To select residues in coagulation factor XIa (FXIa) potentially important for substrate and inhibitor interactions, we examined the crystal structure of the complex between the catalytic domain of FXIa and the Kunitz protease inhibitor (KPI) domain of PN2; the macromolecular substrate (factor IXa) and inhibitor PN2KPI. Analysis of all six Ala mutants demonstrated normal $K_m$ values for S-2366 hydrolysis, indicating normal substrate binding compared with plasma FXIa; however, all except E98A and K192A had impaired values of $k_{cat}$ for S-2366 hydrolysis. All six Ala mutants displayed deficient $K_m$ values for FIX hydrolysis, and all were inhibited by PN2KPI with normal values of $K_i$ except for K192A, and Y5901A, which displayed increased values of $K_i$. The integrity of the S1 binding site residue, Asp$^{189}$, utilizing p-aminobenzenamide, was intact for all FXIa mutants. Thus, whereas all six residues are essential for catalysis of the macromolecular substrate (FIX), only four (Tyr$^{143}$, Ile$^{151}$, Arg$^{3704}$, and Tyr$^{5901}$) are important for substrate and inhibitor interactions. We have therefore utilized this structural information to identify a number of residues within two loop structures (Loop 1 and Loop 2) within the KPI domain postulated to interact with corresponding residues within the catalytic domain of FXIa that are potentially important for both inhibitor and substrate interactions. We have therefore utilized this structural information (Fig. 1) to examine the architecture of residues in close proximity to the catalytic triad and to select a number of residues within the catalytic domain of FXIa (Asp$^{98}$, Lys$^{102}$, Ser$^{195}$, and Tyr$^{370}$) that make the scissile bond between Arg$^{369}$ and Ile$^{370}$ by factor XIIa (FXIla) or thrombin or by autoactivation in the presence of a negatively charged surface (1, 5, 6). Upon activation, each of the identical subunits contains a 50-kDa heavy chain and a 30-kDa light chain. The NH$_2$-terminal heavy chain is composed of four tandem repeat sequences called Apple domains (Apple 1 to Apple 4), and the COOH-terminal light chain contains the catalytic triad residues His$^{413}$, Asp$^{462}$, and Ser$^{557}$ (His$^{57}$, Asp$^{102}$, and Ser$^{195}$), which is characteristic of serine proteases. The NH$_2$-terminal Ile$^{16}$ inserts into the protease domain of FXIa, and the NH$_2$ group forms a salt bridge with the COOH group of Asp$^{194}$. This salt bridge is a defining feature during the formation of FXIa (7).

FXI is the natural macromolecular substrate of FXIa. The Ca$^{2+}$-dependent activation of FIX by FXIa (8, 9) requires the exposure of a substrate-binding site within the Apple 2 and/or Apple 3 domain of FXIa and the γ-carboxyglutamic acid domain of FIX, as well as an extended, macromolecular substrate-binding exosite in the protease domain of FXIa (10–14). The activation of FIX to FIXaβ involves two cleavages by FXIa, one after Arg$^{145}$ and another after Arg$^{180}$, which releases an 11-kDa activation peptide (8, 9, 15). FIXaβ is also produced by the tissue factor-factor VIIa complex (16).

Protease nexin 2 (PN2) is a Kunitz-type protease inhibitor (KPI) secreted by activated platelets (17–19) that has been shown to have high affinity and specificity for FXIa. The interaction between PN2 and FXIa has previously been shown to involve interactions that occur exclusively between the KPI domain of PN2 (PN2KPI) and the catalytic domain of FXIa (FXIac) (20). The isolated KPI domain and the FXIac catalytic domain have been co-crystallized, and their structure has been solved to a resolution of 2.6 Å (21). This structure combined with a mutational analysis of the KPI domain has been used to identify a number of residues within two loop structures (Loop 1 and Loop 2) within the KPI domain postulated to interact with corresponding residues within the catalytic domain of FIXa that are potentially important for both inhibitor and substrate interactions. We have therefore utilized this structural information (Fig. 1) to examine the architecture of residues in close proximity to the catalytic triad and to select a number of residues within the catalytic domain of FXIa (Asp$^{98}$, Lys$^{102}$, Ser$^{195}$, Asp$^{189}$, Gly$^{193}$, Tyr$^{143}$, Ile$^{151}$, Arg$^{3704}$, and Tyr$^{5901}$) that make
intimate contacts with corresponding residues within Loop 1 (Pro3, P3 site; Arg5, P1 site; Met17, P2 site; Ser19, P4 site; and Arg20, P5 site) and Loop 2 (Phe34 and Tyr35) of the KPI domain of PN2 (see Table 1). It should be noted that Arg3704 (alternatively referred to as Arg3705), a residue unique to FXIa, is the fourth amino acid after residue 37 (chymotrypsin numbering) (7), residue 395 in mature FXI, or residue 76 of the catalytic domain of FXIa, whereas similarly Tyr5901 (alternatively referred to as Tyr59A) is the first residue after residue 59. In the present work, we have made selected mutations at these identified exosite residues (i.e. excluding the active site), and examined the resulting enzymes (after activation to FXIa) in the hydrolysis of the peptide substrate S-2366, in the activation of the macromolecular substrate, FIX, and in the regulation of FXIa by PN2. The rationale for selecting these residues for mutational analysis includes the fact that Glu98 is part of the 90s loop (residues 94–100) of FXIa, a surface-exposed loop that varies in length and conformation among serine proteases. In FXIa, the 90s loop folds inward toward the catalytic triad residues and therefore may restrict the accessibility of substrates and inhibitors to this region. Residues Tyr143 and Ile151 are part of the autolysis loop (Tyr143-Thr154) of FXIa. The basic residues within this loop have been previously shown to be important for FXIa serpin specificity (22). A surface-exposed residue unique to FXIa among serine proteases of blood coagulation and highly conserved among various species is Arg3704, which was therefore also chosen for mutational analysis. In this paper, in addition to examining the importance of these selected residues in both substrate hydrolysis and inhibitor (PN2KPI) recognition, we also examined the integrity of the S1 binding site residue, Asp189, utilizing the S1 site probe, p-aminobenzenediazonium (pAB). Our data demonstrate that the S1 site in all mutants is intact. Interestingly, all of the mutant proteins examined demonstrated significant decreases in the $k_{cat}$ values for macromolecular substrate (FIX) catalysis, and all (except for E98A and K192A) displayed significant decreases in the $k_{cat}$ values for small peptide hydrolysis, whereas the $K_m$ values for peptide hydrolysis were normal for all mutant enzymes. Although all of the residues chosen for mutational analysis were selected on the basis of interactions with PN2KPI, all mutants demonstrated abnormal macromolecular substrate recognition. Whereas mutations at four of these sites (Glu98, Tyr143, Ile151, and Arg3704) resulted in normal values of $K_i$ for inhibition by PN2KPI, in contrast, mutations at the other two sites (Lys192 and Tyr5901) resulted in enzymes with impaired interactions with PN2KPI as well as macromolecular substrate catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Reagents**—The site-directed mutagenesis kit (QuikChange™) was purchased from Stratagene (La Jolla, CA). Lipofectamine 2000 reagent was from Invitrogen. The chromogenic substrate S-2366 (L-pyroglutamyl-L-prolyl-L-arginyl-p-nitroaniline hydrochloride) was purchased from Chromogenix (Mölndal, Sweden). The fluorogenic substrate t-butoxyacarbonyl-Glu(O-benzoyl)-Ala-Arg-methylcoumaryl-7-amide was obtained from Peptides International (Louisville, KY). Spectrozyme FIXa substrate MeSO$_2$-d-CHG-Gly-Arg-pNA.AcOH was purchased from American Diagnostica (Hauppauge, NY). Ethylene glycol was purchased from Fisher. Bovine serum albumin, penicillin/streptomycin, glutamine, o-phenylenediamine, aprotinin, and cyanogen bromide-activated-Sepharose 4B were obtained from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Mediatech (Herndon, VA). Genetinc (G418) was purchased from Invitrogen. All other reagents were of analytical grade and were the best quality commercially available.

