped dependence on heparin concentration in both the absence and presence of Ca2+ (Fig. 2). At the optimum heparin concentration (50–300 nM), AT inactivated GDFXa with k2 = 5.1 ± 0.6 × 107 M−1 s−1 and k2,h = 5.9 ± 0.1 × 107 M−1 s−1 in the absence and presence of Ca2+, respectively (Table I). The maximal value and heparin concentration dependence of k2 for E2FXa inactivation were similar to that for GDFXa inactivation (data not shown).

![Fig. 1. Heparin concentration dependence of FXa inactivation by AT in the absence or presence of Ca2+. FXa (0.2 nM) inactivation by AT (5 nM) was monitored at varying concentrations of heparin (0–26 μM) in TBS buffer containing 1 mg/ml BSA and 0.1% PEG 8000 in the presence of either 100 μM EDTA (○) or 2.5 mM CaCl2 (●). The second-order association rate constants were determined as described under “Experimental Procedures.” The solid lines represent fits to Equation 1.](http://www.jbc.org/Downloadedfrom http://www.jbc.org/)

| Table I |
|---|
| The second-order inhibition rate constants (k2) for AT inactivation of FXa, GDFXa, and thrombin in the presence or absence of pentasaccharide (PS) or heparin. |

| Protease | No cofactor | PS | Heparin | Template effect |
|---|---|---|---|---|
| | k2 (106 M−1 s−1) | k2 (106 M−1 s−1) | k2 (106 M−1 s−1) | |
| FXa | 2.0 ± 0.1 | 6.7 ± 0.1 | 7.3 ± 0.1 | 5.2 ± 1.1 |
| GDFXa | 1.7 ± 0.1 | 5.7 ± 0.1 | 8.0 ± 0.1 | 90 ± 12 |
| Thrombin | 6.6 ± 0.6 | 0.1 ± 0.01 | 0.1 ± 0.01 | 5600 ± 1400 |

The k2 values for inactivation of each enzyme in the absence or presence of a saturating concentration of cofactors (1 μM pentasaccharide or 50 nM heparin) were determined in TBS buffer containing 1 mg/ml BSA, 0.1% PEG 8000, and either 100 μM EDTA or 2.5 mM CaCl2 as described under “Experimental Procedures.” The kinetic values are averages of at least three independent measurements ± S.D.
shown). These results suggest that Ca\(^{2+}\) occupancy of the EGF-1 domain or the catalytic domain does not alter the cofactor function of heparin in the reaction; rather the anionic Gla domain or the catalytic domain does not alter the cofactor function of AT in the absence or presence of Ca\(^{2+}\). For GDFXa inactivation, similar values of 11.6 \pm 2.7 nm for \(K_{\text{AT,H}}\) and 2.8 \pm 0.4 \mu M for \(K_{\text{FXa,H}}\) were found in the presence of Ca\(^{2+}\). In this case, lower values of 5.4 \pm 2.7 nm for \(K_{\text{AT,H}}\) and 0.26 \pm 0.08 \mu M for \(K_{\text{FXa,H}}\) were obtained in the presence of EDTA, indicating tighter binding of heparin to GDFXa in the absence of Ca\(^{2+}\).

The heparin chain length dependence of FXa inactivation by AT in the presence of Ca\(^{2+}\) was studied using oligosaccharides containing 6, 10, 14, and 18 saccharides and high affinity heparin fragments containing on average 22, 35, 50, and 64 saccharides. The cofactor effect of the 6–18-residue long oligosaccharides and the 22-residue long heparin fragment were all simple saturation curves (up to 50 \mu M) in both the absence and presence of Ca\(^{2+}\) with only an \(1.7\)-fold enhanced cofactor effect for the 18-residue long oligosaccharide and an \(2.5\)-fold enhanced cofactor effect for the 22-residue long heparin fragment in the presence of Ca\(^{2+}\). A slight improvement in the reactivity of FXa with AT in the presence of Ca\(^{2+}\) was also observed in the absence of heparin (Table I). The maximal \(10\)–13-fold template cofactor effect of heparin displaying a bell-shaped dependence on heparin concentration was observed with any of the 35–64-residue long high affinity heparin fragments used for FXa or GDFXa inactivation (data not shown). The optimal heparin concentration, \(k_2\) values, and the \(K_{\text{FXa,H}}\) and \(K_{\text{AT,H}}\) values for the 50- and 64-residue long heparin fragments were all similar to the values determined for the unfractionated heparin (data not shown).

