Changing Residue 338 in Human Factor IX from Arginine to Alanine Causes an Increase in Catalytic Activity*

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This study was designed to identify functionally important factor IX (FIX) residues. Using recombinant techniques and cell culture, we produced a mutant FIX with arginine at 338 changed to alanine (R338A-FIX). This molecule had approximately 3 times greater clotting activity than that of wild type FIX (wt-FIX) in the activated partial thromboplastin assay. R338A-FIX reacted normally with a panel of three FIX specific monoclonal antibodies and migrated on sodium dodecyl sulfate-polyacrylamide gels indistinguishably from wt-FIX. Using functional assays, we determined that R338A-FIX's $K_m$ for factor VIIIa (FVIIIa) was similar to that of wt-FIXa. Our kinetic analysis, using factor X as substrate, indicated that the mutation's major effects were a 3-fold increase in $k_{cat}$ and a 2-fold decrease in $K_m$ both manifested only in the presence of FVIIIa. R338A-FIXa's increased catalytic efficiency did not result from ablation of a thrombin sensitive site, reported to occur at arginine 338, since in our assays the thrombin inhibitor, hirudin, had no effect on activity of either wt-FIXa or R338A-FIXa. R338A-FIXa and wt-FIXa had equal activity, with or without FVIIIa, toward the synthetic substrate, methylsulfonyl-D-cyclohexylglycyl-arginine-p-nitroanilide. Interestingly, R338A-FIXa had reduced affinity for heparin. Therefore, we propose that R338A-FIXa's increased activity is not due to an allosteric effect on the active site, but that the Arg-338 residue is part of an exosite that binds both factor X and the mucopolysaccharide, heparin.

Factor IX (FIX), which plays a key role in both the intrinsic and extrinsic coagulation pathway, circulates as a 415-amino acid, single-chain plasma zymogen. The zymogen of FIX is activated by FXa or by the tissue factor-FVIIa complex. Specific cleavages between Arg-145 and Ala-146 and between Arg-180 and Val-181 result in a light chain and heavy chain linked by a single disulfide bond between cysteine 132 and cysteine 289 (4). The structural organization of factor IX is similar to that of the vitamin K-dependent blood clotting proteins FVII, FX, and protein C (2). The approximately 45 amino acids of the amino terminus comprise the $\gamma$-carboxyglutamic acid, or Gla, domain. This is followed by two epidermal growth factor homology domains, an activation peptide and the catalytic “heavy chain,” which is a member of the serine protease family (3, 5, 6).

The major physiological function of factor IXa in the blood coagulation cascade is to convert FX to FXa in a process that requires the presence of phospholipid surface, calcium ions, and FIXa's protein cofactor, FVIIIa. FIXa alone is an extremely poor protease, but when bound to FVIIIa to form the "intrinsic FXase" complex it becomes a potent FX activator (7, 8). It has been proposed that, upon binding FVIIIa, FIXa may undergo a conformational change at or near the active site (9). The importance of FIX is exemplified by the fact that patients with defective FIX molecules suffer from hemophilia B, an X-linked, recessive bleeding disorder that is clinically indistinguishable from hemophilia A in patients who have no FVIII function.

Using recombinant techniques, we created mutant FIX molecules designed to locate the residues of FIXa that bind FVIIIa. We have found one mutation, R338A-FIX, whose clotting activity is 2.5–3 times that of wild type FIX. In an attempt to understand the causes contributing to the increased clotting activity of R338A-FIX, we have determined several functional parameters. Our results demonstrate that the increased clotting activity of FVIIIa-dependent and is due to an increased $k_{cat}$ and decreased $K_m$ for FX, the substrate for the FIXa:FVIIIa complex.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides for in vitro mutagenesis were purchased from Life Technologies, Inc. T7 DNA polymerase, T4 DNA ligase and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Sequenase 7-deaza-dGTP DNA sequencing kits were purchased from U. S. Biochemical Corp. The anti-human FIX monoclonal antibodies used in this study were A-1, A-5, and A-7, a gift from Dr. Kenneth J. Smith. Purified normal human plasma FIX, FX, Fxa, FXa, and thrombin were purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Coagulation control level 1, FIX-deficient plasma, FVIII-deficient plasma, aPTT reagent, and standard heparin were obtained from Sigma. Purified normal human plasma FVIII was a gift from Roher Biotechnology Inc. (Springfield, VA). Phosphatidylserine and phosphatidylcholine were purchased from Avanti Polar Lipids (Alabaster, AL). Spectrozyme-FIXa (methylsulfonyl-D-cyclohexylglycyl-arginine-p-nitroanilide) and Spectrozyme-FXa (methoxyoxycarbonyl-N-cyclohexylglycyl-glycyl-arginine-p-nitroanilide) were obtained from American Diagnostica Inc. (Greenwich, CT). Hirudin was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). All other reagents were of the highest purity available.

