Thrombosis can lead to life-threatening conditions such as acute myocardial infarction, pulmonary embolism, and stroke. Although commonly used anti-coagulant drugs, such as low molecular weight heparin and warfarin, are effective, they carry a significant risk of inducing severe bleeding complications, and there is a need for safer drugs. Activated Factor XI (FXIa) is a key enzyme in the amplification phase of the coagulation cascade. Anti-human FXI antibody significantly reduces thrombus growth in a baboon thrombosis model without bleeding problems (Gruber, A., and Hanson, S. R. (2003) Blood 102, 953–955). Therefore, FXIa is a potential target for anti-thrombosis therapy. To determine the structure of FXIa, we derived a recombinant catalytic domain ofFXI, consisting of residues 370–607 (rhFXI370–607). Here we report the first crystal structure of rhFXI370–607, in complex with a substitution mutant of ecotin, a pan-
serine protease protein inhibitor secreted by Escherichia coli, to 2.2 Å resolution. The presence of ecotin not only assisted in the crystallization of the enzyme but also revealed unique structural features in the active site of FXIa. Subsequently, the sequence from P5 to P2’ in ecotin was mutated to the FXIa substrate sequence, and the structures of the rhFXI370–607-ecotin mutant complexes were determined. These structures provide us with an understanding of substrate binding interactions of FXIa, the structural information essential for the structure-based design of FXIa-selective inhibitors.

Crystalllographic analysis and refinement of the rhFXI370–607-ecotin complex refined to a clear 2.2 Å electron density map with an R factor of 23.6% and an Rfree of 26.5%. The model of FXIa includes residues 370–607, 12 residues of the ecotin substrate, and 12 residues of the C-terminal domain of ecotin. The catalytic center of FXIa, with the catalytic triad consisting of Cys391, Asp392, and His394, is conserved relative to FXIIa and FXIIIa, which have been reported to be involved in the catalytic mechanism. The metal ion binding site was determined from the electron density map and refined with high occupancy, suggesting that the metal ion is essential for the catalytic activity of FXIa.

The co-crystallization of FXIa with ecotin provides insights into the structure-based design of FXIa-selective inhibitors. The binding site of ecotin at the catalytic center of FXIa is unique, with the C-terminal domain of ecotin interacting with the catalytic residues of FXIa. This interaction is critical for the catalytic activity of FXIa, as observed in the structure of the rhFXI370–607-ecotin complex. The results of this study provide a structural basis for the development of novel anti-coagulant agents that might be advantageous over existing inhibitors for prophylaxis and treatment of thrombotic disorders.

FXIa is a 160-kDa protein composed of two identical 80-kDa subunits that are linked by a disulfide bond and is activated by cleavage after residue 369 of each of the two subunits,
producing a protein that contains two heavy and two light chains. Each heavy chain contains four tandem repeat sequences, designated “apple” domains, containing binding sites for platelets (12), heparin (13), and many proteins such as thrombin (14), high molecular weight kininogen (15), factor XIIa (16), and glycoprotein Ibα (17). Each light chain, consisting of residues 370–607, contains a catalytic domain. The catalytic domain of FXIa is a trypsin-like serine protease, highly homologous to the catalytic domains of other factors in the blood coagulation cascade, such as FXa, FVa, and thrombin. To develop FXIa-selective inhibitors using structure-based ligand design, it is essential to obtain the three-dimensional structure of FXIa and to identify unique features of the FXIa catalytic domain.

