The Elusive Role of the Potential Factor X Cation-binding Exosite-1 in Substrate and Inhibitor Interactions*

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Coagulation factor X (FX) is a vitamin K-dependent zymogen activated into FXa by cleavage at a single bond (Arg15–Ile16 in the chymotrypsin numbering system) hydrolyzed by either of two complexes: FVIIa bound to tissue factor (TF), which triggers coagulation after injury, or FIXa associated with FVIIIa, which amplifies thrombin production after initiation of coagulation. FXa is the only endogenous prothrombin activator, but substantial thrombin production occurs only within the prothrombinase complex, where FXa binds to FVa in the presence of calcium on an appropriate phospholipid surface. Two inhibitors control FXa activity, viz. the tissue factor pathway inhibitor (TFPI) and antithrombin.

Extended binding sites (exosites) play a crucial role in virtually any substrate, cofactor, or inhibitor recognition within the coagulation cascade. Perhaps best characterized is the case of thrombin taking advantage of two exosites for extended interactions in numerous functions (1). Exosite-1 is formed by a set of basic residues comprising loops 34–40 and 70–80, which neighbor each other in the folded structure (2); it interacts with fibrogenin, thrombomodulin, platelet activator receptor-1, FV, FIXa, heparin cofactor II, and hirudin. Exosite-2, comprising loop 91–102 and helices 165–173 and 233–245, is also formed by a set of basic residues that interact with heparin, with the chondroitin sulfate of thrombomodulin, and within prothrombin with kringle-2 (4). In FVIII, the region corresponding to exosite-1 of thrombin is critical for FX activation (5, 6), in FIXa, this region is also important for FX activation in the absence of FVIII and for its inhibition by antithrombin in the absence of heparin (7). In contrast to thrombin, FVIIa, FIXa, and FXa share a negatively charged patch within segment 70–80, whereas FXa is unique in that both loops 34–40 and 70–80 are negatively charged. Thus, the region of FXa corresponding to exosite-1 of thrombin forms a negative cluster, raising the question as to whether it could constitute a functional exosite. FXa has a unique macromolecule substrate, but exosites may also participate in its inhibition and/or in the activation of its zymogen. In fact, a number of studies have established that exosite(s) must be critical in several FXa functions. Electrostatic interactions involving loop 34–40 of FXa appear to play a significant role in binding to the second Kunitz domain of TFPI (8, 9). FXa also appears to interact with a complementary surface on pentasaccharide-activated antithrombin (10), and the region of FXa topologically equivalent to exosite-2 of thrombin seems to be involved in the binding of heparin and FVa (11). Above all, prothrombin recognition by prothrombinase undoubtedly involves one or more exosites on FXa and/or FVa (12–14).

In a previous study (15), we reported that the catalytic groove of FXa is minimally selective with unconstrained peptides and that FVa has little effect, if any, on this selectivity. These two observations therefore also favor the hypothesis that FXa uses secondary binding sites to improve binding and/or to ensure proper alignment of the substrate scissile bond. In the present study, we have examined the role of the potential cation-binding exosite-1 of FX by expressing mutants of FX in which part of the electrostatic potential of FX was neutralized or reversed. We obtained mixed results in that essential functions of FX and FXa were unaffected by mutations within segment 70–80, whereas prothrombin activation and TFPI inhibition were impaired by the loop 34–40 mutations. Thus,
despite a high conservation between species, the potential exosite-1 of FX appears to be less important than its thrombin counterpart.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Human prothrombin (16) and antithrombin (17) were prepared from outdated plasma as described previously. Human FIXa and FVIIa, bovine FVas, and the FX activator isolated from the Russell’s viper venom (RVV-X) were purchased from Kordia (Leiden, the Netherlands). The concentration of FXa was assessed by titration in FX-depleted plasma (Diagnostica Stago, Anières, France). TFFI was from American Diagnostica (Andersy, France), TF from Dade Behring (Paris la Défense, France), and FVIII from Baxter (Lesnines, Belgium). FVIIIia was converted to FVIIIa by incubation with 0.1 nM thrombin in the buffer described previously (15). Production and purification of recombinant FX and derivatives in milligram quantities were performed by a combination of anion-exchange and affinity (using antibody HPC-4 coupled to agarose) chromatography as described for prothrombin derivatives (17, 23). Final products appeared to be homogeneous by Coomassie Blue staining following SDS-PAGE. One hour prior to use as a zymogen, FX and derivatives were incubated with 1 nM phenylmethanesulfonyl fluoride to neutralize any trace of FXa. Control experiments indicated that, after 30 min in Tris-HCl buffer, phenylmethanesulfonyl fluoride was fully hydrolyzed and would not interfere with other reactions.