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Characterization of Mutant FIX with Greater Clotting Activity

Methods

In Vitro Mutagenesis and Construction of the Expression Plasmid—Site-directed mutagenesis was performed as described by Kunkel (10). The entire cDNA sequence was determined to ensure that the mutation was correct and that no inadvertent mutations had been introduced. Expression and Purification of Recombinant Proteins—Human embryonic kidney 293 cells were grown in a mixture of Dulbecco's modified Eagle's medium and F-12 medium, supplemented with 10% fetal calf serum. FIX and its mutants, in the vector pCMV5, a derivative of pCMV mamalian expression vector (11), were cotransfected into cells with pSV2 neo selection marker, using the calcium phosphate coprecipitation method (12). G418-resistant cell clones were subcloned and expanded. The supernatants from each clone were collected and assayed for FIX expression using a radioimmunometric assay with FIX antibodies. Stable clones with high expression levels of FIX were transferred into 850-cm² roller bottles for protein production in the serum-free Dulbecco's modified Eagle's medium/F-12 medium supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin, 5 µg/ml vitamin K, and 10 mg/liter insulin-transferrin-selenium selenite. The supernatants were collected every 4 h for 10 days and stored at –20 °C. For purification, the frozen supernatants were thawed and EDTA and benzamidine-HCl were added (final concentration of 4 mM and 5 mM, respectively). The supernatants were filtered through a 0.45 µm Millipore filter to remove cell debris and incubated at 4 °C with overnight shaking in Q Sepharose Fast Flow resin (2.5 ml of resin/liter of supernatant). The resin was pre-equilibrated in 20 mM Tris-HCl, 15 mM NaCl, 2 mM cation, the frozen supernatants were thawed and EDTA and benzamidine-HCl were added (final concentration of 4 mM and 5 mM, respectively). The supernatants were filtered through a 0.45 µm Millipore filter to remove cell debris and incubated at 4 °C with overnight shaking in Q Sepharose Fast Flow resin (2.5 ml of resin/liter of supernatant). The resin was pre-equilibrated in 20 mM Tris-HCl, 15 mM NaCl, 2 mM benzamidine-HCl, pH 7.4 (equilibrium buffer). The resin was then loaded in a column, washed with equilibration buffer containing 2 mM EDTA for 4 h, and washed for another 1 h with equilibration buffer without EDTA. FIX was eluted from the column with a 0–60 mM calcium gradient, concentrated in a Centriprep-30, and stored at –80 °C.

Radioimmunometric Assay—Three monoclonal antibodies (A1, A5, and A7) were used in the assay. A5 was employed at 10 µg/ml in 50 mM NaHCO₃ (pH 8.5) to coat a 96-well microtiter plate. Samples were added to the antibody-coated wells. After incubation at 4 °C overnight, the second antibody, A1 or A7, labeled with 125I by IODOBEAAs according to the manufacturer’s instructions (Pierce) was added at about 1 × 10⁵ cpm/well. Unbound radioactive antibody was washed with buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, and 1 mM MgCl₂, pH 7.5) after a 4-h incubation, and the bound radioactive content of each well was measured. The standard curve was obtained using plasma FIX.

Analysis of Recombinant FIXs—SDS-PAGE was performed as described by Laemmli (13). The total protein/well used on SDS-PAGE was about 1 µg. Following electrophoresis, the purified proteins were visualized by silver staining.

Clotting Assay—One-stage activated partial thromboplastin time (aPTT) assays were performed according to the manufacturer’s instructions (Sigma). For partial thromboplastin time (PTT) assays, the rate of generation of FIXa was determined on a microplate reader. The assay was performed by incubation of 25 µl of FIXa with 100–300 nM hirudin, and 5 mM calcium for an additional 5 min at 25 °C. The concentration of FIXa was estimated from its activity in a one-stage aPTT assay, assuming the concentration of FIXa in plasma to be 0.3 nM.

Kᵢ Determination for the FIXa-FIXa Interaction—Freshly prepared 0.4 nM FIXa (25 µl) containing 400 µM PSPC (15) (45% phosphatidylserine, 55% phosphatidylcholine) was incubated with 25 µl of FIXa varying from 0 to 20 nM for 5 min at 25 °C in HEPS buffer plus 5 µM calcium, to form FXase. 200 nM FX and 1 µM Spectrozyme-FXa in 50 µl were added, and the initial rate of FXa generation was determined on the microplate reader.