To obtain the first structure of the catalytic domain of FXIa (rhFXI370–607) and ecotin, we formed a complex between rhFXI370–607 and ecotin. Ecotin is a 142-amino acid pan-serine protease inhibitor found in the periplasm of *Escherichia coli* (18). The protein exists as a dimer and inhibits a broad range of serine proteases. The ecotin dimer interacts with two serine protease molecules to form a tetrameric complex. Several crystal structures of serine proteases in complex with ecotin have been reported (19–23). The unique arrangement of the interactions in the serine protease-ecotin tetramer and the adaptability of the ecotin dimer suggested that ecotin could assist in the crystallization of many serine proteases, including rhFXI370–607. Here we report three rhFXI370–607-ecotin structures. The first is the rhFXI370–607-ecotinM84R structure, in which the P₁ residue of ecotin has been mutated from methionine to arginine. The second is rhFXI370–607-ecotinD structure, in which the ecotin residues P₅ through P₉ have been replaced with residues from the FXIa substrate FIX (9,NDTFRVNY). The last is rhFXI370–607-ecotinP, in which ecotin residues P₂ to P₉ were mutated to the substrate sequence of FXIa (8,NDTFRVNY), which helped to explain structural differences between rhFXI370–607-ecotinM84R and rhFXI370–607-ecotinD. All together, these structures not only reveal three-dimensional structural information for FXIa, but also demonstrate the possible interaction of the enzyme with its substrate.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant FXI Catalytic Domain**—The recombinant FXI catalytic domain, amino acid residues 370–607, was expressed as a secreted protein in the methanolotrophic yeast *Pichia pastoris*. A detailed report on cloning and expression will be presented elsewhere.2 The enzyme was initially separated from conditioned medium using ammonium sulfate precipitation (50–80% saturation). The enzyme was further purified by immobilized Zn^2+^-chelate chromatography (Zn-IMAC, Chelating Sepharose Fast Flow, Amersham Biosciences) and cation exchange chromatography (SP-Sepharose Fast Flow, Amersham Bioscience). To remove high mannose-type oligosaccharides side chains, the enzyme was treated with endoglycosidase H (New England Biolabs) at a concentration of 500 units/mg rhFXI370–607 for 90 min prior to the ion exchange step. The purity of the protein was estimated by SDS-PAGE analysis on 8–16% polyacrylamide gels (Invitrogen) and by RP-HPLC analysis (data not shown). Protein concentrations were also estimated by RP-HPLC using a calibration curve with commercially purchased, native FXIa (Hematologic Technologies, Inc., Essex Junction, VT). The protein was reduced by treatment with 10 mM dithiothreitol to allow separation of the heavy and light chains during chromatography, and only the peak derived from the light chain was used for calibration purposes. Enzymatic activity was measured using a peptide substrate pyroGlu-Pro-Pro-7-methylamidocoumarin. This is a modification of the standard peptide substrate S2266 in which the chromogenic moiety p-nitroaniline was replaced with the fluorogenic reporter, 7-methylamidocoumarin.

2 P. Pandey, K. J. Seidl, F. Bibbins, J. E. Strickler, and D. T. Weaver, manuscript in preparation.
dimer and serine proteases that allows ecotin to facilitate the crystallization of the different serine proteases forming a complex with it. Because the catalytic domain of FXIa is a serine protease, we proposed that ecotin would be an inhibitor for FXIa and that ecotin would facilitate the crystallization of rhFXI370–607. Furthermore, ecotinM84R was designed to interact better with the aspartate residue that all trypsin-like serine proteases possess in the substrate binding site. The ability of each of the mutants to inhibit the peptidolytic activity of FXIa was assessed. The IC50 values for ecotinM84R, ecotinP, and ecotinD were 3, 200, and 600 nM, respectively. However, subsequent results suggest that ecotinP and ecotinD were acting as competitive substrates, see below. The rhFXI370–607 -ecotinM84R tetramer is similar to other serine proteases of the chymotrypsin fold (Fig. 1B). The globular structure is formed by two β-barrels with a few helical segments and many loops. The catalytic triad, composed of Ser195, His57, and Asp102 is located at the junction between the extended C-terminal regions of the two ecotins. The corresponding residue numbers for the FXI sequence and trypsin numbering system are listed in the Supplemental Material (Table S1).