**FX Activation Kinetics**—Activation of FX and derivatives was studied at 25°C under pseudo first-order conditions in kinetic buffer (50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 5 mM CaCl2, 2 mg/ml bovine serum albumin, 0.2% (w/v) polyethylene glycol (M, 8000), and, when required, 16 μM phospholipid vesicles). To ascertain that pseudo first-order conditions were met, two zymogen concentrations were assayed, with all other experimental conditions the same. With RVV-X, the reaction was initiated by the addition of 50 nM activator to 0.5 μM FX or variant (alternatively, 0.25 μM). With FVIIa (100 pM) plus TF (1.3 nM) or with FXa (50 pM) plus FVIIIa (8.6 nM), reactions were initiated by the addition of the mixture to 10 μM FX or variant (alternatively, 5 nM). At time intervals, an aliquot was withdrawn, and the reaction was quenched by the addition of 50 mM EDTA. The progress of FXa (or activated variant) formation was monitored by the rate of S2222 hydrolysis. Pseudo first-order rate constants (k) were estimated by non-linear regression analysis of the amount of activated FX (FXa) upon time (t) using Equation 1,

\[
FXa_{t} = FXa_{0} + FXa_{eq} \left(1 - e^{-kt}\right)
\]

(Eq. 1) in which FXa0 represents the initial FXa concentration (normally zero), k is the rate of FXa formation after full activation. The k/Km values of FXa activation were deduced from the ratio of k to the activator concentration (RVV-X, FVIIa, or FXa).

Large quantities of FXa and derivatives were prepared by passage through a 5-ml HiTrap N-hydroxysuccinimide-activated column (Amersham Biosciences, Orsay, France) coupled to RVV-X. Following activation the active site concentration was determined by titration with H-α-Phe-Arg-Arg-CH2Cl as described previously (15). Before each kinetic determination, the concentration of FXa and derivatives was systematically controlled by measuring the rate of 100 μM S2222 hydrolysis.

**Prothrombin Activation Kinetics**—Prothrombin activation by FXa or derivatives was examined by progress curve kinetics of a coupled reaction in which FXa or derivatives (with or without FVa) were added to a mixture of prothrombin (12 nM to 1.5 μM) and S2238 (100 μM). Reactions were performed at 25°C in kinetic buffer containing 16 μM phospholipid vesicles and were started by the addition of 0.2 nM FXa alone or 0.1 μM FXa plus catalytic amounts of FVa. The rationale behind this approach is that because 100 μM S2238 is only saturating, the rate of the second reaction (pNA release from S2238) is a direct function of thrombin concentration. On the other hand, as long as the initial prothrombin concentration is >10-fold its Km for the activator, activation is steady-state, and thrombin increases linearly with time. Thus, if the thrombin concentration steadily increases, the rate of pNA release steadily increases (i.e., accelerates). As long as <10% of both substrates are consumed, acceleration remains constant and is proportional to the rate of the first reaction. Practically, the increase in pNA with time (t) was monitored, and acceleration coefficients (α) were estimated by fitting data corresponding to <10% prothrombin and S2238 consumption to Equation 2 (24, 25),

\[
pNA_{t} = 0.5a^{2} + \beta + pNA_{0}
\]

(Eq. 2) in which pNA0 represents the initial concentration of pNA (normally zero), β is the rate of pNA release independent of thrombin (also derived from the rate of prothrombin formation), and a is a function of the Km of the activator concentration (FVa or FXa) and of prothrombin for its activator. For any given concentration of S2238 and prothrombin, a is directly proportional to the activator concentration (FVa alone or in complex with FXa). Under the conditions used, the rate of prothrombin formation by FXa alone represented <1% of that catalyzed by FVa FXa and was neglected in the presence of FVa. The FVaFXa concentration was estimated using Equation 3 (19),

\[
FVaFXa = (K_{p} + FVa + FXa) - (K_{p} + FVa + FXa)^{2} - (4FVaFXa)^{2}/2
\]

(Eq. 3) in which Kp represents the dissociation constant of FVa + FXa, and FVa and FXa are the total concentrations of FVa and FXa, respectively. The Kp values were estimated by varying the concentration of FVa at a fixed prothrombin concentration, and the Kp and K′ values of prothrombin activation were estimated by varying the concentration of prothrombin at a fixed FVa concentration.