**Proteins**—FXIa, FIXa, and FXI purified from human plasma were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Kunitz-type protease inhibitor was purified from transfected *Pichia pastoris* as described previously (21). The monoclonal antibody 5F7 (directed against the A1 domain located within the heavy chain of FXI) was initially purified from the ascites fluids in a hybridoma cell line (23) and now is commercially available from Green Mountain Antibodies (Burlington, VT). Corn trypsin inhibitor (coupled to Affi-Gel) columns were purchased from Enzyme Research Laboratories (South Bend, IN).

**Methods**

**FXI Mutant Constructs**—The cDNA for the full-length FXI sequence inserted in pJVCMV vector (a gift from Dr. David Gailani, Vanderbilt University, Nashville, TN) served as a template for the synthesis by PCR of the FXI catalytic domain mutants. The mutations were introduced using a PCR-based site-directed mutagenesis kit (QuikChange™) using the appropriate mutagenic primers. The PCR products containing mutations were inserted into pJVCMV vector and were propagated in XL1-Blue bacteria. Each purified plasmid DNA was sequenced in the forward and reverse directions to verify that the appropriate mutation was incorporated.

**Protein Expression in Human Embryonic Kidney (293) Cells and Purification**—Human embryonic kidney cells (293-HEK) were transfected with 40 μg of the pJVCMV vector containing inserts for FXI mutants and 2 μg of pRSVneo vector (containing the gene that confers resistance to neomycin and allows the selection of positive clones) using Lipofectamine 2000. Positive clones were selected using Genetinc (G418) at a concentration of ~500 μg/ml, and the expression levels were assessed by ELISA (described below). Cells were expanded in 2-liter roller bottles in DMEM containing 10% fetal bovine serum, penicillin/streptomycin, 1-glutamine, and G-418 (~150 μg/ml final concentration) in a 5% CO$_2$ incubator, 37 °C. After the cells reached confluence in the roller bottles, the medium was replaced with serum-free DMEM supplemented with penicillin/streptomycin (50 units/ml penicillin; 50 μg/ml streptomycin), 1-glutamine (0.3 mg/ml), G-418 (~150 μg/ml), insulin-transferring selenium-A, soya bean trypsin inhibitor (10 μg/ml), lima bean trypsin inhibitor (10 μg/ml), and aprotinin (10 μg/ml). Conditioned media were collected after 48–72 h, centrifuged, and filtered through an acetate filter (0.45-μm pore size) to remove any cell debris, were made 5 mM in EDTA and 5 mM in benzamidine to prevent any nonspecific protease cleavage of the protein, and were stored at −20 °C until they were ready to be processed.
Expressed protein from cell supernatant was applied to the 5F7 monoclonal antibody affinity column equilibrated in 25 mM Tris-HCl, 100 mM NaCl, and 5 mM benzamidine, pH 7.4. The column was washed with equilibrium buffer until the $A_{280}$ returned to base line. Adsorbed protein was eluted with 2 M potassium thiocyanate made in the equilibrium buffer. The collected fractions were concentrated and dialyzed extensively against Tris-buffered saline, pH 7.4. The yield of mutant proteins ranged from 0.5 to 1.2 mg/liter of culture fluid. The purity of the fractions was assessed by SDS-PAGE before being pooled and concentrated to 0.25 ml for immediate use in experiments or for storage either at −80 °C or in liquid nitrogen. As shown in supplemental Fig. 1, all FXI mutants were >95% pure as judged from SDS-PAGE (4–15%) and like plasma FXI all migrated at 160 kDa nonreduced and at 80 kDa under reducing conditions (i.e. incubation with β-mercaptoethanol). The activated mutants like FXIa migrated as a single band nonreduced and as two bands of ~50 and ~30 kDa comprising the heavy and light chains, respectively.

*Activation of FXI Mutants*—FXI mutant proteins were activated overnight by FXIIa (10:1 molar ratio of FXI to FXIIa) at 37 °C. The FXIa was subsequently removed using a corn trypsin inhibitor column. Samples of the activated protein were analyzed by SDS-PAGE.

*Active Site Titration*—The active site concentrations of FXIa mutants were measured (24, 25) utilizing the fluorogenic active site titrant 4-methylumbelliferyl 4-guanidinobenzoate hydrochloride monohydrate (MUGB) obtained from Sigma. The fluorescent product 4-methylumbelliferone released by the hydrolysis of MUGB by FXIaWT or pFXIa was determined at excitation and emission wavelengths of 323 and 446 nm, respectively. FXIaWT (0–200 nM) and 500 pM substrate 2366 in 25 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$, 0.5% BSA, pH 7.4. The reaction was performed at 37 °C, and the absorbance was read at 405 nm. Inhibition constants were determined by plotting the residual activity of FXIa and by using the following equation,

$$K_i = 1C_{50}/(1 + ([S]/K_m))$$

(Eq. 1)

where $S$ is the substrate concentration and $K_m$ is the Michaelis constant of FXIa for S-2366 previously determined to be ~0.25 mM (29).

