The Mechanism by Which Heparin Promotes the Inhibition of Coagulation Factor XIa by Protease Nexin-2*

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Previous kinetic studies have shown that protease nexin-2 is a potent, reversible, and competitive inhibitor of factor XIa. Here we show that high molecular weight heparin potentiates the ability of protease nexin-2 to inhibit factor XIa with a parabolic concentration dependence, predominantly because of an increase of the association rate constant with little perturbation of the dissociation rate constant. No effect on factor XIa inhibition by protease nexin-2 was observed with heparin preparations of 6–22 saccharide units (0.1 nm–1 μM), whereas heparin preparations with 32–64 saccharide units potentiated factor XIa inhibition by protease nexin-2 in a size- and concentration-dependent manner. We propose a model wherein heparin exerts this effect by providing a template for the assembly of factor XIa–protease nexin-2 complexes, and only heparin polymers consisting of greater than 32 saccharide units (M, >10,000) are sufficiently long to provide a template to which factor XIa and protease nexin-2 molecules can bind simultaneously. Heparin-mediated enhancement of factor XIa inhibition by protease nexin-2 was partially abrogated by high molecular weight kininogen, suggesting that high molecular weight kininogen may play a role in regulating factor XIa activity.

Protease nexin-2 (PN-2)1 is a ~120-kDa soluble fragment of Alzheimer amyloid β-protein precursor isoforms containing the 56-amino acid Kunitz-type (or Kunin)2 protease inhibitor domain (1–4). PN-2 has been found to be an abundant platelet α-granule protein that is secreted from platelets upon their activation with physiological agonists (5–8). Factor XIa (FXIa) is a homodimeric serine protease (~160 kDa) involved in the intrinsic pathway of blood coagulation.

We have previously conducted detailed kinetic analyses to characterize the inhibition of FXIa by PN-2 using a fluorogenic substrate that is ~1,000-fold more sensitive for FXIa than the most sensitive and specific chromogenic substrates (8). The mechanism of FXIa inhibition by PN-2 is best described as a simple, one-step binding interaction during which PN-2 reversibly inhibits FXIa. Our studies have shown that PN-2 is a slow, tight binding inhibitor of FXIa, with an association rate constant (k(on)) of 2.1 ± 0.2 × 10⁶ M⁻¹ s⁻¹, a dissociation rate constant (k(off)) of 8.5 ± 0.8 × 10⁻⁴ s⁻¹, and a Kᵢ of 400 pm, a value in good agreement with previous reports (6–8).

Further, we found that high molecular weight kininogen (HK) protects FXIa from inhibition by PN-2 with a saturable concentration dependence (the EC₅₀ for HK was essentially identical to the Kᵢ for the binding interaction between FXIa and HK) and that this protection results from the decreased ability of PN-2 to inhibit the FXIa-HK complex (8). Zinc ions, which are known to affect several functions of both FXIa (9, 10) and PN-2 (11), are known to increase the affinity of the FXIa-PN-2 complex (9, 12). We found that Zn²⁺ was able to abolish the protection afforded to FXIa by HK and hypothesized the existence of a zinc-dependent conformation of HK which does not obstruct the interaction between FXIa and PN-2 (8).

Several studies have shown that the capacity of PN-2 to inhibit FXIa is potentiated by heparin (6, 13), a negatively charged glycosaminoglycan that is synthesized by mast cells and is similar to the heparan sulfate glycosaminoglycans expressed on the surface of endothelial cells. In the present studies, we have used highly purified heparin fractions to perform experiments designed to elucidate the mechanism by which heparin enhances the interaction between FXIa and PN-2. Moreover, because Zn²⁺ ions potentiate FXIa inhibition by PN-2 (9, 12) and because previous studies have shown that HK and Zn²⁺ ions regulate the inhibition of FXIa by PN-2 secreted from activated platelets (8), we were prompted to investigate the effects of HK and Zn²⁺ ions on the heparin-potentiated inhibition of FXIa by PN-2.