The rhFXI370–607 Structure in the rhFXI370–607-EcotinM84R Complex—The overall structure of rhFXI370–607 is similar to other serine proteases of the chymotrypsin fold (Fig. 1B). The globular structure is formed by two β-barrels with a few helical segments and many loops. The catalytic triad, composed of Ser195, His57, and Asp102 is located at the junction between the two β-barrel domains. A unique feature of rhFXI370–607 revealed in the rhFXI370–607-EcotinM84R structure is that the side chain of Glu98 (Fig. 1B) points into the active site, where it does not appear to have a complementary polar environment. One of the oxygen atoms of the Glu98 side chain forms a hydrogen bond with its main-chain nitrogen atom, and the other oxygen atom is within hydrogen bonding distance to the carboxyl oxygen of ecotin Leu92. Because Glu98 interacts with ecotin Leu92, this orientation of Glu98 might be ecotin-induced. Another unique feature is the orientation of Tyr59A, located near the catalytic His57 (Fig. 1B), which points away from the protein and is in van der Waals contact with the ecotin 50s loop. Although it was not clear from this structure whether the positioning of Tyr59A in rhFXI370–607 is ecotin-induced, subsequent crystal structures have shown that this is a feature of rhFXI370–607, and is not induced by either ecotin or crystal packing. However, the side-chain position of Glu98 varies from structure to structure.

The EcotinM84R Structure—The modified jellyroll fold of the ecotin monomer is preserved in the rhFXI370–607-EcotinM84R structure. The extended C-terminal regions of the two ecotins form anti-parallel β-sheets and wrap around each other at the

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**Table I**

Data collection and refinement statistics

|                      | rhFXI370–607 | rhFXI370–607-ecotinM84R | EcotinD | EcotinP |
|----------------------|--------------|-------------------------|--------|--------|
| **Diffraction data** |              |                         |        |        |
| Resolution (Å)       | 50–2.2       | 50–2.9                  | 30–2.6 | 30–2.6 |
| (Last shell)         | 2.28–2.20    | 2.69–2.6                | 2.9     | 2.9    |
| Space group          | P2_12_1    | P2_12_1             | P2_12_1| P2_12_1|
| Unit cell dimensions (Å) | a = 44.6 | a = 44.5          | b = 91.4| b = 90.1|
|                      | c = 186.9   | c = 188.6             | b = 91.4| b = 90.1|
| Completeness (%)     | 90.2 (69.1) | 92.9 (97.8)           | 90.5 (90.9)|       |
| Redundancy           | 4.2         | 5.0                    | 5.6    |        |
| Rmerge (%)           | 9.9 (38.6)  | 12.8 (46.1)            | 9.4 (41.7)|       |

**Data collection and refinement statistics**

- **Data collection and refinement statistics**
  - **Resolution (Å)**: 50–2.2 for rhFXI370–607, 50–2.9 for rhFXI370–607-ecotinM84R, 30–2.6 for EcotinD, and 30–2.6 for EcotinP.
  - **Space group**: P2_12_1 for all structures.
  - **Unit cell dimensions (Å)**: a = 44.6 for rhFXI370–607, a = 44.5 for rhFXI370–607-ecotinM84R, b = 91.4 for EcotinD, and b = 90.1 for EcotinP.
  - **Completeness (%)**: 90.2 (69.1) for rhFXI370–607, 92.9 (97.8) for rhFXI370–607-ecotinM84R, 90.5 (90.9) for EcotinD.
  - **Redundancy**: 4.2 for rhFXI370–607, 5.0 for rhFXI370–607-ecotinM84R, 5.6 for EcotinD.
  - **Rmerge (%)**: 9.9 (38.6) for rhFXI370–607, 12.8 (46.1) for rhFXI370–607-ecotinM84R, 9.4 (41.7) for EcotinD.

**Additional statistics**

- **Completeness (%)**: 90.2 (69.1) for rhFXI370–607, 92.9 (97.8) for rhFXI370–607-ecotinM84R, 90.5 (90.9) for EcotinD.
- **Redundancy**: 4.2 for rhFXI370–607, 5.0 for rhFXI370–607-ecotinM84R, 5.6 for EcotinD.
- **Rmerge (%)**: 9.9 (38.6) for rhFXI370–607, 12.8 (46.1) for rhFXI370–607-ecotinM84R, 9.4 (41.7) for EcotinD.