*Hydrolysis of Substrate 2366*—To determine the kinetic parameters of S-2366 hydrolysis by pFXIa and the FXIa mutants, increasing concentrations of S-2366 (0–1.5 mM) were added to pFXIa or FXIa mutants (6.7 nM final concentration). Linear initial rates of generation of p-nitroaniline (pNA) were measured by continuous monitoring of absorbance at 405 nm in a reaction mixture of 100 μl (path length of 3.125 mm) in a microplate reader (Molecular Devices, CA). All our experiments were carried out at a temperature of 37 °C in Tris buffers prepared with a pH of 7.4 at room temperature (~25 °C). Because the calculated pH at 37 °C, based on the $\Delta pK_a$/degree of ~0.031 for Tris and the 12 °C temperature difference is 7.0, the actual pH at which our experiments were carried out is likely to be ~7.0. Concentrations of pNA were determined using a molar extinction coefficient of 9933 M$^{-1}$ cm$^{-1}$. Titration curves of S-2366 hydrolysis were generated by KaleidaGraph (Abelbeck Software, Reading, PA), and the data were analyzed using a nonlinear least squares fit of data points to the equation for a rectangular hyperbola, $y = ax/(b + x)$.

*Activation of FIX by FXIa and FXIa Mutants*—To examine the contributions of individual residues clustered around the active site of FXIa to the activation of FIX, either FXIa or one of the FXIa mutants (2.5 nM final concentration) was added to increasing concentrations of FIX (0–2 μM) in 50 mM Tris-HCl with 150 mM NaCl, 5 mM CaCl$_2$, and 1% BSA. As described previously (30), the reaction was allowed to proceed for 3 min before it was stopped by the addition of aprotinin (0.7 mM). Ethylene glycol (16%, v/v) was added to increase the sensitivity of the substrate for FIXa before the addition of Spectrozyme (2.6 mM). Linear initial rates of release of pNA were monitored by measuring absorbance at 405 nm at 37 °C and analyzed as described previously (30).

* Determination of Equilibrium Inhibition Constants for FXIa Mutants by PN2KPI*—Increasing concentrations of WT PN2KPI domain (0–10 nM) in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl$_2$, 0.5% BSA, pH 7.3, were incubated with pFXIa or FXIa mutants (1 nM final concentration) for 30 min at 37 °C in a microtiter plate to achieve equilibrium between enzyme and inhibitor. Residual FXIa activity in the reaction mixture was determined by hydrolysis of S-2366 (0.5 mM). The absorbance at 405 nm in a microplate reader (Molecular Devices) was measured under pseudo-first-order kinetic conditions, and the results were converted to fraction of amidolytic activity remaining. The concentration at which 50% activity remained ($IC_{50}$) was determined using Kaleidograph. The inhibition constant was then determined using Equation 1 (see above).

* Determination of Inhibition Constants for FXIa Mutants by PN2KPI by Progress Curve Analysis*—For those FXIa mutant proteins determined to be deficient in PN2KPI inhibition (i.e. with significant increases in equilibrium inhibition constants), $K_i$ values were also determined by progress curve analysis as described previously (31). Briefly, the release of a highly fluorescent product from the peptidyl substrate t-butoxycarbonyl-Glu(o-benzoyl)-Ala-Arg-methylcoumaryl-7-amide by FXIa or mutant molecules was monitored in the presence of varying concentrations of PN2KPI. Substrate hydrolysis was initiated by the addition 10 μl of enzyme (25 pm FXIa, final concentration) to 90 μl of a mixture of inhibitor (varying concentrations)
and fluorogenic substrate in TBSB in 96-well black polystyrene microtiter plates. The progress of substrate hydrolysis was monitored for up to 50 min at 15- or 60-s intervals at room temperature (ranging from 24 to 27 °C) in a fluorescence plate reader, and $K_v$ values were determined as described earlier (31).

**Biotinylation of PN2KPI**—PN2KPI was biotinylated using the EZ-Link® Micro sulf-NHS-biotinylation kit obtained from Pierce. The appropriate volume of sulfo-NHS-biotin solution was added to PN2KPI protein solution in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4) and incubated at 4 °C for 2 h. Excess biotin was removed by applying the protein solution to a desalting spin column and centrifuging the column at 1000 × g for 2 min. Purified biotinylated PN2KPI was obtained from the collection of the flow-through solution. The concentration of biotinylated PN2KPI was determined by a BCA assay.

**Determination of FXIa Binding to PN2KPI by ELISA**—100 μl of biotinylated PN2KPI (0.5 μg/ml) was added to each well of a NeutrAvidin-coated plate (Pierce), which was then incubated at 4 °C overnight. Unbound biotinylated PN2KPI was removed, and the wells were washed three times with wash buffer (PBS-Tween, 0.1% (v/v)). 200 μl of blocking buffer (PBS-BSA, 1% (w/v)) was added to each well of the plate, which was incubated at room temperature for 2 h. Blocking buffer was then discarded, and the wells were washed three times with wash buffer. 100 μl of various concentrations of FXIaWT or FXIa mutants (0–35 nM) was added into the wells and incubated at room temperature for 30 min. FXIa protein solutions were then discarded. After the wells were washed three times with wash buffer, the detecting antibody, peroxidase-conjugated polyclonal anti-FXI antibody, diluted 100-fold in sample diluent (HBS-BSA-Tween, 100 mM NaCl, 100 mM HEPES, pH 7.2, 1% BSA, and 0.1% Tween 20) was added to each well and incubated at room temperature for 1 h. After the wells were washed thoroughly with wash buffer, 100 μl of the substrate, o-phenylenediamine (0.42 mg/ml) in citrate-phosphate buffer (27 mM citrate acid, 97 mM Na$_2$HPO$_4$, pH 5) was added to each well for 5–10 min for color development. 50 μl of stopping solution (2.5 mM H$_2$SO$_4$) was added to stop the color development reaction. The plate was read at 490 nm using a Thermomax plate reader (Molecular Devices Corp., Sunnyvale, CA).