**Inhibition Kinetics of FXa and Derivatives**—The “active” concentration of antithrombin (in the presence of 1 unit/ml heparin) and that of

**TABLE I**

| Cation-binding Exosite of FX |
|-----------------------------|
| Mutated residues are shown in boldface. Numbering is based on homologous sequence in human prothrombin (20, 21). Following these modifications, the vector was used as a template to prepare vectors for variants E36Q/E37Q/E39Q, E36K/E37K/E39K, E74Q/E76Q/E77Q, and E74K/E76K/E77K of FX (Table I). Possible superimposition of 3D-structures of FX and derivatives were observed for the three FX Loop 34

\[
\text{Disintegrin loop } 34 \text{ in } \alpha_{v} \beta_{3} \text{ integrin}
\]

\[
\text{Disintegrin loop } 34 \text{ in } \alpha_{v} \beta_{3} \text{ integrin}
\]
PAI-1 were estimated as described previously (18, 18, 26) by titration by progress curve kinetics, except that kinetic buffer contained 5 mM EDTA instead of CaCl₂. The reaction was initiated by the addition of 20%—500 pM FXa or derivatives such that significant inhibition occurred over the time period of the experiment (<1 h). For inhibition by antithrombin, the reaction was performed in the presence of 5 μg/ml Polybrene (Sigma) to neutralize any trace of heparin. Reactions were performed at 25 °C in kinetic buffer and initiated by the addition of 200—500 pM FXa (or derivative) such that <10% of the substrate (S2222, 100 μM) was hydrolyzed over the time period of the experiment. The observed rate constants of inhibition (kobs) were estimated by fitting the data to Equation 4 for slow binding inhibition (27),

\[
A_{\text{cat}} = v_f + (v_i - v_f)(1 - \exp(-k_{\text{obs}}/A_{\text{cat}}))
\]

where \(A_{\text{cat}}\) and \(A_{\text{uncat}}\) represent \(A_{\text{cat}}\) initially (before the addition of FXa) and at time \(t\), respectively, and \(v_f\) and \(v_i\) are the initial and final (normally zero with serpins) rates of S2222 hydrolysis, respectively. The \(k_i\) was deduced from \(k_{\text{obs}}\) using Equation 5 to account for the competition introduced by the substrate,

\[
h_a = k_a(1 + S2222/K_a)\chi_0
\]

where S2222 is the initial S2222 concentration, \(K_a\) is its Michaelis constant for FXa (or derivatives), and \(I_0\) is the active concentration of added serpin. The k_i of TFPI for FXa (or derivatives) was also estimated by progress curve kinetics, except that kinetic buffer contained 5 mM EDTA instead of CaCl₂. The reaction was initiated by the addition of 200 μM FXa or derivatives to a mixture of 2—150 nM TFPI and 100 μM S2222. The \(k_i\) values were estimated using Equations 4 and 5 in which the initial concentration of TFPI was substituted for \(I_0\).

The polysaccharide-catalyzed association rate constant (\(k_{\text{cat}}\)) of activated antithrombin for FXa (or derivatives) was also estimated by analysis of the data from progress curve kinetics completed under pseudo first-order conditions. Reactions were started by the addition of 250 pM FXa to a mixture of antithrombin (2.5—10 nM), 100 μM S2765 (used instead of S2222 to slow down the reaction by competition), and a limiting amount of heparin (5–25 nM) or a saturating amount of pentasaccharide (150 nM). With a saturating amount of pentasaccharide, \(h_{\text{cat}}\) was estimated using Equations 4 and 5 in which \(h_{\text{cat}}\) was substituted for \(h_a\) and the total (saturated) antithrombin concentration was substituted for \(I_0\). In the presence of saturating heparin, the reaction was too fast to allow accurate analysis even with recordings as frequent as every second. Thus, inhibition reactions were performed in the presence of a catalytic amount of heparin, and \(h_{\text{cat}}\) and \(h_{\text{uncat}}\) were estimated using Equation 6 (28),

\[
k_{\text{obs}} = k_{\text{cat}}\text{AT}_{\text{free}} + k_{\text{uncat}}\text{AT}_{\text{PS}}\]

where \(\text{AT}_{\text{free}}\) and \(\text{AT}_{\text{PS}}\) represent the concentrations of free and polysaccharide-bound antithrombin, respectively, and \(k_{\text{uncat}}\) is the \(k_i\) in the absence of polysaccharide estimated as described above. The antithrombin-polysaccharide concentration was estimated using Equation 7 (which in which antithrombin-polysaccharide was substituted for FVaFXa, and the concentration of heparin was substituted for FVa, and that of antithrombin for FVb). The \(K_p\) of the antithrombin-polysaccharide complex was determined by intrinsic fluorescence measurement as described by Olson et al. (28).

