Studies of the mechanisms of blood coagulation zymogen activation demonstrate that exosites (sites on the activating complex distinct from the protease active site) play key roles in macromolecular substrate recognition. We investigated the importance of exosite interactions in recognition of factor IX by the protease factor XIa. Factor XIa cleavage of the tripeptide substrate S2366 was inhibited by the active site inhibitors p-aminobenzamidine (K_i = 28 ± 2 μM) and aprotinin (K_i = 1.13 ± 0.07 μM) in a classical competitive manner, indicating that substrate and inhibitor binding to the active site was mutually exclusive. In contrast, inhibition of factor XIa cleavage of S2366 by factor IX by aprotinin (K_i = 0.33 ± 0.05 μM) was non-competitive, whereas inhibition by active site-inhibited factor IXaβ was competitive (K_i = 38 ± 14 μM) but was not inhibited by factor IX, consistent with loss of factor IX-binding exosites on the non-catalytic factor XI heavy chain. The results support a model in which factor IXA binds initially to exosites on the factor XIa heavy chain, followed by interaction at the active site with subsequent bond cleavage, and support a growing body of evidence that exosite interactions are critical determinants of substrate affinity and specificity in blood coagulation reactions.

Factor IX (fIX) is the zymogen precursor of a trypsin-like plasma protease, factor IXaβ (fIXaβ), that contributes to fibrin clot formation through proteolytic activation of factor X (1, 2). fIX conversion to fIXaβ is achieved through two proteolytic cleavages after Arg^{145} and Arg^{180}, releasing an 11-kDa activation peptide (3–5). During hemostasis, fIX activation occurs through two distinct pathways mediated by the protease factors VIIa and XIa (fXIa) (3, 4, 6–8). Plasma coagulation is initiated when factor VIIa binds to the integral membrane protein tissue factor (TF) at a wound site (8–10). fIXaβ generated by factor VIIa/TF converts factor X to Xa and is probably involved in initial fibrin formation and sustained thrombin production. Activation of fIX by fXIa likely occurs after initial fibrin formation (11, 12) and is required for maintenance of clot stability, particularly in tissues rich in fibrinolytic activity that would otherwise quickly degrade the clot (13, 14).

fXIa differs from other coagulation proteases in several important aspects. fXI is a homodimer (15, 16), whereas other coagulation proteases are monomers (2). Although the C-terminal portion of the fXI polypeptide is a typical trypsin-like protease domain, the N-terminal non-catalytic region contains four repeats called apple domains (A1–A4 from the N terminus) not found on other coagulation proteases (16, 17). Furthermore, fXIa lacks the phospholipid-binding GlA domain characteristic of vitamin K-dependent coagulation proteases. Indeed, although phospholipid lowers K_m for most coagulation protease reactions several orders of magnitude, it has little effect on fIX activation by fXIa (4, 7, 18). The molecular mechanism by which fXIa activates fIX is not completely understood, and the unusual structural features of fXIa cited above make it difficult to extrapolate from data obtained for other coagulation proteases.

Enzymes involved in fibrin formation are members of the chymotrypsin family of serine proteases (2, 19, 20). Despite having relatively similar catalytic domains, these enzymes exhibit specific substrate recognition (21). Substrate specificity and affinity for many serine proteases involved in digestive or degradative processes are governed primarily by interactions between the protease catalytic domain and sites on the substrate near the protease cleavage site (22–24). These interactions, which involve active site groups (primarily the S1–S3 substrate-binding subsites) and surface loops on the catalytic domain, are critical for proper alignment of substrate with the active site. More specialized proteases, including those involved in coagulation, frequently have domains outside of the protease domain that are required for proper function (1, 2, 25). A substantial body of evidence obtained by structural biology approaches indicates that binding interactions outside the protease domain are important determinants of substrate affinity and specificity in coagulation reactions (26–30).