**Molecular Modeling**—In the modeling procedure, the energy-minimized light chain of FXIa was compared with the energy-minimized light chain of FXIa with mutations at residues Ile$^{151}$, Glu$^{28}$, Tyr$^{143}$, and Arg$^{2704}$ (chymotrypsin numbering). The energy minimization method utilized SYBYL software (version 7.2, from Tripos Inc., St. Louis, MO) with the charge type being AMBER7 F99 and ligands having Gasteiger-Hückel charges. Energies of the models were all minimized by ~85,000 kcal/mol. The cut-off point for the calculation was the point at which the force gradient became less than 0.05 kcal mol$^{-1}$ Å$^{-1}$ or until 10,000 iterations were completed.

**Data Analysis**—$K_m$, $k_{cat}$, and $K_v$ values for FXIa mutants were compared with the values for pFXIa and analyzed for their statistical significance using an unpaired Student’s t test. The Bonferroni adjustment was applied to the $p$ values obtained from the $t$ test in order to correct for a type I error of level 0.05 (an error made by incorrectly declaring an error due to chance). Values were considered statistically significant if $p < 0.05$.

**RESULTS**

Initially we mutated the six residues identified from the crystal structure (Fig. 1 and Table 1) to alanine (Table 2) and examined their interactions with pAB, S-2366, FIX, and PN2KPI. Some of these residues were further examined for the effects of charge and size of side chains by additional mutations as shown in Table 2.

**Inhibition by pAB of FXIa Mutants**—The inhibition of FXIa and mutant proteins by pAB was examined in order to assess the integrity of the S1 substrate-binding site of FXIa (27). The results are presented for plasma FXIa and all of the mutant proteins in supplemental Fig. 2, and the derived $K_v$ values are summarized in Table 2. Control values throughout the paper are reported for plasma-derived FXIa (referred to as pFXIa), and these values were not significantly different from those obtained with recombinant wild-type FXIa. Therefore, for the sake of conciseness, control values for FXIaWT are not reported.

FXIa was inhibited by pAB with an inhibition constant ($K_i$) of 51.3 ± 1.14 μM. All alamine mutants, with the exception of the Y143A mutant ($K_i = 21.1$ μM) and the K192A mutant ($K_i = 152.9$ μM), displayed $K_v$ values ranging from 36.2 to 73.9 μM, which were not significantly different from the $K_v$ value for pFXIa. The $K_v$ value for the E98V mutant ($K_v = 39.6$ μM) was also within this range, whereas the $K_v$ value for the E98D mutant ($K_v = 29.6$ μM) and the K192E mutant ($K_v = 24.5$ μM) were only slightly lower, and the K192Q mutant ($K_v = 79$ μM) was only slightly higher. We do not regard these minor differences as biologically relevant because inspection of the inhibition data (supplemental Fig. 2) indicate only minor deviations from control curves. Because the $K_v$ value for some of these mutants were slightly lower than that of the wild-type protein, suggesting slightly more potent inhibition, these results provide no evidence for a defect in pAB binding to any of the mutant proteins, except possibly minor defects for the K192A and the K192Q mutants. Combined with the results of active site titrations, demonstrating that the mutant proteins retained 62–124% (mean = 81%) of their active site concentration (see “Methods”), these data suggest that the S1 substrate-binding sites of most if not all mutant proteins were largely intact.

**Cleavage of Synthetic Substrate S-2366 and FIX by FXIa and FXIa Mutants**—The ability of each of the FXIa mutants to cleave the small synthetic substrate, S-2366, was examined at varying substrate concentrations, and the results are presented in supplemental Fig. 3. The $K_m$ and $k_{cat}$ values for substrate hydrolysis were determined for each FXIa mutant and are summarized in Table 2. The $K_m$ values for FXIa mutants were not significantly different from those for plasma FXIa (0.34 ± 0.10 mM), thereby indicating that the binding affinity of S-2366 to the FXIa mutant proteins was normal. With the exception of FXIa/E98A and FXIa/K192A, all of the FXIa mutants showed marked decreases in $k_{cat}$ values ($6–60$ s$^{-1}$), which were significantly different from that of plasma FXIa (201 ± 20.3 s$^{-1}$). The E98A and K192A mutants displayed ~2-fold decreases in $k_{cat}$ values, which were not significantly different from that of the normal protein. The decreased $k_{cat}$ values and normal $K_m$ val-
FXIa Substrate Catalysis and Inhibition by PN2

FIGURE 1. Structure of the FXIa catalytic domain in complex with the KPI domain of PN2 (Protein Data Bank code 1ZJD). The ribbon structure shown in green is the catalytic domain of FXIa, whereas the ribbon structure shown in purple is the KPI domain of PN2. The active site residues of FXIa (His57, Asp102, and Ser195), with side chains shown as sticks are highlighted and labeled in yellow. The KPI residues (Pro13, Arg15, Met17, Ser19, Asp189, Phe34, and Tyr35) previously identified (21) as closely interacting with FXIa exosite residues clustered around the active site are shown and labeled in red with side chains depicted as sticks. The corresponding FXIa residues therefore selected for mutational analysis (Asp98, Lys192, Ser195, Asp189, Tyr143, Ile151, Arg3704, and Tyr5901) are shown and labeled in purple with side chains depicted as sticks. These interactions, together with measured distances and types of interactions are depicted in Table 1.

TABLE 1

| KPI domain | FXIa | Å | Type of interaction |
|------------|------|---|-------------------|
| Pro13 (P3 site) | Gly196 | 4.38 | Hydrophobic |
| Pro13 (P3 site) | Lys192 | 3.48 | Ionic |
| Arg15 (P1 site) | Ser195 | 3.10 | Hydrogen bond |
| Arg15 (P1 site) | Asp189 | 2.57 | Ionic |
| Arg15 (P1 site) | Gly193 | 2.80 | Hydrogen bond (main chain) |
| Met17 (P2 site) | Tyr143 | 3.73 | Hydrogen bond |
| Met17 (P2 site) | Ile151 | 3.96 | Hydrophobic |
| Met17 (P2 site) | Arg3704 | 3.12 | Hydrogen bond |
| Ser19 (P4 site) | Arg3704 | 3.12 | Hydrogen bond |
| Arg15 (P5 site) | Tyr5901 | 3.55 | Ionic |
| Phe34 (loop 2) | Tyr143 | 3.76 | Hydrophobic |
| Tyr35 (loop 2) | Tyr5901 | 3.83 | Hydrophobic |

values indicated that all of the mutations, with the exception of FXIa/E98A and FXIa/K192A, resulted in normal binding to S-2366 but impaired turnover of S-2366 hydrolysis by FXIa. The observation that the K192E mutation had only a minor effect on the $k_{\text{cat}}$ of S-2366 hydrolysis compared with FXIa/K192A, whereas the K192R mutation had a major effect on $k_{\text{cat}}$ is counterintuitive and not subject to definitive rational interpretation.