### Table II

| FX variant | Oligonucleotides (5’ → 3’) |
|------------|--------------------------|
| E36Q/E37Q/E39Q(a) | 5’-GGGACCGGAACACGAGGCGTCGTTTGGTGTA-3’ |
| E36Q/E37Q/E39Q(b) | 5’-GGGACCGGAACACGAGGCGTCGTTTGGTGTA-3’ |
| E36Q/E37Q/E39K(a) | 5’-GGGACCGGAACACGAGGCGTCGTTTGGTGTA-3’ |
| E74Q/E76Q/E77Q(a) | 5’-GGGACCGGAACACGAGGCGTCGTTTGGTGTA-3’ |

### RESULTS AND DISCUSSION

**Potential Exosite-1 of FX and Zymogen Activation—**Human FX exposes three acidic residues in loop 34—40 (Glu80, Glu76, and Glu77) and five in loop 70–80 (Asp70, Glu4, Glu1, Glu71, and Glu80), with Asp70 and Glu80 being involved in calcium binding (31). In the folded protease, loops 34—40 and 70–80 are adjacent and constitute a negatively charged cluster, which is conserved in human, bovine, rat, mouse, and chicken FX. Considering that the topologically equivalent region in thrombin is of opposite charge and constitutes exosite-1, which...
is critical for most functions, we wondered if the negative cluster of FX constitutes a secondary binding site (Fig. 1). To investigate this, we prepared a series of mutants in which sets of three glutamates were replaced with glutamine or lysine, viz. E36Q/E37Q/E39Q, E36K/E37K/E39K, E74Q/E76Q/E77Q, and E74K/E76K/E77K (Table I). Glu36, Glu37, and Glu39 of loop 30–40 were replaced with glutamine in E36Q/E37Q/E39Q and with lysine in E36K/E37K/E39K. Similarly, Glu 74, Glu76, and Glu77 of loop 70–80 were replaced with glutamine in E74Q/E76Q/E77Q and with lysine in E74K/E76K/E77K. Wild-type FX and its derivatives were expressed in BHK-21 cells and purified to homogeneity by affinity chromatography.

To be functional, an exosite evidently requires a complementary motif to exist on the macromolecular substrate, inhibitor, or cofactor (32, 33). Because FX is initially a zymogen, it constitutes the substrate during its activation to FXa and thus may display complementary motif(s) to activator(s). In our search for a role for the potential exosite-1 of FX, we first determined the susceptibility of FX mutants to activation by RVV-X, TF-FVIIa, and FVIIa-FIXa (Fig. 2). FX and derivatives were activated by RVV-X with comparable kcat/Km values (Table III), thus excluding the potential exosite-1 of FX as a player in this reaction. Undoubtedly, secondary binding site(s) are implicated in FX activation by TF-FVIIa because uncleavable FX (with glutamine substituted for the arginine of the scissile bond) has unchanged affinity for TF-FVIIa (34). The impact of the FX mutations on activation by TF-FVIIa was, however,
Cation-binding Exosite of FX

The kinetic constants ($k_{\text{cat}}$ and $K_m$) of prothrombin activation were estimated by nonlinear regression analysis of the dependence of the acceleration of S2238 hydrolysis by increasing thrombin concentrations (estimated as described in the legend to Fig. 3) upon initial prothrombin concentration using the Michaelis-Menten equation and are given together with their S.E. Measuring accelerations amplified experimental uncertainties, with the result that final S.E. values were relatively high (up to 60% for the $K_m$ values).

| FVa | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}$ | $K_m$ |
|-----|--------------|------|--------------|------|
| Wild-type | (6.5 ± 0.3) × 10^{-3} | 306 ± 42 | 5.7 ± 0.8 | 86 ± 40 |
| E36Q/E37Q/E39Q | (7.4 ± 0.9) × 10^{-4} | 293 ± 96 | 0.6 ± 0.09 | 57 ± 29 |
| E36K/E37K/E39K | (6.9 ± 0.4) × 10^{-4} | 299 ± 47 | 0.6 ± 0.04 | 45 ± 9 |
| E74Q/E76Q/E77Q | (4.5 ± 0.2) × 10^{-3} | 181 ± 22 | 10.5 ± 2.1 | 129 ± 77 |
| E74K/E76K/E77K | (5.3 ± 0.4) × 10^{-3} | 519 ± 104 | 7.3 ± 1.4 | 111 ± 64 |

Prothrombin activation by prothrombinase was performed with a saturating concentration of FVa (250 pm); prothrombinase concentration was calculated according to the estimated $K_m$ of each FVa variant for FVa (Fig. 4).