FX Activation by FIXa in the Presence of Phospholipid—wt-FIXa or mutant R338A-FIXa (50 µl, 50 nM) was incubated with 200 µM PSPC vesicles, 10 mM calcium, and 0.5 mM Spectrozyme-FXa in a microplate well for 5 min at 25 °C. Varying concentrations of FX (50 µl, between 0 and 400 nM) were added to this mixture, and the absorbance at 405 nm was measured for 30 min on the microplate reader. The final concentrations were 25 nM FXa, 100 µM PSPC, 0–200 nM FX, and 0.25 mM Spectrozyme FXa.

The Effect of Hirudin on FIXa-FIXa Binding—The experiments were performed as described for the assay of FIX binding to FIXa, except that in one group, 100 nM hirudin was added to completely inhibit thrombin activity; in another group, no hirudin was added.

Hydrolysis of Synthetic Substrate Spectrozyme-FXa—FX was activated using FIXa at a molar ratio 100:1 in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) with 5 mM CaCl₂ at 37 °C for 2 h or at a molar ratio 2000:1 in the same buffer at 37 °C for 4 h. The process of activation was followed by removing aliquots at specific time points. The reaction was stopped by adding SDS and 2-mercaptoethanol, after which the samples were immediately boiled for 5 min and run on SDS-PAGE. Completely activated samples, judged by SDS-PAGE and Western blot, were aliquoted and stored at –80 °C.

Antigenicity, γ-carboxyglutamic acid, and clotting activity of the mutant R338A and wild type factor IX were measured for 30 min on the microplate reader. The final concentrations were 50 nM FIXa and 0–4 nM Spectrozyme FXa.

The Effect of Hirudin on FIXa-FIXa Binding—The experiments were performed as described for the assay of FIX binding to FIXa, except that in one group, 100 nM hirudin was added to completely inhibit thrombin activity; in another group, no hirudin was added.

Electrophoretic Analysis of FIXa’s Effect on FIXa—Purified plasma wt-FIXa (2000 nM), FIXa (0.15 nM), FXa (300 nM), and calcium (5 mM) were mixed and incubated at 25 °C. At different time points, samples were removed, subjected to SDS-PAGE, and then silver-stained.

Hydrolysis of Synthetic Substrate Spectrozyme-FXa—FX was activated using FIXa at a molar ratio 2000:1 (FIXa-FIXa) and the concentrations of activated proteins were confirmed using antithrombin III titration (14). The assay was performed by incubation of 25 µl of FIXa with 100 nM wt-FIXa or R338A-FIXa in HEPES buffer plus 33% ethylene glycol and 5 mM calcium was added to a 96-well microplate. Substrate cleavage was initiated by the addition of another 50 µl of varying concentrations of Spectrozyme-FXa (0–8 mM) diluted in the same buffer, and the plate was immediately assayed by monitoring the absorbance at 405 nm. The final concentrations were 50 nM FIXa and 0–4 nM Spectrozyme FXa.
The Expression and Purification of Mutant R338A-FIX or Recombinant wt-FIX—The expression level of the carboxylated Fix varied from 500 to 1000 ng/10^6 cells/24 h. SDS-PAGE inhibitor. K_i is the dissociation constant for enzyme-substrate complex, and the dissociate constant of enzyme-inhibitor complex is K_{i1}. S is the concentration of substrate, and I is the concentration of inhibitor. The data were fit with KaleidaGraph.

RESULTS

The Expression of Mutant R338A-FIX or Recombinant wt-FIX—The expression level of the carboxylated FIX varied from 500 to 1000 ng/10^6 cells/24 h. SDS-PAGE

In the presence of FVIIIa, 5 µl of 120 nM wt-FIXa or R338A-FIXa was mixed with 35 µl of about 17 nM FVIIIa containing 142 µM PSPC. The mixture was incubated at room temperature for 5 min to form FXase. The final concentrations were 12 nM FIXa, 12 nM FVIIIa, 5 mM calcium, 100 µM PSPC, and 0–4 mM Spectrozyme-FIXa. The absorbance values at 405 nm were converted into molar concentration of product, using an extinction coefficient of 9.65 × 10^5 M^-1 cm^-1. Data were fit (nonlinear least squares) to the Michaelis-Menten equation using KaleidaGraph published by Synergy Software (PCS Inc.).