None of the FXIa mutants was able to catalyze the activation of the macromolecular substrate FIX as efficiently as pFXIa (supplemental Fig. 4 and Table 2). Thus, all of the mutants examined displayed 4–10-fold decreased values of $k_{\text{cat}}$ compared with pFXIa (0.73 s$^{-1}$). As is apparent from inspection of the saturation curves (supplemental Fig. 4), we were unable to calculate reliable values of $K_m$ for all of the mutant proteins examined. Thus, all FXIa mutants displayed saturation curves truncated at very low values of $V_{\text{max}}$. For a number of these mutants (e.g., especially for those depicted in supplemental Fig. 4, A (E98D and E98V), B (K192Q and K192R), C (R3704A and Y143A), and D (Y5901V)), due to the insensitivity of the chromogenic substrate (Spectrozyme in the presence of ethylene glycol), the amounts of product (FIXa) generated at low concentrations of FIX are too low to be reliably measured. Therefore, we have not calculated values of $K_m$, and measured values of $k_{\text{cat}}$ should be regarded as overestimates. Thus, the $k_{\text{cat}}$ values listed in Table 2 most likely underestimate the defects in $k_{\text{cat}}$ for many of the mutants studied. For this reason, the values of $k_{\text{cat}}$ <0.2 s$^{-1}$ for FIX activation have been listed as such to reflect the insensitivity of the assay and to acknowledge that the rates were too low to be quantified reliably. Some of the other mutants studied (e.g. supplemental Fig. 4, A (E98A), B (K192A and K192E), C (I151A), and D (Y5901A)) displayed saturation curves truncated at very low values of $V_{\text{max}}$, suggesting decreased values of apparent $K_m$, which typically reflects tighter binding to FIX. It should be noted, however, that the measured $K_m$ is not the true substrate dissociation constant ($K_d$ or $K_p$) and that the rate of the reaction contributes to the $K_m$ (i.e. $K_m = K_d + k_{\text{cat}}/k_{\text{on}}$). Therefore, the decreased reaction rates that were observed for some of these FXIa mutants most likely resulted in decreased values of substrate concentration at saturation in the absence of enhanced enzyme-substrate affinity that would be manifested by decreased values of $K_p$. Because these decreased values of $K_m$ most likely reflect the defects in $k_{\text{cat}}$ rather than enhanced substrate binding, as reported previously (32, 33), we have not listed values of $K_m$ for FIX activation in Table 2 and have included a notation that reliable values of $K_m$ for FIX activation are not available. The major conclusion to...
be drawn from the $k_{\text{cat}}$ values for the mutants in Table 2 is that the values listed are overestimates, and therefore the defects in $k_{\text{cat}}$ are underestimated, thereby strengthening our argument that all of the mutations have deleterious effects on the capacity of FXa to catalyze FIX activation.

**Inhibition of FXa and FXa Mutants by PN2KPI—FXa and each of the mutant proteins were incubated with various concentrations of PN2KPI (supplemental Fig. 5) to determine $K_i$ values, summarized in Table 2. The equilibrium inhibition constant measured for plasma FXa was 1.5 nM, in close agreement with the $K_i$ value determined by progress curve analysis (0.610 nM). As shown in Table 2, mutations at four of these sites (Glu98, Tyr143, Ile151, and Arg3704) resulted in normal values of $K_i$, ranging from 1.0 to 1.4 nM, comparable with that of pFXa, demonstrating that these residues are not essential for inhibition by PN2KPI. In contrast, mutations at the other two sites (Lys192 and Tyr5901) resulted in significantly increased $K_i$ values or no discernible inhibition utilizing the equilibrium inhibition assay. In each instance, these results were confirmed utilizing progress curve analysis, which demonstrated significantly increased $K_i$ values (Table 2). The sole exception to this result was observed with the K192Q mutant, which displayed normal $K_i$ values using both methods, suggesting that the presence of an amino group in the side chain of either lysine or glutamine of residue 192 of FXa is important for inhibition by PN2KPI.

**Binding of FXa and FXa Mutants to PN2KPI—A possible explanation for the failure of PN2KPI to inhibit FXa molecules mutated at Tyr5901 and Lys192 is the failure of the mutant proteins to bind to PN2PKI. To examine this possibility, we selected four of the FXa molecules studied here to examine their capacity to bind to PN2KPI in a microtiter plate equilibrium binding assay. The results are shown in supplemental Fig. 6. Two of these proteins (FXa wt and FXa/Y143A) were inhibited with normal $K_i$ values (1.5 and 1.3 nM, respectively; see Table 2) by PN2KPI and were shown to bind with $K_D$ values (1.63 and 1.97 nM, respectively; see Table 3) very close to the measured $K_i$ values. The other two proteins (FXa/K192E) were not inhibited by PN2KPI in the equilibrium binding assay and were characterized by highly significant increases in $K_i$ values by progress curve analysis (see Table 2). In the direct binding assay, the FXa/Y5901A mutant interacted with PN2KPI with normal affinity ($K_i = 1.22$ nM), whereas the FXa/K192E mutant bound to PN2KPI with a $K_i$ value (4.75 nM) only ~3-fold higher than FXa wt. Thus, we conclude that the impaired susceptibility of molecules mutated at Tyr5901 and Lys192 to inhibition by PN2KPI arises not from a defect in the binding of KPI to the catalytic domain of FXa but rather from the fact that these two residues play an essential role in both substrate catalysis and inhibitor recognition.