TABLE VI

Kinetics of fluorescence-quenched substrate cleavage by FVa or derivatives

The kinetic constants ($k_{\text{cat}}/K_m$) for substrate hydrolysis were estimated by nonlinear regression analysis of the increase in fluorescence upon time using a pseudo first-order equation as described previously (15) and are given together with their S.E. The amino acid specific to each substrate is shown in boldface. ABz, ortho-aminobenzoyle; EDDnp, N-(2,4-dinitrophenyl)ethylenediamine.

| Substrate | $k_{\text{cat}}/K_m$ | FVa | E36Q/E37Q/E39Q | E36K/E37K/E39K |
|-----------|---------------------|-----|----------------|----------------|
| ABz-VQFR-TLGDQ-EDDnp | (1.5 ± 0.06) × 10^{-4} | (1.9 ± 0.09) × 10^{-4} | (2.3 ± 0.12) × 10^{-4} |
| ABz-VQFR-ILGDQ-EDDnp | (3.6 ± 0.21) × 10^{-4} | (7.6 ± 0.40) × 10^{-3} | (7.6 ± 0.34) × 10^{-3} |
| ABz-VQFR-SAGDQ-EDDnp | (1.2 ± 0.05) × 10^{-4} | (1.9 ± 0.06) × 10^{-4} | (2.2 ± 0.09) × 10^{-4} |
| ABz-VQFR-SVGDQ-EDDnp | (1.5 ± 0.07) × 10^{-4} | (1.7 ± 0.06) × 10^{-4} | (1.9 ± 0.09) × 10^{-4} |
| ABz-VQFR-SLTDQ-EDDnp | (3.0 ± 0.13) × 10^{-4} | (2.7 ± 0.13) × 10^{-4} | (2.6 ± 0.10) × 10^{-4} |
| ABz-VQFR-SLEDQ-EDDnp | (1.7 ± 0.03) × 10^{-4} | (2.1 ± 0.05) × 10^{-4} | (3.6 ± 0.09) × 10^{-4} |

Clearly, our results suggest that the potential exosite-1 of FX is nonessential for its activation by RVV-X, TF:FVIIa, or FVIIa:FVa. The contribution of loops 34–40 and 70–80 of FIX to its activation by TF:FVIIa has not been studied in detail, but its calcium-binding site (within segment 70–80) is not involved in the reaction (35). In contrast, positive charges within proexosite-1 of prothrombin are essential for its activation by prothrombinase (36, 37), whereas E39K prothrombin is normally activated (17). In addition, proexosite-1-specific ligands such as the sulfated C-terminal tail of hirudin block prothrombin activation by prothrombinase (13, 38).

Role of Loop 34–40 of FVa in Prothrombin Activation—We next considered whether the potential exosite-1 of FX contributes to enzyme catalysis. FX variants were activated in large quantities by RVV-X; their active site content was assessed by $H_{\text{o}}$-Phe-Phe-Arg-CH$_2$Cl titration; and their amidolytic activity was characterized. The $k_{\text{cat}}$ and $K_m$ values of S2222 and S2765 were comparable for FVa, E36Q/E37Q/E39Q, and E36K/E37K/E39K, but were slightly altered (<2.5-fold) for E74Q/E76Q/E77Q and E74K/E76K/E77K (Table IV). Because the pNA substrates do not have a C-terminal extension that could directly interact with the potential exosite-1 of FX, this was perhaps not surprising. The small changes observed could reflect an allosteric coupling with the active site of FVa, which could result from an alteration of the electrostatic potential of FXs, and/or could originate from a newly created, nonproductive, low affinity binding site for pNA substrates such as the one described in thrombin (39).

A number of studies have established that the specific cleavage of the two peptide bonds in prothrombin by prothrombinase is governed by interactions remote from the catalytic groove (13, 40). To explore if the potential cation-binding exosite-1 of FXs is important in prothrombin activation, we recorded progress curves of pNA release and evaluated the acceleration of S2238 hydrolysis due to increasing thrombin concentrations (Fig. 3). Initial velocities of thrombin production at various
initial prothrombin concentrations were deduced from the acceleration coefficients to estimate $K_{\text{cat}}$ and $K_m$ values for each FXa variant. Kinetic analyses were performed in the presence of phospholipids and calcium with or without FVa (Table V). Overall, the $K_m$ of prothrombin was little affected (changed $\leq 2$-fold) whether or not FVa was present, ruling out the possibility that FXa utilized its potential exosite-1 to capture thrombin. The loop 70–80 mutations possibly increased the $k_{\text{cat}}$ of the reaction, whereas rates were severely impaired by the loop 34–40 substitutions; with or without FVa, the $k_{\text{cat}}$ values decreased by $\leq 10$-fold for E36Q/E37Q/E39Q and E36K/E37K/E39K (Table V). A profound alteration of the FXa catalytic groove is unlikely to be the cause of the generalized defect in prothrombin activation because the amidolytic activities of the loop 34–40 mutants were normal. To explore the possible influence of the mutations on the prime side of the scissile bond, we measured the hydrolysis rate of fluorescence-quenched substrates with representative $P_1^\text{cat}$, $P_2^\text{cat}$, and $P_3^\text{cat}$ residues (15). The catalysis of these substrates was very little affected by FVa, the rate of cleavage of the fluorescence-quenched peptides by the variants was increased or decreased by 2-fold at most (Table VI). In the presence of FVa, the defect in prothrombin activation by the loop 34–40 mutations could originate from a decreased affinity for the cofactor. We estimated the $K_p$ values of the FXa variants for FVa by measuring the rate of thrombin production with increasing FVa concentrations, but the mutations had only limited impact: values of $0.3 \pm 0.1$, $0.6 \pm 0.2$, and $0.7 \pm 0.4$ nM were observed for FXa, E36Q/E37Q/E39Q, and E36K/E37K/E39K, respectively (Fig. 4).