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3. L. Jin, P. Pandey, R. E. Bahine, D. T. Weaver, S. S. Abdel-Meguid, and J. E. Strickler, unpublished data.
dimer interface of ecotin (Figs. 1A and 2). When the Cα/H9251 atoms of an ecotinM84R monomer (chain C, residues 9–60, 71–75, and 105–134) are compared with other ecotin monomers derived from the structure of the ecotin dimer (PDB code: 1ECZ), the trypsin-ecotin complex (1EZS), the collagenase-ecotin complex (1AZZ), the granzyme B-ecotin complex (1FI8), the thrombin-ecotin complex (1ID5), and the FXa-ecotin complex (1P0S), the root mean square deviation is between 0.53 and 1.19 Å. The ecotin monomer in the rhFXI370–607-ecotinM84R complex is most similar to that in the thrombin-ecotin structure and most divergent from the granzyme B-ecotin structure. As illustrated in Fig. 2, most of the differences are located in ecotin residues 80–100 to accommodate the uniqueness of the active sites in different proteases. There is a slightly altered spatial orientation of the primary and secondary binding sites with respect to each other. When the Cα atoms of one ecotin monomer in the different ecotin dimers structures described above were superimposed, the relative positions of the second monomer diverged considerably (Fig. 2). The dimericity of rhFXI370–607-ecotinM84R is much higher than that of the granzyme B-ecotin complex. The root mean square deviation is between 0.53 and 1.19 Å. The dimeric structure of a protein is responsible for the ability of ecotin to inhibit a variety of serine proteases.

The structural differences in the ecotin dimer are also reflected in the differences in the protease-ecotin tetrameric structures. In fact, molecular replacement searches did not produce a reasonable solution using either the tetrameric structures of trypsin-ecotin or collagenase-ecotin as search models against the diffraction data of rhFXI370–607-ecotinM84R. The tetrameric structure of granzyme B-ecotin, however, yielded an unambiguous solution from the rotational and translational search of molecular replacement.  

**Interactions between EcotinM84R and rhFXI370–607 at the Primary Binding Site**—The primary binding site of ecotinM84R interacts extensively with the active site of rhFXI370–607 in a substrate-like manner (Fig. 3). Eight residues of the ecotin 80s loop, residues 81–88, on both sides of the scissile bond, are in direct contact with rhFXI370–607. The binding pockets of serine proteases are defined according to the residues of its substrate, thus the P1 residue of the substrate (located C-terminal to the scissile bond) occupies the S1 site of the protease, P1 (located C-terminal to the scissile bond) re-

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**Figure 1. Stereoview of the Crystal Structure of rhFXI_{370–607}-ecotinM84R.** A, a trace of rhFXI_{370–607}-ecotinM84R tetramer. The ecotinM84R molecules are colored in blue and yellow, and the rhFXI_{370–607} molecules are colored in green and red. The N and C termini of each molecule are labeled in the same color as the molecule. The primary and secondary binding sites between rhFXI_{370–607} and ecotinM84R are indicated by arrows. B, a ribbon diagram of the catalytic domain of FXa in the rhFXI_{370–607}-ecotinM84R crystal structure. The α helices are colored in purple and β sheets are in green. A few key residues, discussed in the text, are presented in a ball-and-stick representation with CPK coloring. The N and C termini are indicated. All figures were prepared with DINO (www.dino3.org) and Povray (www.povray.org).
sides in the S₁ site, and so on. Cys^{50} of ecotin forms a disulfide bond with ecotin Cys^{87} (P₃) to anchor the 80s loop, whereas many hydrogen bonds between the ecotin 50s and 80s loops provide additional support.