**DISCUSSION**

The mechanism by which serine proteases recognize and cleave their substrates and inhibitors involves the formation of a catalytic triad (His$^{37}$, Asp$^{102}$, and Ser$^{195}$), located at the

### TABLE 2

**Substrate catalysis by FXa and mutants and inhibition by pAB and PN2KPI**

Values represent the mean ± S.D. of independent measurements (n = 3). The Bonferroni adjustment was applied to calculate $p$ values.

| Enzyme     | Substrate | $K_a$ | $K_{\text{cat}}$ | Inhibitor | $K_i$ |
|------------|-----------|-------|------------------|-----------|-------|
|           |           | μM   | s⁻¹              |           | μM   |
| pFXa       | S-2366    | 339 ± 100 | 201 ± 20.3 | pAB (μM) | 51.3 ± 1.14 |
| E98A       | S-2366    | 60.0 ± 0.04 | 0.73 ± 0.02 | PN2KPI (μM) | 1.5 (0.610) |
| E98D       | S-2366    | 60.0 ± 0.04 | 0.73 ± 0.02 | pAB (μM) | 41.6 ± 1.20 |
| E98V       | S-2366    | 383 ± 20 | 5.8 ± 0.1 | pAB (μM) | 29.6 ± 4.65 |
| I151A      | S-2366    | 525 ± 60 | 21 ± 1.0 | pAB (μM) | 1.2 (ND) |
| Y143A      | S-2366    | 538 ± 100 | 7 ± 0.1 | pAB (μM) | 39.6 ± 2.03 |
| R370A      | S-2366    | 268 ± 10 | 8 ± 0.1 | pAB (μM) | 37.3 ± 3.89 |
| K192A      | S-2366    | 852 ± 20 | 95 ± 9.5 | pAB (μM) | 152.9 ± 6.73 |
| K192E      | S-2366    | 315 ± 50 | 86 ± 4.3 | pAB (μM) | 24.5 ± 1.00 |
| K192Q      | S-2366    | 603 ± 230 | 30 ± 5 | pAB (μM) | 79.0 ± 6.32 |
| Y5901A     | S-2366    | 292 ± 40 | 29 ± 1.1 | pAB (μM) | 49.7 ± 0.88 |
| Y5901V     | S-2366    | 336 ± 40 | 42 ± 1.6 | pAB (μM) | 41.9 ± 0.62 |

* The numbers listed under $K_a$ (μM) for PN2KPI represent equilibrium constants for FXa inhibition by PN2KPI, whereas the numbers in parentheses represent inhibition constants determined by progress curve analysis. NI, no inhibition observed.
* a Not significant when compared with pFXa.
* b ND, not determined; reliable values of $K_a$ could not be obtained.
* d p < 0.05 compared with pFXa.
entrance of the substrate/inhibitor-binding pocket, the geometry of which is stabilized by hydrogen bonds (34). The formation of a tetrahedral transition state intermediate that is stabilized by hydrogen bonds between an oxyanion intermediate and the amido groups of Ser195 and Gly193 is required for the hydrolysis of peptide bonds by serine proteases. Because the architecture of residues located adjacent to the active site is an important determinant of substrate and inhibitor specificity and enzyme activity, we took advantage of the crystal structure of the enzyme-inhibitor complex between the catalytic domain of FXIa and the KPI domain of protease nexin 2 to identify residues potentially important for substrate hydrolysis and/or inhibitor recognition. We also utilized the primary sequence of FXI and a highly homologous protein, PK, to identify residues unique to FXIa, whose macromolecular substrate (i.e. FIX) and major inhibitor (i.e. PN2) are poorly recognized by plasma kallikrein. Finally, residues that are important for enzyme function, including substrate and inhibitor interactions, are characteristically highly conserved among various species (35, 36).

As demonstrated in Table 2, mutations to alanine of four surface-exposed FXIa residues (Tyr143, Ile151, Tyr5901, and Arg3704) resulted in significant reductions in the rate of S-2366 hydrolysis, whereas effects on $k_{\text{cat}}$ by the other two mutants (E98A and K192A) were minimal. In the FIX activation assay, however, mutations at all of the six residues resulted in severely reduced rates of FXIa generation. Therefore, although all six residues were essential for optimal rates of macromolecular substrate (FIX) cleavage to generate FXIa, only four residues (Tyr143, Ile151, Tyr5901, and Arg3704) are involved in hydrolysis of the peptidyl substrate S-2366.

Interestingly, although each of the six FXIa residues chosen for mutational analysis was selected on the basis of its structural interactions with specific KPI residues demonstrated to be important for FXIa inhibition, mutations at four of these sites (Glu98, Tyr143, Ile151, and Arg3704) resulted in normal values of $K_i$ (1.0–1.4 nM), demonstrating that these residues are not essential for inhibition by PN2KPI. In contrast, mutations at the other two sites (Lys192 and Tyr5901) resulted in significantly increased $K_i$ values or no discernible inhibition, demonstrating that residues Lys192 and Tyr5901 are essential for interaction with PN2KPI. It is worth emphasizing that whereas three mutants, K192A, K192E, and K192R, showed impaired inhibition, the mutant K192Q inhibited FXIa with $K_i$ similar to PN2KPI (Table 2). A plausible explanation is that the presence of an amino group in the side chain of either lysine or glutamine of residue 192 of FXIa is important for inhibition of FIXa by PN2KPI.

It is not surprising that the four mutants Y143A, I151A, Y5901A, and R3704A were found to be deficient in assays of both FIX activation and small peptide hydrolysis because enzyme molecules with impaired catalytic activity against a small tripeptide substrate such as S-2366 would also be expected to have impaired catalytic activity against the normal macromolecular FXIa substrate. However, both E98A and K192A mutants were characterized by only a ∼53% (insignificant) reduction in the $k_{\text{cat}}$ for S-2366 cleavage with a highly significant decrease in FIX substrate catalysis, suggesting that both Glu98 and Lys192 are essential for cleavage of the macromolecular substrate FIX. Thus, mutations at either of these two residues may disrupt FIX binding to a FXIa substrate-binding exosite (14).

Glu98 is part of the 90s loop (comprising residues 94–100) that varies slightly in length and conformation among serine proteases. Comparison of the 90s loop structure of FXIac (94YKMAESG100) (Fig. 2A) with that of PK (94YKVSEG100)
chains are positioned differently (Fig. 3 because the conformation of the 90s loop is different, the side
structures, the aliphatic portion of the side chain of Glu98 forms
interaction. The function of the Ser$^{214}$ residue is not fully
interactions restrict the region and block solvent accessibility to
the indole side chain. Mutation of Glu98 to Asp or Val disrupted
interactions and adversely affected the catalytic function of FXIa; thus, compared with pFXIa, the $k_{\text{cat}}$ values for S-2366
hydrolysis by the E98D and E98V mutants were reduced by
35-fold and 10-fold, respectively. The fact that the mutation of
Glu$^{98}$ to an alanine resulted in a minor (2-fold) decrease
in $k_{\text{cat}}$ against S-2366, whereas all three Glu$^{98}$ mutants caused a
highly significant decrease in $k_{\text{cat}}$ against FIX suggests that resi-
due Glu$^{98}$ may be important for macromolecular substrate
selectivity. Thus, the striking differences between FXIa and kal-
lkrein in the positions of Glu$^{98}$ (Figs. 2B and 3A) and His$^{174}$
(Fig. 3A) most likely account partially for their striking differ-
ces in catalytic activity and explain the essential role of Glu$^{98}$
in catalysis of the macromolecular substrate, FIX.