Overall, the defect in prothrombin activation of the loop 34–40 mutants resulted in deficient hydrolysis of prothrombin that was essentially independent of FVa and prothrombin binding and did not involve a major remodeling of the catalytic groove of FXa. Thus, the defect would result from a conformation that prevents the scissile bond from obtaining the optimal geometry for turnover. Consistent with this picture, mutations D70K and E80K in FXa moderately affect prothrombin activation (41); peptide 58–97 of FXa (which binds CaCl$_2$ with high affinity) does not compete with FXa for FVa binding (31); and a monoclonal antibody directed against nearby residues 82–91 and 102–116 blocks prothrombin activation without affecting prothrombinase assembly (14). In addition, FVa itself does not seem to induce major allosteric remodeling of the catalytic groove of FXa (15, 42). The core FVa-binding region of FXa is localized within the positively charged region of FXa, comprising Arg$_{125}$, Arg$_{165}$, Lys$_{169}$, and Lys$_{230}$ within the region topologically equivalent to exosite-2 of thrombin, diametrically opposed to loop 34–40 (11, 43–46). In this model, the potential cation-binding exosite-1 of FXa would remain, within prothrombinase, exposed to the solvent and therefore accessible, conceivably to interact with the anion-binding proexosite-1 of thrombin.

### TABLE VII

| FXa                        | $k_{\text{cat}}$ for antithrombin | $k_{\text{cat}}$ for heparin-activated antithrombin | $k_{\text{cat}}$ for pentasaccharide-activated antithrombin | $k_{\text{cat}}$ for TFPI |
|----------------------------|----------------------------------|----------------------------------------------------|----------------------------------------------------------|--------------------------|
| Wild-type                  | $(3.2 \pm 0.11) \times 10^7$     | $(2.3 \pm 0.13) \times 10^7$                      | $(4.4 \pm 0.04) \times 10^7$                            | $(1.7 \pm 0.08) \times 10^7$ |
| E36Q/E37Q/E39Q             | $(2.7 \pm 0.12) \times 10^7$     | $(3.5 \pm 0.41) \times 10^7$                      | $(2.5 \pm 0.09) \times 10^7$                            | $(7.9 \pm 0.88) \times 10^7$ |
| E36K/E37K/E39K             | $(1.1 \pm 0.02) \times 10^7$     | $(1.1 \pm 0.09) \times 10^7$                      | $(2.8 \pm 0.21) \times 10^7$                            | $(1.2 \pm 0.09) \times 10^7$ |
| E74Q/E76Q/E77Q             | $(1.4 \pm 0.05) \times 10^7$     | $(1.7 \pm 0.05) \times 10^7$                      | $(1.5 \pm 0.40) \times 10^7$                            | $(7.8 \pm 0.53) \times 10^7$ |
| E74K/E76K/E77K             | $(1.2 \pm 0.03) \times 10^7$     | $(1.3 \pm 0.07) \times 10^7$                      | $(1.7 \pm 0.19) \times 10^7$                            | $(4.9 \pm 0.58) \times 10^7$ |

1. Inhibition by heparin-activated antithrombin was analyzed using Equation 6 to estimate $k_{\text{cat}}$; free and bound antithrombin concentrations were estimated using Equation 3.
2. Inhibition by pentasaccharide-activated antithrombin (with a saturating amount of pentasaccharide) was estimated using Equations 4 and 5 in which $k_{\text{cat}}$ was substituted for $k_{\text{cat}}$, and $v_0$ was set to zero.
3. Inhibition by TFPI was analyzed using Equations 4 and 5.