The Inhibition of wt-FIXa and R338A-FIXa by Heparin—wt-FIXa (8 nM, 25 µl) or R338A-FIXa was mixed with 25 µl of 1.6 nM FVIIIa in HEPES buffer with 5 mM calcium and 200 µM PSPC and incubated at 25 °C for 5 min to form FXase. Varying concentrations (12–10,800 nM, 25 µl) of heparin (assuming that the molecular weight of standarded heparin is about 15,000) in the same buffer plus 5 mM calcium was added and incubated for another 5 min. The initial rate of FX activation was determined by addition of 25 µl of a mixture containing of 800 nM FX, 2 mM Spectrozyme-FIXa, 5 mM calcium, and 1.0 mg/ml albumin.

Data Analysis—FIXa Activity—Absorbance of initial rate of FX activation mediated by FIXa was converted to nanomolar Xa/min by the following equation (16, 17).

Absorbance = at^2 + bt + c (Eq. 1)
a gives the rate at which FXa cleaves the chromogenic substrate or the rate at which FX is cleaved, b gives the amount of FIXa present in the zymogen FX, and c gives the amount of cleaved substrate at time 0.

FIXa Binding to FVIIIa—The K_d for FIXa binding to FVIIIa was determined using Equation 2, where it is assumed that the rate of FIXa cleavage is directly proportional to the FIXa:FVIIIa complex concentration.

\[ \frac{([\text{FIXa}]_0 + [\text{FVIIIa}]_0 + K_d) - \sqrt{([\text{FIXa}]_0 + [\text{FVIIIa}]_0 + K_d)^2 - 4([\text{FIXa}]_0([\text{FVIIIa}]_0)}}}{2} \]

(Eq. 2)

Inhibition of Intrinsic FXa by Heparin—The data from inhibition experiments were fit to Equation 3, described as partial noncompetitive inhibition by Segel (18).

\[ v = \frac{V_{max}[S]}{K_v + V_{max}[S][I]} \]

(Eq. 3)

v is the initial velocity of FX activation. V_{max} is the maximal velocity at a saturating concentration of FX. V_{max,i} is the V_{max} in the presence of inhibitor. K_i is the dissociation constant for enzyme-substrate complex, and the dissociate constant of enzyme-inhibitor complex is K_{i1}. S is the concentration of substrate, and I is the concentration of inhibitor. The data were fit with KaleidaGraph.

![Figure 2](image1.png)

**Fig. 2. Binding of R338A-FIXa and wt-FIXa to their cofactor, FVIIIa.** The effect of binding R338A-FIXa (open squares) and wt-FIXa (closed squares) to FIX's cofactor, FVIIIa, was assessed in the presence of 100 µM PSPC vesicles at 5 mM CaCl_2. FVIII (0.1 nM) was activated by thrombin and after 5 min the thrombin was inhibited by 100 nM hirudin. Binding was measured by FIXa:FVIIIa complex mediated FXa generation as described in the Experimental Procedure section. Data represent the mean ± SD of 5 experiments. The K_d and B_{max} values of the R338A-FIXa were 0.75 ± 0.10 nM, and 3.56 ± 0.15 nM FXa/min, respectively. For wt-FIXa, the K_d was 1.01 ± 0.24 nM and B_{max} was 1.30 ± 0.10 nM FXa/min.

![Figure 3](image2.png)