Our molecular modeling studies also predict perturbations of the interactions of neighboring amino acids due to mutations
at residues identified herein to be important for substrate hydrolysis but not inhibitor interaction. The positions of the
various mutants at positions Arg$^{3704}$, Glu$^{98}$, Tyr$^{143}$, and Ile$^{151}$
with respect to active site residues (Ser$^{195}$, His$^{57}$, and Asp$^{102}$)
are shown in Fig. 4A along with Ser$^{214}$, Trp$^{215}$, Asp$^{194}$, and the
NH$_2$-terminal residue Ile$^{16}$. These interactions are important
for determining the architecture of the FXIa active site, which is
perturbed when mutations of some of these crucial residues are
introduced, resulting in impaired catalysis. Glu$^{98}$ lies within 4 Å
of Trp$^{215}$, with which it appears to have a weak van der Waals
interaction. The function of the Ser$^{214}$ residue is not fully
accounted for. In thrombin, mutation of Ser$^{214}$ to alanine
caused a loss in activity (39), whereas in trypsin, a significant
increase in activity occurred (40). The backbone of Ser$^{214}$
is known to be important in substrate binding because it forms a
hydrogen bond with the P1 residue of the substrate (41). Mutations
of Glu$^{98}$ in the model (Fig. 4B) showed movement of the
relative position of the α-carbon of the mutated residue and the
Trp$^{215}$ ring and the relative position of Ser$^{214}$, the hydroxyl
group of which forms a hydrogen bond with the carboxyl oxy-
gen of the Asp$^{102}$. Although it is difficult to predict from the
model the extent of perturbation of the architecture of the active
site by different residues at position 98, it is reasonable to
assume that it is dependent on the size as well as charge of the
mutated residue. As shown in Table 2, the E98D mutant
showed the largest decrease in amidolytic activity (~35-fold),
valine showed a slightly lower decrease (~10-fold), and the ala-
nine mutant displayed a statistically insignificant decrease (~2-
fold), whereas all these mutants manifested major decreases in
$k_{\text{cat}}$ for FIX activation. Although all of the residues mutated are
on the surface of the molecule, serine proteases are very sensi-
tive to even small changes. This is demonstrated by their large
presence in biological systems, as they comprise the largest pro-
tease family, mainly resulting from mutations in only ~50 resi-
dues. This illustrates the wide range of functions and unique
interactions they are able to perform from a shared structure
from only small variations (41, 42).

The autolysis loop (Tyr$^{143}$-Thr$^{154}$) is a surface-exposed loop
structure that has previously been shown to be important for
substrate and inhibitor specificity in FIXa (43), FXa (44), FXIa
(45), thrombin (46), urokinase-type plasminogen activator (47),
and activated protein C (48). In FIXa, residues Arg$^{143}$ and
Lys$^{147}$ have been shown to be involved in substrate (FX) recog-
nition, and Arg$^{150}$ has been shown to be involved in interaction
with antithrombin when in the heparin-activated conforma-
tion (43). Residues Tyr143 and Ile151 of FXIa are both contained within this loop (Fig. 1A). Tyr143 is not conserved among serine proteases; however, in all species for which the FXI sequence is known, Tyr at position 143 is conserved. In the species for which the FXI sequence is known, residue 151 is typically an Ile or a Val. In Homo sapiens, residue 151 is an Ile. Based on the crystal structure of FXIa in complex with PN2KPI (21), Tyr143 of FXIa forms a van der Waals interaction with Met17 of PN2KPI, and Ile151 is within close proximity to Met17. We therefore anticipated the possibility that mutation of Tyr143 and Ile151 to alanine might impair the interaction of the resulting mutants with PN2KPI. However, in experiments measuring the residual activity of Y143A after preincubation with PN2KPI, there was no observed difference in the inhibition compared with that of pFXIa. In assays measuring amidolytic activity, however, the Y143A mutant demonstrated a normal $K_m$; however, the $k_{cat}$ value was decreased ~30-fold compared with pFXIa. Likewise, Y143A was defective in catalyzing FIX activation (Table 2). Likewise, the $K_m$ for hydrolysis of S-2366 by FXIa I151A was not significantly different from that for pFXIa. Catalysis of S-2366, however, was affected by this mutation with a ~3.5-fold decrease in $k_{cat}$ value compared with pFXIa. Similarly, the $k_{cat}$ value for catalysis of FIX was decreased (Table 2). The $K_i$ value for the inhibition of Y143A or I151A by pAB was not significantly different from that for pFXIa, indicating that there was no gross structural rearrangement of the S1 specificity site. Tyr143 is within hydrogen bonding distance to Lys192 (Fig. 3B), a residue that has been demonstrated to be important for inhibitor and substrate specificity in trypsin, FXa, activated protein C, and thrombin (49–53). Also within close proximity is Ile16, the residue that forms a salt bridge with Asp194 upon activation of FXI (Fig. 4A). By changing Tyr143 or Ile151 to Ala, the interaction of residue 143 with Lys192 may be adversely affected, thereby causing the overall structure of the oxyanion hole, formed by the adjacent residues Gly193 and Ser195, to be affected as well. The amide nitrogen of Gly193 may no longer be