It is known that the bridging effect of heparin on the thrombin-AT reaction is dependent on the ionic strength of buffer, but the cofactor effect, which is mediated by a conformational change in the reactive site loop of AT, is relatively insensitive to the ionic strength as long as AT is saturated with heparin (5). Similarly, the additional cofactor effect of heparin in FXa inactivation in the presence Ca\(^{2+}\) or in GDFXa inactivation in both the absence and presence of Ca\(^{2+}\) decreased as a function of increasing salt concentration such that the differences in the reactivities of FXa derivatives was completely abolished at 0.4 m NaCl (Table II).

### DISCUSSION

The anticoagulant effect of heparin is primarily mediated through the acceleration of the rate of inactivation of coagulation proteases (mainly thrombin and FXa) by AT. It is thought that formation of a heparin-AT-protease ternary complex by a bridging or template mechanism accounts for the bulk of the

### TABLE II

| [NaCl] (m) | \(k_2\) (M\(^{-1}\)s\(^{-1}\)) | \(Ca^{2+}\) | EDTA | \(k_2\) (M\(^{-1}\)s\(^{-1}\)) | \(Ca^{2+}\) |
|-----------|-----------------|-----------|------|-----------------|-----------|
| 0.10      | 3.5 \pm 0.7 \times 10^6 | 4.4 \pm 0.4 \times 10^7 | 5.1 \pm 0.6 \times 10^7 | 5.9 \pm 0.1 \times 10^7 |
| 0.15      | 4.6 \pm 0.1 \times 10^6 | 2.3 \pm 0.1 \times 10^7 | 4.4 \pm 0.1 \times 10^7 | 3.0 \pm 0.1 \times 10^7 |
| 0.20      | 3.6 \pm 0.4 \times 10^6 | 9.7 \pm 0.6 \times 10^6 | 2.2 \pm 0.2 \times 10^7 | 1.3 \pm 0.1 \times 10^7 |
| 0.25      | 2.3 \pm 0.4 \times 10^6 | 4.4 \pm 0.6 \times 10^6 | 8.0 \pm 0.3 \times 10^6 | 6.3 \pm 1.0 \times 10^6 |
| 0.30      | 1.4 \pm 0.4 \times 10^6 | 2.3 \pm 0.3 \times 10^6 | 3.3 \pm 0.5 \times 10^6 | 2.5 \pm 0.1 \times 10^6 |
| 0.40      | 5.3 \pm 0.5 \times 10^5 | 6.3 \pm 0.2 \times 10^5 | 7.2 \pm 1.5 \times 10^5 | 7.1 \pm 1.1 \times 10^5 |

**Fig. 2.** Heparin concentration dependence of GDFXa inactivation by AT in the absence or presence of Ca\(^{2+}\). GDFXa (0.2 nM) inactivation by AT (5 nM) was monitored at varying concentrations of heparin (0–26 \mu M) in TBS buffer containing 1 mg/ml BSA and 0.1% PEG 8000 in the presence of either 100 \mu M EDTA (○) or 2.5 m CaCl\(_2\) (●). The second-order association rate constants were determined as described under “Experimental Procedures” and fitted by Equation 1 (solid lines).

**TABLE I**

Salt concentration dependence of the cofactor function of heparin in FXa and GDFXa inactivation by AT in the presence of EDTA or Ca\(^{2+}\).

The \(k_2\) values were determined by incubating each FXa derivative (0.2 nM) with AT (5 nM) and optimal concentration of heparin (300 nM) in TBS buffer containing 0.1–0.4 m NaCl, 1 mg/ml BSA, 0.1% PEG 8000, and either 100 \mu M EDTA or 2.5 m Ca\(^{2+}\) as described under “Experimental Procedures.” The kinetic values are the average of at least three independent measurements \pm S.D.
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accelerating effect of heparin in thrombin inactivation (5, 6, 9). In FXa inactivation, however, it is believed that a heparin-induced conformational change in the reactive site loop of AT is responsible for virtually all of the accelerating effect and that heparin bridging AT and protease in a ternary complex has a minimal or no effect on acceleration of the reaction (4, 7–10).