**Fig. 3. Kinetics of FX activation by R338A-FIXa and wt-FIXa.** A, in the absence of FVIIIa. Twenty-five nM R338A-FIXa (open squares) and wt-FIXa (closed squares) in 5 mM CaCl_2 and 100 µM PSPC vesicles were incubated with FX (0 to 200 nM). Data represent the mean ± SD of 2 experiments with duplicate wells. The K_{i1} and V_{max} values for R338A-FIXa were 53.50 ± 7.44 nM, 2.58 ± 0.13 nM FXa/min, respectively. For wt-FIXa, the K_{i1} was 28.45 ± 3.56 nM, and V_{max} was 2.47 ± 0.04 nM FXa/min. In the presence of 0.1 nM FVIIIa, 0.25 nM R338A-FIXa (open squares) and wt-FIXa (closed squares) was used to activate FX in the presence of 0.1 nM FVIIIa. Each concentration of FX is the mean ± SD of 3 experiments. The K_{i1} for R338A-FIXa was 7.72 ± 0.05 nM, and V_{max} was 0.24 ± 0.04 nM FXa/min; For wt-FIXa, the K_{i1} was 18.38 ± 3.37 nM, and V_{max} was 0.63 ± 0.03 nM FXa/min. (C). In the presence of 0.4 nM FVIIIa, FVIIIa (0.4 nM) was incubated with 0.25 nM R338A-FIXa (open squares) or wt-FIXa (closed squares), then used to activate FX. Each concentration of FX was the mean ± SD of 3 experiments. The K_{i1} and V_{max} were 12.23 ± 0.84 nM, 5.42 ± 0.08 nM FXa/min for R338A-FIXa, and 19.64 ± 1.79 nM, 2.46 ± 0.06 nM FXa/min for wt-FIXa. All of the curves were determined from fitting the data to the Michaelis-Menten equation.
analysis demonstrated that both purified R338A-FIX and recombinant wt-FIX migrated as a single band with the same molecular weight as plasma FIX (Fig. 1), and Gla analysis showed full carboxylation of both mutant R338A-FIX and recombinant wt-FIX (Table I), further indicating the high purity of the expressed proteins. In addition, both R338A-FIX and recombinant wt-FIX bound to three FIX-specific monoclonal antibodies with the same efficiency as did plasma-purified FIX or pooled normal plasma (Table I).

**Clotting Activity**—The initial characterization of recombinant FIX was a one-stage aPTT assay. Purified plasma FIX and normal pooled plasma were employed as standards. The results, shown in Table I, indicate that R338A-FIX had 2.6-fold higher clotting activity than normal FIX. To rule out the possibility that the increased clotting activity was due to trace amounts of FIXa generated during protein purification, all the FIX were activated and then assayed in FIX-deficient plasma (PTT assay). As shown in Table I, the PTT clotting activity of activated R338A-FIX was 2.8-fold higher than activated plasma FIX. This result agrees well with results of the aPTT assay and suggests that the increased activity is not the result of activated FIXa contaminating the R338A-FIX preparation.

**FIXa Binding to FVIIIa**—We assumed that FXase activity is directly proportional to the concentration of FIXa-FVIIIa complex (17). This is a reasonable assumption because, in the conditions that we use, addition of FVIIIa to FIXa results in a 300–1000-fold increase in the FXase reaction rate. In the assay of FIXa binding to FVIIIa, binding affinity was monitored through a FXa generation mediated by FIXa-FVIIIa complex.

The results of five experiments, each done in duplicate, are shown in Fig. 2. When all the data were fit to Equation 2, the apparent dissociation constant (K_d) for binding of R338A-FIXa to FVIIIa was 0.75 ± 0.10 nM compared with 1.01 ± 0.24 nM for plasma FIXa. This is consistent with the published binding constants (17). The maximum rate of FX cleavage achieved, however, was 2.7-fold greater for activated R338A-FIXa than for wt-FIXa (3.56 ± 0.15 nM FXa/min and 1.30 ± 0.10 nM/min, respectively.)

**FX Activation by FIXa in the Absence or Presence of Cofactor FVIIIa**—The kinetic parameters for cleavage of FX by R338A-FIXa, plasma FIXa (data not shown), or recombinant wt-FIXa were investigated in the absence or presence of their cofactor, FVIIIa. In the absence of FVIIIa, the k_cat values for R338A-FIXa and wt-FIXa were 1.72 × 10^{-3}s and 1.70 × 10^{-3}s, respectively, while the K_m for R338A-FIXa was about 2-fold higher compared with that of recombinant FIXa (Fig. 3A, Table II). When FVIIIa at either 0.1 nM (Fig. 3B) or 0.4 nM (Fig. 3C) was included in the reaction, the k_cat of R338A-FIXa was 1.65 and 1.20/s, respectively, compared with 0.53 and 0.63/s for recombinant wt-FIXa (Table II). Moreover, in contrast to the situation in the absence of FVIIIa, the K_m of R338A-FIXa was about 2-fold lower compared with wt-FIXa (Table II).

**The Effect of Hirudin on the Mutant R338A-FIXa and wt-FIXa Binding to Their Cofactor, FVIIIa**—One obvious possibility for the increased activity of R338A-FIX is that replacing the arginine by alanine eliminates a potential proteolytic cleavage site. It has been reported, for example, that thrombin can cleave FIX after residue 338 to render the FIX molecule inactive (19, 20). This did not seem likely because the experiments that demonstrated this phenomenon required a very large concentration of thrombin. Because hirudin is a potent inhibitor of thrombin we repeated our binding experiments in the presence and absence of hirudin. As shown in Fig. 4, hirudin had no detectable effect on the maximum velocity achieved with recombinant wt-FIXa. V_max was 1.27 ± 0.12 nM FXa/min in the absence of hirudin, and 1.35 ± 0.15 nM FXa/min in its presence. Thus, the cleavage of FIXa at Arg-338 by thrombin does not explain the increased catalytic activity of R338A-FIXa.