The S₁ binding pocket of rhFXI_{370–607} is the only deep pocket in rhFXI_{370–607} used for substrate binding (Fig. 3). Ecotin Arg^{84} fits into the S₁ pocket of rhFXI_{370–607}. In one pair of the rhFXI_{370–607}–ecotinM84R complexes, the guanidinium group of ecotin Arg^{84} (chain C) forms salt bridges with both oxygen atoms of Asp^{180} of rhFXI_{370–607} (chain A). The conformation of ecotin Arg^{84} and its interactions with rhFXI_{370–607} involving two water molecules, are very similar to the corresponding residues in the structures of trypsin-ecotin and thrombin-ecotin complexes. In the other pair of the rhFXI_{370–607}–ecotinM84R complexes, the guanidinium group of ecotin Arg^{84} (chain D) forms only a single salt bridge with one of the oxygen atoms of Asp^{180} (chain B). Furthermore, there is only one water molecule mediating the interaction between ecotin Arg^{84} and the S₁ pocket. In both pairs, the main chain oxygen of ecotin Arg^{84} is stabilized by the oxyanion hole of rhFXI_{370–607} formed by the main-chain nitrogen atoms of Gly^{193} and Ser^{195}.

The S₂, S₃, and S₄ binding sites are less well defined (Fig. 3), although the S₂ loop of rhFXI_{370–607} (residues 96–100, including Glu^{98}) is in a unique conformation compared with other serine proteases (see discussion below, Fig. 4). Ecotin Thr^{83} (P₃) is in van der Waals contact with His^{57} of the S₂ area but does not form any direct polar contacts with rhFXI_{370–607}. The side chain of ecotin Ser^{82} (P₃) is facing the solvent and makes van der Waals interactions with the side chain of ecotin His^{83}. The main-chain nitrogen and oxygen atoms of ecotin Ser^{82} form two hydrogen bonds with the main-chain oxygen and nitrogen atoms of Gly^{216} of rhFXI_{370–607}, respectively. These main-chain to main-chain interactions have been observed in numerous other peptide or peptide-mimetic compounds interacting with serine proteases (27). Ecotin Val^{81} (P₄) is in van der Waals contact with Trp^{215} and His^{174} of the S₃ pocket, as well as Glu^{98} of the S₂ loop. The ecotin 80s loop interacts with not only the ecotin 80s loop, but also the primary binding site of rhFXI_{370–607}. Ecotin Leu^{82} occupies the space above the S₃ loop.

The four ecotin residues on the prime side are in van der Waals contact with rhFXI_{370–607}. The main-chain carbonyl oxygen of ecotin Ala^{86} (P₂) is within hydrogen bonding distance of the NH₁ of Arg^{37D} of rhFXI_{370–607} in both chains C and D. The main-chain nitrogen of ecotin Ala^{86} in chain D forms a hydro-
tional bond with the carbonyl oxygen of Leu$^{29}$ of rhFXI$_{370-607}$ in chain B. The guanidinium groups of Arg$^{171}$ and Arg$^{173}$ in both chains A and B of rhFXI$_{370-607}$ pack against ecotin Pro$^{28}$ (P$_4$). FXIa is very selective for its substrate. More research is required to identify which site or sites in FXIa are the key selectivity elements for its substrate binding.

**Structure Comparison of rhFXI$_{370-607}$ with Other Serine Proteases**—To identify features specific to FXIa, we compared the structure of rhFXI$_{370-607}$ to human trypsin (1TRN) and other related proteases in the coagulation cascade: thrombin (1H8I), FVIIa (1QFK), FIXa (1RFN), and FXa (1HCG) (Fig. 4). All the structures were aligned with rhFXI$_{370-607}$ by superimposing the Ca atoms of the catalytic triads. The core structure is similar and superimposes well in all of these serine proteases; however, the lengths and conformations of surface loops are quite different. The following discussion is based on the orientation shown in Fig. 4.

The conformation of the S$_1$ pocket of FXIa resembles that of the other trypsin-like proteases. Asp$^{189}$, at the base of the S$_1$ pocket, interacts with an arginine residue of the substrate. The residues forming the S$_1$ pocket are conserved except for three residues at 190, 192, and 213 (Table II). It is useful to distinguish three classes of trypsin-like serine proteases: those with an ala, serine, or threonine at position 190. The subtle difference between these residues changes the hydrogen-bonding network in the S$_1$ pocket and influences inhibitor binding and selectivity as determined for urokinase-type plasminogen activator, a trypsin-like serine protease that has Ser$^{190}$ (28–30).