In the absence of Ca\(^{2+}\), an experimental condition that all other previous studies have used to compare the extent of the cofactor effect of heparin and pentasaccharide in the acceleration of FXa inactivation. In the presence of Ca\(^{2+}\), however, it was found that heparin-catalyzed FXa inactivation was at least 13-fold higher than the inactivation in the absence of Ca\(^{2+}\).

FXA belongs to a family of vitamin K-dependent coagulation proteases, which all contain a highly anionic N-terminal Gla domain with several \(\gamma\)-carboxylated glutamic acid (Glu) residues that are involved in Ca\(^{2+}\)-dependent membrane binding (22, 23). In FXa, this domain contains 11 \(\gamma\)-carboxylated Glu residues, which are in random disordered conformation in the absence of Ca\(^{2+}\) but fold properly to their native conformation in the presence of Ca\(^{2+}\) (15). The observation that the accelerating effect of heparin in FXa inactivation in the presence of Ca\(^{2+}\) was \(\sim 13–15\)-fold higher than FXa inactivation in the absence of Ca\(^{2+}\) suggests that the anionic Gla domain prevents the binding of heparin to FXa and thereby the formation of a ternary bridging complex. The Gla domain can interfere with heparin binding to FXas in the absence of Ca\(^{2+}\) by either folding on itself and masking the positively charged residues of the heparin binding site and/or simply by creating a highly electronegative molecule preventing the approach of heparin by repulsive interactions. The interference of an unfolded Gla domain with heparin binding in the absence of Ca\(^{2+}\) may be a universal phenomenon occurring in other vitamin K-dependent Gla-containing coagulation proteases, because in a recent study it was also noticed that heparin dramatically accelerates protein C activation by thrombin and FXa in the presence but not in the absence of Ca\(^{2+}\) (24). In the absence of Ca\(^{2+}\), however, heparin accelerated the activation of the Gla-domainless protein C by thrombin, suggesting that similar to FXa, the Gla domain of protein C when not stabilized by Ca\(^{2+}\) ions interferes with the cofactor function of heparin in the activation reaction. The existence of a heparin-binding site in FXa and protein C homologous to that in thrombin is suggested by the conservation of the basic site of thrombin in FXa and activated protein C (25, 26). Interestingly, the observed affinity of FXa for heparin in the presence of Ca\(^{2+}\) is comparable with that measured for thrombin, albeit in the absence of Ca\(^{2+}\) (27). This is the first study to show that the cofactor effect of heparin in FXa inactivation by AT at physiologic Ca\(^{2+}\) exceeds the cofactor effect of heparin in thrombin inactivation and that this effect is mediated by a combination of an \(-300\)-fold enhancement by the pentasaccharide-induced conformational change in the reactive site loop of AT and a \(-60\)-fold enhancement by a template mechanism. The results of this study further suggest that heparin fragments of 18 saccharides long are required to observe a template effect for heparin in FXa inactivation in the presence of Ca\(^{2+}\), similar to the chain length requirement in thrombin inactivation. Low molecular weight heparins (\(M_t = 4000–6000\)) are used as antithrombotic agents, and a number of studies suggest that the antithrombotic effect of these types of heparins can be accounted for primarily by their anti-FXa activity, because oligosaccharides less than 18 residues long that are devoid of anti-thrombin activity still exhibit significant antithrombotic activity (28). Relative to unfraccionated heparin, however, these oligosaccharide fragments show less antithrombotic activity despite the existence of a comparable \(ex vivo\) and \(in vitro\) anti-FXa activity for both types of heparins (17). Based on the oligosaccharide chain length dependence of the antithrombotic effect it has been concluded that in addition to the anti-FXa activity, the longer chain heparins in low molecular weight preparations also provide an additional anti-thrombin activity. Results of the current study now suggest that the reduced antithrombotic effect of low molecular weight heparins may be accounted for solely by their anti-FXa activity, because this activity is expected to show a chain length dependence in the presence of Ca\(^{2+}\) like that of thrombin. Because all current \(in vitro\) assays measure the anti-FXa activity of heparin in the absence of Ca\(^{2+}\), they would miss this additional chain length-dependent cofactor effect of heparin in FXa inactivation.

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Alireza R. Rezaie

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