**EXPERIMENTAL PROCEDURES**

Materials—PN-2 was purified from fibroblast culture media using techniques of heparin affinity chromatography and immunoaffinity chromatography as described previously (7). Human FXIa was purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Human HK was from Enzyme Research Laboratories, Inc. (South Bend, IN). Fluorescein monop-guanidinobenzoate hydrochloride, fluorescein, and unfractionated heparin from bovine lung (average M, ~10,000) were purchased from Sigma. Purified heparin fractions consisting of 6, 10, 14, 18, 22, 32, 48 and 64 saccharide units (1.74, 2.97, 4.2, 5.43, 7, 10, 15, and 20 kDa, respectively) were purified and kindly donated by Dr. Steven T. Olson (University of Illinois, Chicago) and Dr. Ingemar Bjork (Uppsala Biomedical Center, Uppsala, Sweden). Their preparation and characterization are described elsewhere (14, 15). The fluorogenic substrate Boc-EAR-AMC was from Peptide International, Inc. (Louisville, KY). The chromogenic substrate S-2366 was purchased from Chromogenix.
RESULTS from the solution of the nonlinear, separable differential equation, curve data measured at multiple PN-2 concentrations. Equation 2 re-squares fits to Equation 2 of the association and dissociation progress

\[ P(t) = \frac{k_m S_i}{K_m + S_i} \cdot \left( E_0 + \frac{b + \gamma}{2} \cdot t \right) + \frac{1}{h_{man}} \cdot \ln \left( \frac{Q_0 - \exp(-h_{man} \cdot \gamma \cdot t)}{Q_0 - 1} \right) \]  

where \( Q_0 \) is the enzyme-inhibitor complex present at the start of the monitored reaction.

For association experiments \((E_1)_0 = 0\).

For dissociation experiments,

\[ (E_1)_0 = \frac{1}{2} \left( E_T + I_T + \frac{k_{off}}{K_m \cdot D} - \sqrt{(E_T + I_T + \frac{k_{off}}{K_m \cdot D})^2 - 4 \cdot E_T \cdot I_T} \right) \]  

where \( D \) is the factor by which the preincubated enzyme-inhibitor mixture is diluted into the final, monitored reaction.

Determination of Equilibrium Inhibition Constant \((K_i)\)—The \( K_i \) was either calculated as the ratio of the forward and reverse rate constants determined from progress curve analysis or obtained from equilibrium measurements as described below.

In the equilibrium method, increasing amounts of PN-2 (0–10 nM in the absence of heparin and 0–1 nM in the presence of heparin) were incubated with a constant amount of FXIa (125 pM) at 37 °C for 1 h to allow the establishment of an equilibrium between the inhibitor and enzyme. After this preincubation step, a small volume of substrate S-2366 (0.5 mM) was added, and the residual FXIa activity was determined by measuring the initial velocity of substrate cleavage for 10 min. This time period was sufficiently short, and the substrate concentration was sufficiently low to ensure that the equilibrium was not perturbed during the measurement period. Measurements were made in the presence of varying concentrations of unfractionated heparin or purified heparin fractions. The measurements were also made in the presence of HK (50–500 nM) or ZnCl\(_2\) (25 μM) with and without unfractionated heparin (0.1 μg/ml) or the 64 saccharide heparin fraction (100 nM).

After equilibration, the inhibition constant, \( K_i \), was obtained by the least squares fit to Equation 8 of the ratio of the velocity of substrate cleavage measured at varying inhibitor concentrations, \( V_1 \), to the velocity measured in the absence of inhibitor, \( V_0 \).

\[ \frac{V_1}{V_0} = 1 - \frac{(E_T + I_T + K_i) - \sqrt{(E_T + I_T + K_i)^2 - 4 \cdot I_T \cdot E_T}}{2E_T} \]  

RESULTS

Molecular Weight and Concentration-dependent Heparin Effect on FXIa Inhibition by PN-2—We have measured the equilibrium inhibition constant \((K_i)\) of FXIa inhibition by PN-2 in the presence of varying concentrations of unfractionated heparin and well characterized, purified heparin subfractions (14, 15). We found that high molecular weight heparin (fractions containing 32–64 saccharide units) potentiated FXIa inhibition by PN-2 with a parabolic concentration dependence, whereas heparin fractions with a shorter length (fractions containing 6–22 saccharide units) were unable to affect the PN-2/FXIa interaction (Fig. 1). Each of the active heparin subfractions possessed a broad optimal concentration range occurring between 0.01 and 1 μM. Moreover, the extent to which each active heparin species was able to potentiate the ability of PN-2 to inhibit FXIa was directly related to its strand length.