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### Potential Exosite-1 of FXa and Interaction with Unactivated Antithrombin

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**Potential Exosite-1 of FXa and Interaction with Unactivated Antithrombin**—Despite being its main inhibitor in plasma, the reactivity of antithrombin for FXa depends only weakly on the sequence of its reactive site loop, suggesting that one or more exosite(s) govern the interaction (10). Without polysaccharide, FXa and E36Q/E37Q/E39Q were inhibited by antithrombin with comparable $k_{\text{cat}}$ values; E36K/E37K/E39K, E74Q/E76Q/E77Q, and E74K/E76K/E77K had $k_{\text{cat}}$ values 2–3-fold lower (Table VII). Thus, the potential exosite-1 of FXa appears to be only moderately involved in the interaction with antithrombin alone. The same is true for thrombin: mutations in segment 70–80 do not impede inhibition by antithrombin (47), and the E39K mutant is inhibited with a $k_{\text{cat}}$ value comparable with that with thrombin (17). A direct participation of segment 70–80 is also unlikely because it is positively charged in the anticoagulant activated protein C (as in thrombin), which is resistant to antithrombin inhibition (contrary to thrombin). Conversely, direct participation of the charged loop 34–40 is unlikely because it is either neutral or negatively charged in thrombin, FXa, and FIXa (three targets of antithrombin). As a consequence, our observation renders it improbable that the positively charged loop 34–40 of activated protein C is one of the locks preventing its inhibition by antithrombin.

**Role of Loop 34–40 of FXa in the Interaction with Heparin-activated Antithrombin**—Heparin enhances the rate of FXa inhibition by antithrombin by $\approx 1000$-fold (28). Structural data suggest that residues of the reactive site loop of antithrombin have a non-optimal conformation for interacting with FXa, but that binding of heparin to antithrombin alters this conformation and/or frees the loop, allowing it to fit into the catalytic groove of the target (48). In addition, heparin chains that are long enough enhance interaction with thrombin and FXa through a template mechanism (49). Binding of a specific pentasaccharide to antithrombin effectively accelerates FXa inhibition, but is ineffective in enhancing thrombin inhibition. The pentasaccharide therefore allows a distinction between effects due to antithrombin remodeling compared with heparin bridging. Kinetic data also suggest that a region outside the reactive site loop (i.e. an exosite) may primarily cause the enhanced potency of pentasaccharide-activated antithrombin for FXa (50). That the pentasaccharide enhancement is detectable with FXa and not with thrombin implies that a complementary exosite exists on FXa that is absent on thrombin. Specifically,
a striking difference between thrombin and FXa is exosite-1: positively charged in thrombin and negatively charged in FXa. Compared with FXa, the \( k_{\text{cat}} \) for inhibition of the FXa derivatives by pentasaccharide-activated antithrombin decreased by 3-fold (Table VII). Thus, as with unactivated antithrombin, the potential exosite-1 of FXa appears to be unrelated to the mechanisms of inhibition by antithrombin: the potential exosite-1 of FXa is somewhat dispensable. Consistent with this conclusion, mutations D70K and E80K in FXa have little consequence on the potency of heparin-activated antithrombin (41), whereas E37Q or E39Q affects it only moderately (52). In FIXa, loop 34–40 seems to be more important: a FIXa variant carrying loop 34–40 of FX is virtually insensitive to antithrombin inhibition, although heparin restores, in part, the ability of the serpin to neutralize the chimera (7).

Overall, the striking difference between exosite-1 of FXa and thrombin appears to be unrelated to the mechanisms of inhibition by antithrombin: the potential exosite-1 of FXa is somewhat dispensable. Consistent with this conclusion, mutations D70K and E80K in FXa have little consequence on the potency of heparin-activated antithrombin (41), whereas E37Q or E39Q affects it only moderately (52). In FIXa, loop 34–40 seems to be more important: a FIXa variant carrying loop 34–40 of FX is virtually insensitive to antithrombin inhibition, although heparin restores, in part, the ability of the serpin to neutralize the chimera (7).

Role of Loop 34–40 of FXa in TFPI Binding and in Preventing Inhibition by PAI-1—TFPI is a more effective inhibitor of FXa compared with unactivated antithrombin, but a low

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**FIG. 5.** Affinity for heparin of FX and derivatives. FX or derivatives were loaded onto a 7.5 x 75-mm heparin-5PW TSK column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl and 5 mM calcium, and the column was developed by increasing the salt concentration (dashed line, right scale). The graph represents the elution profiles of wild-type FX (●), E36Q/E37Q/E39Q (○), and E36K/E37K/E39K (▲). FX variants E74Q/E76Q/E77Q and E74K/E76K/E77K eluted essentially as did FX. Compared with their zymogens, all activated FX and derivatives eluted from the heparin-5PW TSK column at ~50 and 80 mM higher NaCl concentrations in two overlapping peaks that probably corresponded to the α and β forms, respectively.