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Several lines of evidence suggest that FIX activation involves exosite interactions with the FIXa heavy chain. $K_m$ for FIX activation by the isolated FIXa catalytic domain is ~25-fold higher than for intact FIXa (36). Recombinant FIXa in which the A3 domain is replaced with the homologous domain from plasma kallikrein also activates FIX with a substantially greater $K_m$ when compared with wild type FIXa (37, 38). Although these studies clearly show that the FIXa heavy chain interacts with FIX, the importance of these interactions, relative to those at the protease active site, in productive substrate recognition is not clear. Here, we investigated the importance of exosite binding to substrate recognition of FIX by FIXa.

**EXPERIMENTAL PROCEDURES**

**Materials**—S299 (methyl-sulfonyl-p-cyclo-hexyl-glycyl-glycyl-arginine-p-nitroanilide) was from American Diagnostics (Greenwich, CT), and S2366 (L-pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide) was from DiaPharma (West Chester, OH). Aprotinin and $p$-aminobenzamidine (pAB) were from Sigma.

**Plasma Proteins**—FX was prepared from human plasma collected into acid-citrate-dextrose. Plasma (2 liters) at 4 °C was supplemented with human antithrombin to 20 nm, and 160 ml of 1 mM BaCl$_2$ was slowly added with stirring. After 1 h, the precipitate was pelleted at 10,000 g for 20 min, washed twice with 1 liter of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 20 mM benzamidine, 10 mM BaCl$_2$, and resuspended in 100 ml of 10 mM Tris–HCl, pH 7.5, 20% saturated ammonium sulfate, 20 µg/ml soybean trypsin inhibitor. The suspension was dialyzed against 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 20 mM benzamidine, 5 mM EDTA and then against 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 20 mM benzamidine, 2.5 mM CaCl$_2$. After centrifugation, FIX was purified from supernatant by antibody-affinity chromatography by using the calcium-dependent anti-human FIX monoclonal IgG SB 249417 (Dr. John Toomey, GlaxoSmithKline) linked to Affi-Gel-10 (Bio-Rad) (30). After loading, the column was washed with 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM CaCl$_2$, 5 mM benzamidine and then eluted with 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 25 mM benzamidine. Protein-containing fractions were concentrated by ultrafiltration and dialyzed against 50 mM Tris–HCl, pH 7.5, 100 mM NaCl (TBS). Purity was determined by SDS-PAGE and concentration by colorimetric assay (Bio-Rad). Human FIXa, FIXaβ, and FIXai (FIXaβ with the active site inhibited by Glu-Gly-Arg-chloromethyl ketone) were from Hematologic Technologies (Essex Junction, VT).

**Recombinant FIXa Catalytic Domain**—The human FIX cDNA (17) was altered by using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA), converting the TGT triplet for Cys482 to AGC (Ser). The construct (FIX-Ser$^{362,482}$) codes for a protein lacking the disulfide bond that connects the FIXa catalytic domain to the heavy chain. FIX-Ser$^{362,482}$ DNA was ligated into vector pUC19 (38), and 50 µg of plasmid (ATCC CRL 1573) was cotransfected by electroporation (Electrocell Manipulator 600 BTX, San Diego, CA) with 40 µg of construct and 2 µg of pRSVneo (38). Cells were grown in Dulbecco’s modified Eagle’s medium, 5% fetal bovine serum, 500 µg/ml G418. Supernatants from G418-resistant clones were tested by enzyme-linked immunosorbent assay by using goat anti-human FIX antibody (Affinity Biologicals, Hamilton, Ontario, Canada). Expressing clones were expanded in 175-mm$^2$ flasks, and conditioned medium was collected every 48 h. FIX-Ser$^{362,482}$ was purified from medium on an anti-FX IgG 1G5 affinity column (38). Purified FIX-Ser$^{362,482}$ (~300 µg/ml) was activated with 5 µg/ml factor XIIa (Enzyme Research Laboratories, South Bend, IN) at 37 °C, and complete activation was confirmed by SDS-PAGE. Activated protein was reapplied to the 1G5 antibody column to separate the FIXa$^{362,482}$ heavy chain, which flow through the column, from medium on an anti-fX IgG 1G5 affinity column (38). Purified FIX-Ser$^{362,482}$ was used as a macromolecular ligand (S2) interacts at the exosite during the first step, and after a conformational rearrangement governing the recognition is not clear. Here, we investigated the importance of exosite binding to substrate recognition of FIX by FIXa.