Kinetic Mechanism of FXIa Inhibition by PN-2 in the Presence of Heparin—Our previous studies (8) demonstrated that in the absence of heparin the kinetics of FXIa inhibition by PN-2 conform to a one-step second-order reaction mechanism. Heparin does not appear to affect this mechanism. We analyzed pseudo first-order data obtained in the presence of an optimal concentration of fractionated heparin (100 nM, 64 saccharide units) using methods described previously (8) and equations developed by Cha (18) and extended by Morrison and Walsh (19, 20). Consistent with the reaction proceeding via a simple
As shown in Fig. 5, heparin strands affect the forward rate constant in a manner that is both concentration- and chain length-dependent but do not significantly affect the reverse rate constant at any concentration. As would be expected from a one-step mechanism, the pseudo first-order rate constant observed, $k_{obs}$, was linearly dependent upon the inhibitor concentration (Fig. 2). We also found that the stoichiometry of the FXIa/PN-2 interaction is not changed by the presence of heparin (data not shown). Effect of Heparin on the Rate Constants with Which PN-2 Inhibits FXIa—To investigate the mechanism by which heparin increases the affinity of the interaction between PN-2 and FXIa, progress curve measurements were performed and the kinetic rate constants ascertained. The forward ($k_{\text{on}}$) and reverse ($k_{\text{off}}$) rate constants were obtained by fitting families of association (e.g. Fig. 3) or dissociation (e.g. Fig. 4) progress curves simultaneously to the appropriate form of Equation 2. Rate constants derived from the data shown in Fig. 3 demonstrate that the increased potency with which PN-2 inhibits FXIa in the presence of an optimal concentration (100 nM) of long chain heparin molecules (64 saccharide units) is the result of an increased forward rate constant ($k_{\text{on}}$) increased from 3.2 to $4.3 \times 10^6$ M$^{-1}$ s$^{-1}$ in the presence of heparin); the reverse rate constant, $k_{\text{off}}$, was not significantly perturbed ($k_{\text{off}}$ was 0.0013 s$^{-1}$ in the absence of and 0.0014 s$^{-1}$ in the presence of heparin). The rate constants derived from analysis of dissociation progress curves were very similar to those obtained from the association progress curves and confirm that heparin, when sufficiently long, brings about an increase in the second-order association rate constant ($k_{\text{on}}$ increased from 2.0 to $27 \times 10^6$ M$^{-1}$ s$^{-1}$) but has little discernible effect on the reverse rate constant ($k_{\text{off}} = 8.5$ and $7.8 \times 10^4$ in the absence and presence of heparin, respectively) (see Table I for average values).

We conducted experiments similar to those presented in Fig. 3 to ascertain the effect of increasing concentrations of each heparin subfraction (containing 6, 10, 14, 18, 22, 32, 40, and 64 saccharide units) on the kinetics of FXIa inhibition by PN-2 (see Fig. 5). The results presented in Fig. 1, only the heparin fractions greater than 32 saccharide units in length bring about an increase in the forward rate constant. This effect is maximal for the longest heparin molecules (64 saccharide units) and has a distinctive parabolic concentration dependence.