**Electrophoretic Analysis of FIXa’s Effect on FIXa**—Another potential source of proteolysis in our experimental system is the FXa generated during the reaction. To test the possibility that FXa might cleave the heavy chain of FIXa at arginine 338, we incubated purified plasma FIXa with FXa and FVIIIa. If FXa can cleave FIXa at arginine 338, which is responsible for the difference in activity between wt-FIXa and R338A-FIXa, it would be easily observed by SDS-PAGE. As shown in Fig. 5, no cleavage in the heavy chain of FIXa (M, 28,000) was observed when incubated for up to 120 min. Thus, reduced proteolysis of

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**Table II**

| Enzyme | k_cat | h_cat/K_m |
|--------|-------|-----------|
| wt-FIXa | 28.45 ± 3.56 | 2.55 ± 0.09 | 25 | 1.70 × 10^{-3} | 5.98 × 10^4 |
| R338A-FIXa | 53.50 ± 7.44 | 2.58 ± 0.13 | 25 | 1.72 × 10^{-3} | 3.22 × 10^4 |
| With FVIIIa 0.1 nM | 18.38 ± 3.37 | 0.63 ± 0.03 | 0.020 | 0.53 | 2.88 × 10^7 |
| R338A-FIXa | 7.72 ± 0.65 | 2.47 ± 0.04 | 0.025 | 1.65 | 2.14 × 10^8 |
| With FVIIIa 0.4 nM | 19.64 ± 1.79 | 2.46 ± 0.06 | 0.065 | 0.63 | 3.21 × 10^7 |
| R338A-FIXa | 12.23 ± 0.84 | 5.42 ± 0.08 | 0.075 | 1.20 | 9.81 × 10^5 |

* k_{cat} = V_{max}/[enzyme], in the presence of FVIIIa, the enzyme concentrations of FIXa · FVIIIa complex were calculated based on Equation 2 and observed K_d values.

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**FIG. 4.** The effect of hirudin on R338A-FIXa and wt-FIXa binding to its cofactor FVIIIa. In parallel experiments, the effect of hirudin on the binding of R338A-FIXa or wt-FIXa to FVIIIa was determined. The methods were as described in Fig. 2. In the presence (cross) or absence (filled squares) of 100 nM hirudin, the K_d and B_max of wt-FIXa were similar; 0.85 ± 0.29 nM and 1.35 ± 0.15 nM FXa/min in the presence of hirudin, and 0.38 ± 0.15 nM, 1.27 ± 0.12 nM FXa/min in its absence. For R338A-FIXa, the K_d and B_max in the presence of 100 nM hirudin (filled circles) were 0.51 ± 0.11 nM and 3.38 ± 0.19 nM FXa/min while in the absence of hirudin (open squares) the values were 0.40 ± 0.06 nM, 3.39 ± 0.12 nM FXa/min. Each point is the mean of 4 determinations with the standard deviations shown.
R338A-FIXa by FXa compared with wt-FIXa does not explain the increase in catalytic activity.

**Hydrolysis of Synthetic Substrate by FIXa Alone or by the FIXa:FVIIIa Complex**—Whether in the absence or presence of FVIIIa, the \( K_m \) of both R338A-FIXa and wt-FIXa for synthetic peptidyl substrate, Spectrozyme-FXa was similar (Table III). Based on our experiments, the \( K_d \) for FIXa for FVIIIa is about 1 nM. Thus, the concentration of FIXa:FVIIIa complex in these experiments was \(-9 \) nM (Equation 2), which was high enough to measure any differences caused by changes in the active site between R338A-FIXa and wt-FIXa. Although we used FIXa concentration (12 nM) as an enzyme concentration in calculating \( k_{cat} \) (Table III), there was no obvious difference in \( k_{cat} \) if the FIXa:FVIIIa complex concentration (9 nM) was used. Interestingly, we observed a small increase in \( K_m \) for both enzymes in the presence of FVIIIa.