FIGURE 4. Intramolecular interactions of active site (Ser195, His57, Asp102) and exosite (Ile151, Glu98, Tyr143, and Arg3704) residues involved in substrate (but not inhibitor) hydrolysis within the catalytic domain of FXIa (Protein Data Bank code 1ZJD). A, overview of the architecture of residues in close proximity to the active site. B, energy-minimized structures of Glu98 mutations. C, energy-minimized structure of Y143A mutation. D, energy-minimized structure of R3704A mutation. See “Results” for explanation and discussion.
properly oriented, thereby not allowing the complete formation of the oxyanion hole. From these experiments, we conclude that Tyr\textsuperscript{143} and Ile\textsuperscript{151} have no important role in inhibition by PN2KPI but are important for the normal catalysis of both FIX and S-2366. In our molecular modeling studies (Fig. 4C), no major conformational alteration in the interaction of Ile\textsuperscript{16} with Asp\textsuperscript{194} for FXIa/Y143A or FXIa/I151A could be demonstrated. However, a significant increase in energy (35.4 kcal/mol as a consequence of the Y143A mutation and 3.46 kcal/mol as a consequence of the I151A mutation) was observed in the autolysis loop (Tyr\textsuperscript{143}–Thr\textsuperscript{154}), reflecting disordered structure. Upon inspection of the crystal structure of zymogen FXI (54), it was found that the autolysis loop contains a high amount of thermal movement, as indicated by \(b\)-factors of 140–143 in Lys\textsuperscript{145} and 103–117 in Glu\textsuperscript{142} whereas, in contrast, in the crystal structure of the enzyme FXIa (21), the \(b\)-factors of Lys\textsuperscript{145} (79–83) and Gly\textsuperscript{142} (63–65) were significantly lower. This suggests that the movement of the N-terminal amine of the Ile\textsuperscript{16} residue into close proximity (2.8 Å) of the backbone oxygen of Tyr\textsuperscript{143} has a stabilizing effect upon the autolysis loop. The change in energy effected by the I151A mutation is postulated to result in altered dynamics within the autolysis loop before Ile\textsuperscript{151} has a chance to interact with and stabilize it. Because it is known that the autolysis loop undergoes a significant movement after activation in serine proteases and because it is in the activation domain (41), the mutation of Ile\textsuperscript{151} to alanine may reduce the likelihood of successful active site formation and the resultant impaired activity of the mutant enzyme.

Previous studies focusing on the contributions of basic residues within the autolysis loop of FXIa to serpin specificity (45) determined that mutagenesis of basic residues within the autolysis loop (Arg\textsuperscript{144}, Lys\textsuperscript{145}, Arg\textsuperscript{147}, and Arg\textsuperscript{149}) has no adverse effects on the conformation of the FXIa S1–S3 substrate-binding sites or the capacity of these autolysis loop mutants to cleave the chromogenic substrate S-2366. Our present studies demonstrate that mutation of two additional residues within the autolysis loop of FXIa, Tyr\textsuperscript{143} and Ile\textsuperscript{151}, have no adverse effects on the conformation of the FXIa S1–S3 substrate-binding sites or the PN2KPI binding site. However, these two residues are required for normal substrate hydrolysis, as shown in Table 2 and depicted in Figs. 1 and 4A, in which a hydrophobic interaction between Tyr\textsuperscript{143} and Ile\textsuperscript{151} is demonstrated to be part of interactions involving Lys\textsuperscript{192}, Asp\textsuperscript{194}, and Ile\textsuperscript{16} that are essential for maintaining the normal architecture of the catalytic site.

Arg\textsuperscript{3704} is a residue unique to FXI and is located on a surface-exposed loop region (residues 36–3704, chymotrypsin numbering, or 390–395 in mature FXI or 71–76 of the catalytic domain of FXIa) that is slightly longer than that in other serine proteases. We investigated this unique residue to assess its importance in FXIa activity. The S1 site of the R3704A mutant was intact, with a \(K_i\) value for pAB binding comparable with that of pFXIa. The value of \(k_{cat}\) was significantly decreased for S-2366 cleavage (\(\sim 25\)-fold) compared with pFXIa and was also decreased in FIX catalysis (Table 2). However, it is unclear from inspection of the FXIa crystal structure (Figs. 1 and 4D) exactly what contribution Arg\textsuperscript{3704} makes to substrate hydrolysis. From the FXIa-PN2KPI structure (Fig. 1 and Table 1), we observed that Arg\textsuperscript{3704} is within hydrogen bonding distance with the main-chain nitrogen atom of Met\textsuperscript{17} and the O\(\gamma\) of Ser\textsuperscript{19} of PN2 (21). Despite the proximity of both Met\textsuperscript{17} and Ser\textsuperscript{19} to Arg\textsuperscript{3704}, a loss of inhibition of this mutant by PN2KPI was not observed. Experiments with alanine substitutions at Met\textsuperscript{17} and Ser\textsuperscript{19} of PN2KPI showed a minimal loss of inhibitory function toward pFXIa with a 1.3- and 1.5-fold decrease in activity, respectively (21). In our molecular modeling studies, the mutation of the Arg\textsuperscript{3704} residue to alanine had a significant effect on the local backbone position (Fig. 4D), which was accompanied by a 4.3-kcal/mol rise in energy associated with the surrounding loop structure. This is probably a consequence of an alteration in the interaction between the side chains of Ile\textsuperscript{151} and His\textsuperscript{38}. However, unlike the small decrease in activity that arises from the I151A mutation, the R3704A mutant displayed a large (25-fold) decrease in activity. Therefore, there is likely to be another mechanism accounting for the relatively large effect of the R3704A mutation that is not displayed in the model.

In summary, based on the structure of the complex between the catalytic domain of FXIa and the KPI domain of PN2, we have identified six residues clustered around the active site of FXIa for mutational analysis. All 12 mutant molecules prepared were characterized by reasonably normal S1 specificity site binding to pAB and normal binding affinity to the small peptide substrate S-2366, suggesting normal active site architecture. Mutations at four sites (Glu\textsuperscript{98}, Tyr\textsuperscript{143}, Ile\textsuperscript{151}, and Arg\textsuperscript{3704}) produced enzymes that were normal in inhibitor (PN2KPI) recognition, whereas mutations at the two remaining sites (Lys\textsuperscript{192} and Tyr\textsuperscript{901}) produced enzymes that were deficient in inhibitor (PN2KPI) recognition. In addition, mutations at all six residues (Glu\textsuperscript{98}, Tyr\textsuperscript{143}, Ile\textsuperscript{151}, Arg\textsuperscript{3704}, Lys\textsuperscript{192}, and Tyr\textsuperscript{901}) resulted in enzymes that were deficient in macromolecular (FIX) substrate catalysis. Of the six residues, only four (Tyr\textsuperscript{143}, Ile\textsuperscript{151}, Arg\textsuperscript{3704}, and Tyr\textsuperscript{901}) were found to be important for small substrate hydrolysis. Impaired cleavage of macromolecular substrate (FIX) and not of the small peptide (S-2366) hydrolysis by the two mutants FXIa/E98A and FXIa/K192A indicates that mutations at these two residues (Glu\textsuperscript{98} and Lys\textsuperscript{192}) disrupt exosite interactions of the enzyme with FIX, suggesting that these two residues are part of an exosite for macromolecular (FIX) but not small peptide substrate activation.

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FXIa Substrate Catalysis and Inhibition by PN2

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