**Data Analysis**—The apparent steady-state kinetic parameters $K_m$ and $k_{cat}$ for FIX activation were obtained both by non-linear least-squares fitting of full progress curves for FIX activation at substrate concentrations ranging from 25 to 1000 nM FIX (0.3–11 µM) and by the classical competitive inhibition model (Scheme 1) and by the classical competitive inhibition model in which no ESI complex is formed (41). The hyperbolic mixed-type inhibition model describes the conversion of S2366 (S) to product (P) by FIXa (E) in the presence of S2366 (I). $K_m$ and $k_{cat}$ for hydrolysis of S2366 by FIXa were obtained by initial rate analysis of $p$-nitroaniline generation as a function of FIX concentration. $K_i$ for binding of pAB and aprotinin to FIXa were obtained by fitting the substrate and inhibitor dependences of the initial rates of S2366 hydrolysis both by the general hyperbolic mixed-type inhibition model (Scheme 1) and by the classical competitive inhibition model in which no ESI complex is formed (41).
Exosite-dependent Recognition of Factor IX by Factor XIa

RESULTS

Inhibition of fXIa-catalyzed Hydrolysis of S2366 by fIX and fIXai—In surface plasmon resonance studies, fIX and fIXai bind to fXIa with similar affinity (30). The effect of fIX and active site-blocked fIXβ (fIXai) on fXIa hydrolysis of S2366 was investigated. During reactions with fIX, it is expected that some fIXβ will be generated. Preliminary experiments showed that 2 μM fIXβ does not hydrolyze S2366 appreciably. If fIX interacts with fXIa exclusively at the active site, it would be expected to behave as a competitive inhibitor of fXIa hydrolysis of S2366. In this case, fIXai interaction at the active site may be weak because of the absence of the activation peptide. On the other hand, if exosite interactions are involved in fIX and fIXai binding to fXIa, mixed-type inhibition of S2366 cleavage would be expected for both molecules. fIX and fIXai inhibited fXIa cleavage of S2366 in a concentration-dependent manner (Fig. 2), and substantial residual fXIa activity remained at saturating fIX or fIXai concentrations. The data were fit well by the hyperbolic mixed-type inhibition model (Scheme 1). Binding of fIX resulted in a 2.7-fold increase in Km and a 50% reduction in kcat for fXIa cleavage of S2366. Kf for binding of fIX to free and S2366-bound fXIa was 0.22 ± 0.05 and 0.59 ± 0.09 μM, respectively (Table I). Binding of fIXai caused a similar 2.5-fold increase in Km and a less explicit (14%) decrease in kcat for S2366 cleavage. Kf for binding of fIXai to free fXIa and to the fXIa:S2366 complex was 0.11 ± 0.02 and 0.28 ± 0.02 μM, respectively (Table I). The Kf for binding of fIXai to fXIa was in good agreement with the Km for product inhibition obtained from progress curves of fIX hydrolysis by fXIa (0.075 ± 0.015 μM, Fig. 3).

The model was expanded to contain a reversible step for exosite binding followed by docking to the active site, governed by an equilibrium constant K* = [ES]/[ES*] in which [ES] is the fXIa:fIX complex engaged only at the exosite, and [ES*] is the complex engaged at the exosite and active site. A similar model has been described by Boskovic and Krishnaswamy (33) (Scheme 2). Using the fixed Kf values from the mixed inhibition model allowed the estimation of a lower limit for K* of ~5 for fXIa:fIX and fXIa:fIXai binding. In the latter case, ES2* is a dead-end complex. Ternary complex formation clearly affected Km and kcat, and omitting linkage (assuming α = β = 1 (33)). The initial rate of hydrolysis of chromogenic substrate in Michaelis-Menten form is given by Equation 2 and the initial rate of cleavage of S2366 in a concentration-dependent manner (Fig. 2). The K^m is given by Equation 3. Least squares fitting was performed with Mathematica software (MicroMath Scientific Software, Salt Lake City, UT), and reported estimates of error represent ± 2 S.D.
Kinetics of inhibition were determined from initial velocity measurements using eight concentrations of S2366 or fIX. Values for $K_i$, and $K_{cat}$ for cleavage of S2366 or fIX were fixed for the purpose of determining $\alpha$, $\beta$, and $K^*$ inhibition models. $\alpha$ and $\beta$ were derived from the general hyperbolic mixed-type inhibition model (41) and were fixed when determining $K^*$ in the two-step model. All ranges are ± 2 standard deviations (95% confidence intervals).