**FIG. 6.** Inhibition of E36K/E37K/E39K by PAI-1. The graph represents progress curves of inhibition of 0.25 nM E36K/E37K/E39K in the presence of zero (●), 11 (□), and 22 (▲) mM PAI-1. Under the same conditions, inhibition of FXa and variants was undetectable. Curves were obtained by nonlinear regression analysis using Equation 4 in which \( v \) was set to zero, yielding an apparent rate of inactivation that was used to calculate the value of \( k_a \) according to Equation 5.

**FIG. 7.** Half-lives of FXa, E36Q/E37Q/E39Q, and E36K/E37K/E39K in plasma. The graph represents progress curves of the remaining FXa activity (percent of the initial maximum) of 50 nM FXa (●), E36K/E37K/E39K (○), and E36Q/E37Q/E39Q (□), and E74Q/E76Q/E77Q (▲), and E74K/E76K/E77K (◆) added to non-clottable plasma. Solid lines were obtained by nonlinear regression analysis using Equation 7 to yield an observed rate constant, allowing calculation of the half-life. FXa activity plateaued to ~25% of the initial activity, and this inhibition-resistant activity remained stable for at least 1 h (without added FXa, S2222 hydrolysis was undetectable).

**FIG. 8.** Ability of FX and derivatives to promote thrombin formation in FX-deficient plasma. The graph represents the rates of S2238 hydrolysis (directly proportional to thrombin concentration) as a function of time after the addition of 80 nM FX (●), E36Q/E37Q/E39Q (○), E36K/E37K/E39K (▲), E74Q/E76Q/E77Q (◆), or E74K/E76K/E77K (◆) to FX-depleted plasma containing 8 mM Gly-Pro-Arg-Pro-amide (to prevent fibrin polymerization), 80 µM phospholipids, 20 mM calcium, and 250 pM TF. Without the addition of exogenous FX, no thrombin formation could be detected (◆).
plasma concentration limits its impact. Molecular modeling suggests that electrostatic fields drive, in part, the interaction of FXa with TFPI (8, 9). Basic groups of TFPI would secure a charge region in the distal part of the reactive site loop to confirm its role (53). Compared with FXa, the kₜ values for E36Q/E37Q/E39Q, E36K/E37K/E39K, E74Q/E76Q/E77Q, and E74K/E76K/E77K inhibition by TFPI decreased 11-, 6-, 3-, and 3-fold, respectively (Table VII). Thus, loop 34–40 of FXa appears to be important for interacting with TFPI, whereas the potential of the loop 70–80 mutations is limited. Consistent with this observation, mutations D70K and E80K in FXa have little effect on sensitivity to TFPI (41).

The serpin PAI-1 is unable to inhibit FXa, its natural target being tissue-type plasminogen activator (t-PA). In contrast to FXa, loop 34–40 of t-PA comprises three basic residues, viz. Arg²⁷⁸, Arg²⁸¹, and Arg²⁸⁹, which interact with a negatively charged region in the distal part of the reactive site loop of PAI-1 (54, 55). It was therefore of interest to examine charged region in the distal part of the reactive site loop of the potential exosite-1 of FX played only a critical role in the interaction with TFPI, in preventing PAI-1 binding, and possibly in tempering inhibition by antithrombin. Finally, the results suggest that the electrostatic potential of FX may influence its procoagulant properties. However, mutations within the potential exosite-1 of FX do not result in the major outcome that would be expected for a crucial exosite such as exosite-1 of thrombin, in which a single amino acid change can result in almost complete loss of function (2).

**General Conclusion**—Mutations within loops 34–40 and 70–80 of FX induced subtle modulations of FXa activity, with possible opposing effects. Our results suggest that the potential exosite-1 of FX is unlikely to constitute a critical exosite for FX activation. Nevertheless, our study implicates loop 34–40 of FXa in prothrombin activation, albeit excluding it from FVa binding: loop 34–40 also seems to be important in the interaction with TFPI, in preventing PAI-1 binding, and possibly in tempering inhibition by antithrombin. Finally, the results suggest that the electrostatic potential of FX may influence its procoagulant properties. However, mutations within the potential exosite-1 of FX do not result in the major outcome that would be expected for a crucial exosite such as exosite-1 of thrombin, in which a single amino acid change can result in almost complete loss of function (2).
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The Elusive Role of the Potential Factor X Cation-binding Exosite-1 in Substrate and Inhibitor Interactions
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