The Inhibition of wt-FIXa and R338A-FIXa by Heparin—We measured the effect of heparin on FXase at fixed concentrations of FIXa, FVIIIa, FX, and phospholipids. The initial rate of FX activation is indicated as a function of varying concentrations of the inhibitor. The results shown in Fig. 6 represented means from three different experiments and, as expected, revealed that heparin inhibited FIXa generation both by R338A-FXase and wt-FXase. However, we observed a 2-fold difference in \( K_i \) values (26 \( \pm \) 6.10 nM for R338A-FIXa and 12 \( \pm \) 1.66 nM for wt-FIXa), and a 7-fold difference in \( V_{max(i)} \) values (3.55 \( \pm \) 0.41 nM FXa/min for R338A-FIXa and 0.49 \( \pm \) 0.09 nM FXa/min for wt-FIXa) for the two enzymes. At saturating concentrations of heparin, the enzymes had residual activities of \(-5\%\) for wt-FIXa and 17\% for R338A-FIXa. This result is consistent with heparin’s partial noncompetitive inhibition of FXase reported previously (21).

**DISCUSSION**

Our original aim was to identify residues critical for the interaction between FIXa and FVIIIa. Instead we found a mutation, arginine 338 (C170) to alanine, that resulted in a FIX molecule with 3 times more coagulant activity than recombinant wild type or plasma FIX. In assays using various components of the intrinsic FXase complex, the increased activity was manifested only toward the macromolecular substrate FX and only in the presence of FVIIIa. This increase is the result of a 3-fold increase in \( k_{cat} \) and a 2-fold decrease in \( K_m \) relative to wild type.

There are several possible explanations for our observations. First, changing arginine at 338 to alanine might increase the affinity of FIXa for FVIIIa so that at a given concentration of proteins, more FIXa would be complexed with FVIIIa, thus increasing the effective enzyme concentration. A chimeric factor IXa with factor VIIa’s first epidermal growth factor-like domain had increased activity for this reason (22). This could account for the increase in \( k_{cat} \), but not the decrease in \( K_m \).

Inasmuch as we observe no significant difference for the \( K_d \) between R338A-FIXa and FVIIIa relative to normal FIXa, this model seems unlikely to fit our results.

Second, FVIIIa, through residue 338, may exert an allosteric effect on the active site of FIXa. Complex formation between FVIIIa and FIXa results in at least a 300-fold (Table II) and, in some conditions, much greater (8) increase in \( k_{cat} \) for FX activation. This has usually been interpreted in terms of a Michaelis complex formation preceded only by a bimolecular binding of substrate to enzyme. The \( k_{cat} \) is proportional to the difference in free energy between the ground state (Michaelis complex) and the transition state. Therefore, one possibility is that FVIIIa binding alters the active site environment of FIXa and changes the energy required to reach the transition state. Mutation of R338A-FXa could increase the \( k_{cat} \) allosterically by affecting this process. There is some evidence, not only for factor IXa-factor VIIIa (9), but also for tissue factor-factor VIIa (23) and thrombin-thrombomodulin (24), suggesting that cofactor binding alters the enzyme’s active site configuration. On the other hand, one previous report stated that FVIIIa had no effect on FIXa’s small substrate cleavage (25). To test this, we

![Image](image.png)

**FIG. 5.** SDS-PAGE analysis of FIXa cleavage by FXa. Purified plasma wt-FIXa (2000 nM), FXa (300 nM) and FVIIIa (0.15 nM) were incubated together at 25 °C for the indicated intervals. Aliquots were removed at each time point and subjected to SDS-PAGE analysis. The bands were visualized by silver staining. Lane 1. wt-FIXa control; lane 2. molecular weight marker (97, 66, 45, 31, 21, 14); lane 3–7, experimental samples of FIXa taken at different time points.

![Image](image.png)

**FIG. 6.** Inhibition of R338A-FIXa and wt-FIXa by heparin. Either wt-FIXa (closed squares) or R338A-FIXa (open squares) (2 nM) was further incubated with the varying concentrations of heparin (from 3 nM to 2.7 \( \mu \)M), and then with a fixed concentration of FXa (200 nM) as described in the Experimental Procedures section. Data represent the mean \( \pm \) SD of 3 experiments. The inhibition constants (\( K_i \)) were \( 12.16 \pm 1.66 \) nM for wt-FIXa, \( 25.93 \pm 6.10 \) nM for R338A-FIXa and the \( V_{max(i)} \) values were \( 3.55 \pm 0.41 \) nM FXa/min for R338A-FIXa, \( 0.49 \pm 0.09 \) nM FXa/min for wt-FIXa when all of the data were fit to Equation 3.