### Table I

<table>
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<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Inhibition type</th>
<th>$K_i$ ($\mu M$)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$K^*$ ($\mu M$)</th>
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<tr>
<td>S2366</td>
<td>None</td>
<td>Competitive</td>
<td>233 ± 78</td>
<td>117 ± 10</td>
<td>28 ± 2</td>
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<td>233 ± 78</td>
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<td>0.5 ± 0.1</td>
<td>0.22 ± 0.05</td>
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<td>fIX</td>
<td>Two step conformational</td>
<td>233 ± 78</td>
<td>117 ± 10</td>
<td>2.7 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>≥5</td>
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<tr>
<td>S2366</td>
<td>fIXai</td>
<td>Mixed hyperbolic</td>
<td>233 ± 78</td>
<td>117 ± 10</td>
<td>2.5 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.11 ± 0.02</td>
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<tr>
<td>S2366</td>
<td>fIXai</td>
<td>Two step conformational</td>
<td>233 ± 78</td>
<td>117 ± 10</td>
<td>2.5 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>≥5</td>
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<td>Non-competitive</td>
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<tr>
<td>fIX</td>
<td>Aprotinin</td>
<td>Competitive</td>
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<td>0.49 ± 0.05</td>
<td></td>
<td></td>
<td>0.33 ± 0.05</td>
</tr>
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</table>

**Inhibition of fXa Catalyzed Activation of fIX by fIXai**

Inhibition of fXa catalyzed activation of fIX by fIXai involves cleavage at two distinct sites, values for $k_{cat}$ represent an overall constant for cleavage at both sites. If binding affinity for this reaction is mediated primarily by exosites on fIXai, pAB would be expected to behave as a non-competitive inhibitor of fIX activation. Technical considerations with the fIXai chromogenic substrate assay precluded the use of pAB in these experiments (pAB interferes with fIXai cleavage of chromogenic substrate). Instead, aprotinin, which inhibits fXa without affecting fIXai activity, was used as an active site inhibitor. Initial velocity studies of fIX activation showed that aprotinin inhibits fIXai activation of fIX in a non-competitive manner with $K_i 0.89 ± 0.52 \mu M$ for the fIXai-aprotinin complex (Fig. 4A and Tables I and II) and $\alpha K^* 5.6 ± 2.1 \mu M$ for the ternary fIXai-fIXa-aprotinin complex. The results imply that the fIXai-fIXa-aprotinin complex is a dead-end complex ($\beta = 0$) and that aprotinin binding to fIXai weakens the affinity for fIXai by ~6-fold.

**Discussion**

We used a strategy based on characterizing the effects of active site and macromolecular inhibitors of fIXai to investigate competitive inhibitor of fIX activation by fIXai, if product and substrate recognize the same exosite. Such competitive product inhibition has been demonstrated for factor X activation by factor VIIa/TF (28) and for prothrombin activation by the prothrombinase complex (32, 33). The effects of active site-inhibited fIXai (fIXai) on fXa activation by fIXai were studied (Fig. 4B). fIXai inhibits fXa activation in a competitive manner, with $K_i 0.33 ± 0.05 \mu M$ (Table I) supporting the premise that fIX and fIXai bind to fIXa in a mutually exclusive manner.