Effect of HK and Zn$^{2+}$ Ions on the Inhibition of FXIa by PN-2 in the Presence of Heparin—In the absence of heparin, HK and Zn$^{2+}$ ions affect the inhibition of FXIa by PN-2 in opposite ways (8). HK partially protects FXIa from PN-2, whereas Zn$^{2+}$ ions enhance the ability of PN-2 to inhibit it. We also found (8) (Fig. 6) that only the effect on Zn$^{2+}$ is seen when both HK and Zn$^{2+}$ are present: Zn$^{2+}$ completely abrogates the effect of HK in the absence of heparin. In the present study, we have investigated the effect of HK and Zn$^{2+}$ in both the absence and presence of heparin using the same kinetic approach used in their absence (Table I). Determination of association and dissociation rate constants (Table I) shows that Zn$^{2+}$ ions, like heparin, enhance the inhibitory effect of PN-2 on FXIa (i.e. decrease the $K_I$) by increasing the association rate constant without affecting the dissociation rate constant. Similarly, the protective effect of HK is caused by its capacity to decrease the association rate constant without affecting the dissociation rate constant, and the absence of heparin results in a distinctive parabolic concentration dependence.
as predicted from previous studies (8), Zn$^{2+}$ completely abrogates the protective effect of HK when both are present.

Contrary to what was seen in the absence of heparin, in its presence, zinc ions have very little effect on the rate constants with which FXIa is inhibited by PN-2; the effect of heparin predominates (Table I). Nonetheless, even in the presence of heparin, HK induces a saturable but partial reversal of the effect of heparin on $k_{\text{on}}$. This effect of HK is saturated at its plasma concentration (HK $\sim$ 640 nM in plasma) where the ability of heparin to potentiate PN-2 is approximately halved (see Fig. 6 and Table I). Because HK also binds to heparin, a plausible mechanism by which HK could reverse the effect of heparin would be by displacing FXIa and PN-2 from their heparin binding sites. As shown in Fig. 7, it is unlikely that this mechanism pertains because the optimal heparin concentration is not shifted to a higher value when HK is present. In any event, the protective effect of HK is canceled when Zn$^{2+}$ is added to the reaction mixture even in the presence of heparin (Fig. 6 and Table I).

**DISCUSSION**

The goal of this study was to determine the effect of heparin on the kinetics of FXIa inhibition by PN-2. Using computational methods to analyze progress curve data, we obtained reliable estimates of the individual rate constants governing the reaction between FXIa and PN-2 in the presence of heparin. We found that 1) the overall reaction mechanism can be well described by a simple second-order model; 2) the increased inhibitory potency of PN-2 in the presence of heparin results entirely from an increase in the forward rate constant and not from a decrease in the reverse rate constant; 3) the heparin effect is concentration-dependent, showing little effect at both low and high heparin concentrations and possessing an optimal concentration at which maximum potentiation is observed; and 4) only heparin preparations at least 32 saccharide units in length are able to affect the kinetics of FXIa inhibition by PN-2.

We believe that heparin brings about its effect by providing a template sufficiently long (at least 32 saccharide units) to facilitate the rapid assembly of a high affinity protease-inhibitor complex. Our “template model,” similar to that of the antithrombin III/thrombin interaction in the presence of heparin (21–25), suggests that a heparin-bound FXIa molecule can rapidly and efficiently react with PN-2 molecules bound to the same heparin strand. This model provides a qualitative explanation for all of the observed effects of heparin on the interaction between FXIa and its most potent inhibitor, PN-2. 1) Our data show that high affinity interactions of FXIa and PN-2 with heparin occur more rapidly than the interaction between FXIa and PN-2, as evidenced by an increased second-order rate constant ($k_{\text{on}}$) without any change in the first-order dissociation rate constant. 2) The parabolic concentration profile observed for heparin is also consistent with the template model. At low heparin concentrations, there are not enough heparin strands to saturate all protein binding sites, and the rate of FXIa inhibition is determined mainly by the interaction between the free proteins, whereas excessively high concentrations of heparin saturate FXIa and PN-2 molecules individually, thus decreasing the likelihood of their binding to the same heparin strand. Only at the optimal heparin concentration is there the highest probability of FXIa and PN-2 molecules being bound to the same heparin strand. 3) This model can also explain why only heparin strands longer than a discrete threshold are able to potentiate the ability of PN-2 to inhibit FXIa. Preparations less than 32 saccharide units in length are too short to provide a template to bind both FXIa and PN-2 molecules simultaneously (Figs. 1 and 5). We have observed, as predicted by the model, that as the length of the heparin strand increases, so does its ability to potentiate PN-2. The fact that both the concentration and size of heparin molecules are critical to the degree to which it can enhance the inhibitory capacity of PN-2 supports the view that one heparin strand interacts with both FXIa and PN-2 molecules.