FXIa has an ala, which lacks the polar group of serine or threonine at that position, but provides a slightly larger S$_1$ pocket and is preferred for hydrophobic interactions with ligands. The residue at 192 is involved in binding to ligands and protein inhibitors. The Gln or Glu at position 192 contributes to inhibitor binding in trypsin, FXa, and thrombin (31–33). FXI, as well as FVIIa, has a lysine at position 192. Lys$^{192}$ could be important for substrate specificity and as a potential selectivity element. Residue 213, in close proximity to residue 192, forms the back wall of the S$_1$ pocket. FXIa has a threonine at this position rather than the valine or isoleucine residue found in the other enzymes. However, the O$_\gamma$ points away from the S$_1$ pocket, and the side chain adopts the same rotamer as valine in other enzymes. Therefore, Thr$^{213}$ is unlikely to be useful as a selectivity element for FXIa.

Directly facing the S$_1$ pocket in rhFXI$_{370-607}$ and perpendicular to the catalytic triad, there is a shallow channel lined with hydrophobic residues that we have named the prime-side channel (Fig. 4). It is formed by residues 34–39 (the $37s$ loop) and residues 59–65 (the 60s loop). The lengths of the $37s$ and 60s loops vary among different serine proteases (Table III), and the amino acids in the loops are different. These variations contribute to the distinct sizes, shapes, and properties of the region. Although it is not clear what role the prime-side channel plays, it may be related to substrate recognition and protein-protein interactions for each protease. Of particular interest is the positioning of Tyr$^{59a}$ of FXIa at the mouth of this channel forming an opened door allowing access to the catalytic triad; however, additional structural analysis is required to confirm that this is the natural position of this residue. At least for this complex, this residue helps to define the shape of S$_2$ in FXIa.

The prime-side channel of FXIa may provide selectivity elements for designing specific inhibitors.

The S$_2$ loop of FXIa folds toward the active site cleft from the bottom of the catalytic triad (Fig. 4). The numbers of residues forming this loop do not vary significantly (Table III), but the conformation of the loop differs among the serine proteases. The S$_2$ loop of FXIa is most similar to that of trypstatine (34), with Ala$^{97}$ and Glu$^{98}$/Gln$^{98}$ (rhFXI$_{370-607}$/trypstatine). In both enzymes, Glu$^{98}$/Gln$^{98}$ orients toward the center of the active site cleft. Although the position of the acidic head group of Glu$^{98}$ varies in different FXIa structures, the aliphatic portion of the Glu$^{98}$ side chain is always in van der Waals contact with Trp$^{215}$ and obstructs solvent access to its indole side chain. The unique position of Glu$^{98}$ reduces the size of both the S$_2$ and S$_4$ regions in FXIa and could be a key selectivity element for FXIa.

FXIa has a small S$_4$ pocket with charged and polar amino acids. Trp$^{215}$ is conserved in many trypsin-like serine proteases and forms the back wall of the S$_4$ subsite; however, the numbers and properties of the residues forming the front of the S$_4$ site (residues 170–174, part of the S$_4$ loop consisting of residues 170–176 in FXIa) differ in serine proteases. These differences, in turn, determine the different shapes and properties of the region. FXIa cleaves FIX at two locations that have either a lysine or an aspartate at P$_4$. In the rhFXI$_{370-607}$/ectinM84R structure, ectin has a valine at P$_4$. From this structure, it is not clear how the charged P$_4$ residue in the natural substrate interacts with the S$_4$ pocket.

There are several loops, most of which are remote from the active site, that are different in FXIa compared with other serine proteases. The 80s loop (residues 71–81 of FXIa) is the calcium-binding loop in trypsin, FVIIa and FXIa. FXIa does not bind calcium and does not have calcium-chelating residues; therefore, the 80s loop of FXIa is in a different conformation. Residues 146 and 147 of FXIa are located on the 146s loop (residues 144–149) and protrude over the S$_1$ pocket, creating a small area that has been demonstrated to participate in inhibitor binding in urokinase (35), as well as in thrombin (36). This loop shows a high degree of flexibility in the FXIa structures reported in this report, as well as in FXIa structures in complex with small molecule ligands. Similarly, this loop is disordered in some thrombin (36) and FXa structures (37). The different shape and properties of this region in serine proteases provide opportunities to design inhibitors specific for FXIa versus other serine proteases.