Effects of pAB and fIX on S2366 Hydrolysis by Isolated fIXa Catalytic Domain (fIXaCD)—fIX-Ser362,482 lacks the disulfide bond that connects the catalytic domain and heavy chain after proteolytic cleavage event (16, 17). fIX-Ser362,482 and plasma fXI are 160-kDa disulfide-linked dimers under non-reducing conditions (Fig. 5A), and 80-kDa monomers when reduced (Fig. 5B). After activation, plasma fXI is still a dimer on non-reducing SDS-PAGE (Fig. 5A, lane 3), whereas reduction reveals the separate heavy chains and catalytic domains (Fig. 5B). In contrast, unreduced fIXa-Ser362,482 runs as two bands (Fig. 5A), with the 100-kDa band representing the separate heavy chains dimers connected by a disulfide bond, and the 35-kDa band representing the catalytic domain (fIXaCD), which separate from the heavy chains in the absence of the Cys362-Cys482 disulfide bond. The absence of the Cys362-Cys482 bond allows fIXa to be isolated from the heavy chains by antibody affinity chromatography (Fig. 5C).
the mechanism by which FXa recognizes FIX. The results support the conclusion that recognition of FIX as an FXa substrate is mediated by exosites on FXa. Our results with active site inhibitors are in agreement with work by Pedicord et al. (46), who used an enzyme-linked immunosorbent assay-based system to measure FIX activation. Their work showed mixed inhibition of FIX activation by the inhibitors aprotinin and leupeptin. These findings and the more extensive results of our studies are consistent with a two-step model first proposed for prothrombin activation (31), and subsequently, for factor X activation (28). Initial binding of macromolecular substrate occurs at an exosite(s) on the enzyme followed by a docking step at the active site and catalysis (Fig. 7). The finding that aprotinin is a non-competitive inhibitor of FIX activation by FXa demonstrates that exosite binding is primarily responsible for substrate affinity (approximated $K_m \approx K_s$) and specificity. FIX might have been expected to behave as a competitive inhibitor of FXa cleavage of S2366, similar to the active site inhibitors $p$AB and aprotinin. However, data for FIX-mediated inhibition of S2366 cleavage are fit best by a mixed-type inhibition model, supporting the concept that

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**Fig. 2. Inhibition of FXa hydrolysis of S2366 by fIX and fIXai.**

A, initial velocities of S2366 hydrolysis (0.5 mM) by 6 nM FXa (v) as a function of FX concentration ([fIX]$_0$). B, initial velocities of S2366 hydrolysis by 6 nM FXa active sites (v) as a function of S2366 concentration ([S2366]$_0$) in the presence of fixed FX concentrations, 0 (○), 50 (●), 100 (□), 250 (■), 500 (△), 1000 (▲), and 2000 (○) nM. C, initial velocities of S2366 hydrolysis (0.5 mM) by 6 nM FXa (v) as a function of FXai concentration ([fIXai]$_0$). D, initial velocities of S2366 hydrolysis by 6 nM FXa (v) as a function of S2366 concentration ([S2366]$_0$) in the presence of fixed FXai concentrations of 0 (○), 50 (●), 100 (□), 250 (■), 500 (△), 1000 (▲), and 2000 (○) nM. Data shown are means for three separate experiments. The lines represent the least-squares fits to the data with the parameters listed in Table I. Rates were measured and analyzed as described under “Experimental Procedures.”

---

**Fig. 3. Kinetic parameters for fIX cleavage by FXa.**

A, initial rates of fIX hydrolysis by 0.4 nM FXa active sites (v) as a function of fIX concentration ([fIX]$_0$). B, progress curves of FXa generation ([fIXa]$_0$) from fIX at 25 (□), 50 (●), 100 (□), 250 (■), 500 (△), and 1000 nM (○). The lines represent the least-squares fits to the data with the parameters listed in Table I. Rates were measured and analyzed as described under “Experimental Procedures.”

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**Fig. 4. Inhibition of FXa activation of FIX by aprotinin and FXai.**

A and B, initial velocities of activation of FIX by 0.4 nM FXa active sites (v) as a function of FX concentration ([fIX]$_0$) in the presence of aprotinin at 0 (○), 1.2 (●), or 3.8 (□) $\mu$m (A) or factor FXai at 0 (○), 1.5 (●), and 3.0 (□) $\mu$m (B). Lines represent the least-squares fits to the data with the parameters listed in Table I. Data shown are means for two separate experiments. Rates were measured and analyzed as described under “Experimental Procedures.”