![Dissociation progress curves of the inhibition of FXIa by PN-2 in the presence and absence of heparin](image)

**FIG. 4**

Dissociation progress curves of the inhibition of FXIa by PN-2 in the presence and absence of heparin. Equal volumes of FXIa and PN-2 were preincubated in the absence (**panel A**) or presence (**panel B**) of heparin fractions containing 64 saccharide units (100 nM) at 37°C for 1 h. The mixture was then diluted 100-fold into substrate (Boc-EAR-AMC, 25 µM)-containing buffer. The final concentration of FXIa was 20 pm. Final PN-2 concentrations are shown on the right side of each panel. Data were fitted in Equation 2 as described under “Experimental Procedures.”

**TABLE I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Association rate constant ($k_{\text{off}}$)</th>
<th>Dissociation rate constant ($k_{\text{on}}$)</th>
<th>$K_{\text{calc}}$ (µM$^{-1}$s$^{-1}$)</th>
<th>$K_{\text{meas}}$ (µM$^{-1}$s$^{-1}$)</th>
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<tbody>
<tr>
<td>No heparin</td>
<td>$3.2 \times 10^{6}$ ± 0.2</td>
<td>$1.3 \times 10^{-3}$ ± 0.3</td>
<td>410 ± 20</td>
<td>370 ± 45</td>
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<tr>
<td>ZnCl$_2$ alone</td>
<td>$6.8 \times 10^{6}$ ± 0.4</td>
<td>$9.4 \times 10^{-4}$ ± 0.5</td>
<td>140 ± 6</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>HK alone</td>
<td>$1.8 \times 10^{6}$ ± 0.3</td>
<td>$1.3 \times 10^{-3}$ ± 0.2</td>
<td>710 ± 46</td>
<td>740 ± 23</td>
</tr>
<tr>
<td>ZnCl$_2$ + HK</td>
<td>$5.3 \times 10^{6}$ ± 0.6</td>
<td>$8.7 \times 10^{-4}$ ± 0.4</td>
<td>170 ± 5</td>
<td>160 ± 7</td>
</tr>
<tr>
<td>UF Heparin$^a$</td>
<td>$2.4 \times 10^{6}$ ± 0.1</td>
<td>$8.4 \times 10^{-4}$ ± 0.5</td>
<td>35 ± 10</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>F Heparin$^b$</td>
<td>$4.3 \times 10^{6}$ ± 0.5</td>
<td>$1.4 \times 10^{-3}$ ± 0.2</td>
<td>33 ± 2</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>with ZnCl$_2$</td>
<td>$9.2 \times 10^{5}$ ± 0.5</td>
<td>$8.9 \times 10^{-4}$ ± 0.2</td>
<td>97 ± 22</td>
<td>78 ± 13</td>
</tr>
<tr>
<td>with HK</td>
<td>$1.1 \times 10^{5}$ ± 0.7</td>
<td>$1.2 \times 10^{-3}$ ± 1.0</td>
<td>100 ± 15</td>
<td>140 ± 21</td>
</tr>
<tr>
<td>with ZnCl$_2$ + HK</td>
<td>$3.4 \times 10^{5}$ ± 1.2</td>
<td>$7.8 \times 10^{-4}$ ± 0.8</td>
<td>220 ± 30</td>
<td>160 ± 10</td>
</tr>
</tbody>
</table>

$^a$ Second-order rate constants were obtained by computational analysis of progress curve data as shown in Fig. 3 using Equation 2. $K_{\text{calc}}$ was calculated from the relationship $K_{\text{calc}} = k_{\text{on}}/k_{\text{off}}$.

$^b$ $K_{\text{meas}}$ was determined directly with equilibrium measurement.

$^c$ 25 µM ZnCl$_2$.

$^d$ 250 nM HK.