**Table III**

| Enzyme        | \( K_m \) (\( \mu M \)) | \( V_{max} \) (\( \mu M \) pNA) | \( V_{max} \) (\( \mu M \) pNA) | \( k_{cat} \) (\( \mu M \) pNA) |
|---------------|-------------------------|----------------------------------|----------------------------------|-----------------------------|
| wt-FIXa       | 1.62                    | 7.26                             | 0.12                             | 50                          |
| R338A-FIXa    | 1.44                    | 6.45                             | 0.11                             | 50                          |
| wt-FIXa + FVIIIa | 2.50                   | 1.53                             | 0.026                            | 12                           |
| R338A-FIXa + FVIIIa | 3.50               | 1.62                             | 0.027                            | 12                           |

\( a \) \( k_{cat} = V_{max}/[\text{enzyme}] \). In the presence of FVIIIa, FIXa concentration is 12 nM and the FIXa: FVIIIa complex concentration is about 9 nM.
employed the synthetic substrate Spectrozyme-FIXa to probe the active site of R338A-FIXa. The results show that, for both wt-FIXa and R338A-FIXa, the $k_{\text{cat}}$ values were approximately the same (Table III) in the presence or absence of FVIIIa. This indicates that the mutation itself does not alter the catalytic event at the active pocket of FIXa. In addition, R338A-FIXa’s factor VIIIa-dependent increase in catalytic activity is not induced by an allosteric effect on the active site. This means that FVIIIa binding to FIXa does not cause a rearrangement of factor IXa’s catalytic residues, nor does it cause a change in the rate of the catalytic event. Previous fluorescence studies with active site modified FIXa indicate that binding FVIIIa may cause a conformational change in the active site (9). Although our results show no effect on $k_{\text{cat}}$, we do observe a small $K_m$ increase for both enzymes with FVIIIa. This result is consistent with studies of FVa effect on FXa activity (26). Perhaps our results and those of the fluorescence studies can be reconciled by hypothesizing that, upon factor VIIIa binding, there is a change in substrate binding site beyond the catalytic residues. This causes the slight increase in $K_m$ that we observe with the small substrate and the alteration in fluorescence intensity.

Therefore, our results both with the small substrate and factor X are consistent with a model in which residue 338 is a part of an extended macromolecular binding site (exosite). This exosite may be made more accessible to FX when FIXa binds FVIIIa, thus causing the observed changes in catalytic constants. According to this model, when alanine replaces arginine at residue 338, the smaller neutral side chain of alanine would mitigate an unfavorable interaction during the initial binding of FX to intrinsic tenase. This view is supported by our observations that R338A-FIXa-FVIIIa’s $K_m$ for its substrate FX decreased 7-fold compared with R338A-FIXa alone (53.50 nm versus 7.72 nm, Table II), whereas wt-FIXa’s $K_m$ decreased only 1.5-fold with FVIIIa. It is well known that FVIIIa accelerates FX activation mainly by increasing $k_{\text{cat}}$. Based on our results, cofactor binding has little effect on small substrate catalysis. Therefore, we think that cofactor binding affects the positioning of the substrate at the active site resulting in an increased $k_{\text{cat}}$ in the presence of FVIIIa. This phenomenon is further enhanced in R338A-FIXa. This same realignment of substrate binding apparently has a different effect or no effect on the small substrate. That is because when FVIIIa binds FIXa, causing a rearrangement of the substrate binding site, part of this site is involved only in macromolecular substrate binding. This is where residue 338 is located.

Our results indicate that Arg-338 is not only part of a FX binding site, but is also part of a heparin binding site. Heparin is a partial noncompetitive inhibitor of the intrinsic FXase complex (21). A partial noncompetitive inhibitor and substrate can bind to the enzyme at the same time, and the enzyme-substrate-inhibitor complex has some activity (18). Well characterized exosites on thrombin are involved in a multiplicity of functions, including cofactor, substrate, and heparin binding (27–29). The $\alpha$-helix 330–338 (C162–C170) of FIX is close to part of the region designated exosite II in thrombin, which has been implicated in heparin binding (30). The x-ray crystal structure of porcine FIXa also suggests that this $\alpha$-helix might be a heparin binding site (6). Our results are consistent with this prediction. The mutation of arginine to alanine not only increases the affinity for heparin by about 2-fold, but also increases the $V_{\text{max,hep}}$ (Equation 3) by more than 7-fold. The small $K_m$ change may suggest that, as with thrombin, several residues are necessary for heparin binding on FIX molecule (29). Thus, we propose that Arg-338 is part of an exosite on FIXa that interacts both with substrate and heparin. Whether both functions are mediated by identical residues or only overlap remains to be shown.

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