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pNA, p-nitroaniline.
Exosite-dependent Recognition of Factor IX by Factor XIa

Fig. 5. SDS-PAGE of recombinant wild type and Ser362–482 fXI and fXIA. A, non-reducing gel of plasma fXI (lane 1), fXISer362–482 (lane 2), plasma fXIA (lane 3), and fXIA Ser362–482 (lane 4). B, same proteins as in panel A but under reducing conditions. C, fXIA catalytic domain (CD) purified from activated fXISer362–482 by antibody affinity chromatography. The flow-through of the affinity column contains dimers of fXIA heavy chain (HC). Positions of molecular mass standards in kilodaltons are shown at the left of each panel. Proteins were run on 10% SDS-PAGE and stained with GelCode Blue.

Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Inhibition type</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$K_i$</th>
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<tr>
<td>S2366</td>
<td>None</td>
<td>Competitive</td>
<td>$352 \pm 42$</td>
<td>$67 \pm 2$</td>
<td>$38 \pm 14$</td>
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<td>No inhibition</td>
<td>$352 \pm 42$</td>
<td>$67 \pm 2$</td>
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where $K_i$ is fixed by experimental results at 100–300 nM (30). These values are in close agreement with those for $K_m$ for fIX activation by fXIA, suggesting that $K^*$ does not favor the reaction going to product (i.e. $K^*$ is >1). A limitation of our analysis is that we could not establish an upper limit for $K^*$, probably because the conversion of substrate to product is relatively unfavorable. Indeed, the estimated lower limit for $K^*$ (~5) indicates that conversion to product is unfavorable and would reduce the apparent $k_{cat}$. This may explain why the mixed inhibition model that does not take into account the intramolecular $K^*$ step (Scheme 1) fits the data so well. If $K^*$ favored the reaction going to product ($K^* < 1$), a large discrepancy would have been expected between the models in Schemes I and II.

Activation of fIX involves cleavage after Arg145 and Arg180 (4, 5). During activation by factor VIIa/TF, initial cleavage at Arg145–Ala146 results in accumulation of the intermediate fIXa followed by cleavage at Arg180–Val181 to form fIXaβ (4, 47–49). In contrast, Wolberg et al. (49) showed that little intermediate is formed during fIX activation by fXIA, a finding consistent with the progress curves for fIX activation in the present study. This could be explained by a mechanism in which the first cleavage is followed by rapid cleavage at the second site (50). However, this possibility is not supported by the observation that fIX and intermediates fIXa and fIXαβ (cleaved at Arg180–Val181) are converted by fXIA at similar rates (49).

The mechanism involving rapid sequential cleavage of activation sites (intermediates formed) and the processive mechanism (no intermediates formed) are both compatible with exosite-mediated formation of the initial fIX–fXIA encounter complex. Our analysis does not distinguish between these mechanisms but demonstrates that exosite interactions precede docking at the active sites and catalysis. Given the apparent lack of intermediate generation, we treated fIX activation by fXIA as a simple process for the purpose of constructing our models. We appre-
interaction between S and E at the enzyme active site after binding at the exosite, fIX activation site (49).

An alternative model (not shown) could involve both catalytic domains of the dimer acting on one fIX molecule, with each active site cleaving one individual site. The model shows one-half of the fXIa dimer and makes the assumption that each half-dimer activates fIX independently of the other half. An alternative model (not shown) could involve both catalytic domains of the dimer acting on one fIX molecule, with each active site cleaving one fIX activation site (49).