$^e$ Unfractionated heparin (∼10,000 Da), 0.1 µg/ml.

$^f$ Fractionated heparin consisting of 64 saccharide units (100 nM).
results from the binding of HK to FXIa (8). Zn$^{2+}$ both potentiates PN-2 and reduces the ability of HK to protect FXIa from inhibition by PN-2. In the presence of both Zn$^{2+}$ ions and heparin, the protective effect of HK is abrogated. Because there are high affinity heparin binding sites on all three proteins, PN-2 (6), HK (26), and FXIa$^3$ and because FXIa forms a non-covalent complex with both PN-2 (5–8) and HK (27), it is possible that HK itself or a binary complex formed between FXIa and HK on a heparin template could compete with FXIa and PN-2 on a heparin strand. The results presented in Fig. 7 strongly argue against such a hypothesis: if HK protected FXIa from PN-2 in the presence of heparin by displacing FXIa or PN-2 from heparin binding sites, the optimal heparin concentration would have to be shifted rightward, and this was not the case (Fig. 7).

Certain comparisons can be made between the PN-2/FXIa interaction and the antithrombin III/thrombin interaction (21–25). The antithrombin III/thrombin reaction is accelerated dramatically by heparin, which provides a template for the antithrombin III/thrombin-heparin ternary complex to form. A change in antithrombin III conformation has an added effect on rate enhancement. In contrast to antithrombin III, PN-2 is an excellent FXIa inhibitor even in the absence of heparin (9). Our kinetic analysis of the heparin-enhanced PN-2/FXIa interaction showed only a 10-fold increase in rate, in contrast to the 2,000–4,000-fold increase in the rate of thrombin inhibition by antithrombin III brought about by heparin. At least 18 saccharide units are required for heparin to promote rate enhancements for inhibition of thrombin, FXIa, or FXIIa by antithrombin III (22, 25). For the PN-2/FXIa interaction, a heparin strand containing at least 32 saccharide units was both necessary and sufficient to provide this template. This size template may be required to accommodate both the FXIa molecule (120 kDa), whereas a smaller heparin template may be sufficient to accommodate the smaller antithrombin III molecule (58 kDa).

Because PN-2 is secreted from activated platelets (5–7) at

\[ \text{Heparin Effect on Factor XIa Inhibition by Protease Nexin-2} \]

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the site (i.e. platelet membranes) of assembly of the protease-cofactor-substrate complexes that lead to the generation of thrombin and hemostatic thrombi, it is ideally situated to regulate FXIa activity in the vicinity of platelet thrombi. Platelet activation and the secretion of PN-2 from platelets limit the lifetime of FXIa within the locus of activated platelets (8). In the presence of platelets, as in the purified system, HK protects FXIa from inactivation by PN-2, and Zn\(^{2+}\) ions potentiate FXIa inhibition by PN-2 (8). However, when both HK and Zn\(^{2+}\) are present together with platelets, it is the protective effect of HK which predominates and prolongs the lifetime of FXIa after platelet activation (8). In our present study, we found that exogenous heparin does not promote FXIa inhibition by PN-2 secreted from activated platelets (data not shown), probably as a consequence of the neutralization of heparin by platelet factor 4, which is also secreted, along with PN-2, from the α-granules of activated platelets (28–30). Thus, the major regulator of FXIa within the vicinity of activated platelet thrombi is PN-2, a potent, slow, tight binding, reversible inhibitor of FXIa which is much less effective in inhibiting FXIa bound to the platelet surface in the presence of HK (8, 10, 31). Nonetheless, PN-2 may be much more effective in inhibiting FXIa bound to heparin-like molecules such as heparan sulfate on the surface of nonthrombogenic cells such as endothelial cells. The relative contributions of other dominant FXIa inhibitory serine protease inhibitors found in plasma such as antithrombin III (32), α\(_1\)-protease inhibitor, α\(_2\)-antiplasmin, and C1 esterase inhibitor (33) will have to be reexamined in comparison with PN-2 to resolve the question of which regulatory protein predominates under various physiological and pathophysiological conditions.

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REFERENCES