Kd

activation (44, 51). Gly555 forms part of the active site oxyanion hole and S2 substrate-binding site (44, 51, 52). fIXa-Glu355 hydrolyzes S2366, probably because occupancy of the abnormal active site partially restores the conformation required for catalysis (44). However, this protease has a severe defect in fIX activation, manifest as a several hundredfold decrease in apparent kcat without an effect on Ks, likely caused by a steric clash interfering with the S2-P2 interaction (44). Kf for binding of fIX to fIXa-Glu355 in surface plasmon resonance experiments is similar to binding to wild type fIXa (51). Thus, a mutation that interferes with substrate binding near the active site has a primary effect on kcat and not on Ks, supporting the hypothesis that exosite binding is the major determinant of affinity in the fIX-fIXa interaction.

As previously demonstrated for thrombin (33) and factor Xa (28), fIXaβ is a competitive inhibitor of fIX activation by fIXa. By using surface plasmon resonance techniques, Akturk et al. (30) demonstrated that fIX and fIXaβ bind to fIXa with similar Ks (100–150 nM). The present study demonstrates that binding of fIX to active site-inhibited fIXaβ to fIXa is mutually exclusive, indicating that substrate and product bind to a common site on the enzyme. As reported for active site-inhibited factor Xa binding to factor VIIa/TF, the affinity of fIXa is modestly reduced (~3–4-fold) when compared with Ks for fIX activation by fIXa and Kf for product inhibition calculated from analysis of progress curves. The structure of fIXaβ may be altered when the active site is occupied by the chloromethyl ketone, weakening affinity for fIXa. Indeed, we and Pedicord et al. (46) observed that occupation of the fIXa active site by aprotinin causes a modest (~6-fold) increase in Kd for fIX binding. The absence of the activation peptide from the fIXa variant preparation may also have altered the affinity of the protein for the exosite.

The mechanism described above is supported by a large amount of data obtained over the past 20 years on the importance of the fIXa heavy chain in fIX activation. Isolated plasma fIXa catalytic domain (fIXaCD) activates fIX poorly, while retaining the capacity to cleave small chromogenic substrates (36, 48, 53). fIXa and fIXaCD activate fIX similarly in the absence of calcium ions. Ca2+ substantially enhances fIX activation by fIXa but has little effect on activation by fIXaCD (36), indicating that Ca2+-dependent binding of fIX to the fIXa heavy chain is required for fIX activation. This premise is supported by the observation that the Ca2+-binding fIXa Glu domain is required for fIX binding to fIXa (30). In the present study, fIX inhibited fIX cleavage of S2366 by a mixed type of inhibition, indicating that fIX binds to fIXa and the fIXa-S2366 complex at sites remote from the active site. The failure of fIX to inhibit S2366 cleavage by fIXaCD is consistent with loss of an exosite on the heavy chain. Even without exosite binding, fIX might have competed with S2366 for binding to the active site. That this was not observed suggests that binding at the active site is weak in the absence of binding to the heavy chain, possibly because exosite binding changes the conformation of fIX, the fIXa active site, or both to facilitate docking and catalysis.

Areas on the fIXa heavy chain have been identified that may contain fIX-binding exosites. Peptides representing portions of the fIXa A2 domain are competitive inhibitors of fIX activation by fIXa (54). Recombinant fIXa in which apple domains are replaced with corresponding domains from plasma prekallikrein (PK) indicate that the A3 domain is required for fIX activation (37). The chimera fIXa-PKA3 (fIXa with PKA3) activates fIX with a Kcat ~30-fold greater than for wild type fIXa but with similar Ks (37). These results are supported by work with monoclonal antibodies (36, 37, 55). Sinha et al. (36) showed that an antibody against the fIXa heavy chain is a competitive inhibitor of fIX activation by fIXa, whereas an antibody against the catalytic domain is a non-competitive inhibitor. Sun and Gallani (37) demonstrated that anti-A3 antibodies inhibit fIXa activation of fIX in plasma. Cumulatively, published data and the results of the present study support the...
hypothesis that exosites within the A2 and/or A3 domains of fXIa are critical for initial substrate recognition and formation of a productive enzyme-substrate complex with fIX.

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REFERENCES


Exosite-mediated Substrate Recognition of Factor IX by Factor XIa: THE FACTOR XIa HEAVY CHAIN IS REQUIRED FOR INITIAL RECOGNITION OF FACTOR IX

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