**The Heparin Binding Site on rhFXI$_{370-607}$ Is in a Large Positively Charged Area**—Heparin is reported to facilitate the inhibition of rhFXI$_{370-607}$ by the protease nexin II, a multifunctional protein containing a Bowman-Burk type protease inhibitor domain that is secreted by activated platelets and has been suggested to be a physiologically relevant inhibitor of FXIa (38). In the presence of heparin, the K$_I$ of protease nexin II for FXIa decreased from 436 ± 62 to 88 ± 10 pm (39). Through mutation and peptide inhibition studies, the heparin-binding site has been located on the catalytic domain of FXIa and involves residues Lys$^{170}$, Arg$^{171}$, and Arg$^{173}$ (39). In our FXIa structure, these residues are part of the S$_1$ loop on the back side of the S$_1$ pocket. It is likely that the binding of heparin changes the conformation of the S$_1$ loop and the active site, thus facilitating the binding of protease nexin II.

| Residue/enzyme | FXIa | Trypsin | FXa | FIXa | FVIIa | Thrombin |
|----------------|------|---------|-----|------|-------|----------|
| 190            | Ala  | Ser     | Ala | Ser  | Ser   | Ala      |
| 192            | Ser  | Glu     | Gln | Gln  | Glu   | Gly      |
| 213            | Thr  | Val     | Thr | Val  | Val   | Val      |
| 190            | Ala  | Ser     | Ala | Ser  | Ser   | Ala      |
| 192            | Ly$^8$ | Gln    | Gln | Gln  | Lys   | Glu      |
| 213            | Thr  | Val     | Thr | Val  | Val   | Val      |
It is interesting that these three positively charged residues in rhFXI370–607 are part of a large, positively charged area formed by residues Lys 170, Arg 171, Arg 173, Arg 185, Arg222, and Arg224. The large size of this positively charged area is unique in FXIa compared with other serine proteases.

Negatively charged glycosaminoglycans have been shown to affect the activation of FXI and inactivation of FXIa by serine protease inhibitors. FXI can be activated on immobilized dextran sulfate and heparin sulfate (4, 40). In addition, glycosaminoglycans have been shown to enhance the inhibition of FXIa by C1 inhibitor, anti-thrombin III, and proteasenexin II (39, 41, 42). Polyanions can inhibit the hydrolysis reaction of the peptide substrate S2366 (pyroGlu-Pro-Arg-p-nitroanilide) and FIX activation by FXIa in a concentration-dependent manner (43). Based on the fluorescence characteristics of 5-(dimethylamino)-1-(naphthalenesulfonyl)-glutamylglycylarginyl-FXIa in the presence or the absence of a negatively charged surface, it has been suggested that binding of FXIa to the polyanions heparin and dextran sulfate results in an allosteric modification of its functional activity (43). It is very likely that the function of this large positively charged patch on FXI is to interact with negatively charged surfaces and, in turn, to regulate FXIa activity. Another patch of positive residues (Arg144, Lys145, Arg147, and Lys149) has been shown not to be involved in heparin binding (39).

**rhFXI370–607-EcotinD Structure**—Most of the 18 known families of macromolecular serine protease inhibitors have a cysteine involved in a disulfide linkage located somewhere between P5 and P2/H11032 (44). Ecotin, along with the Kunitz soybean trypsin inhibitor and potato-I inhibitor, does not have this disulfide. These classes of inhibitors provide the opportunity to introduce the sequence of a natural substrate into a substrate-like macromolecular inhibitor. In a previous study, the natural P4 to P1 substrate sequence for the serine protease granzyme B was engineered into ecotin (21). We designed EcotinD, with its P5 to P2/H11032 residues mutated to match the corresponding sequence of the FXIa substrate FIXP580NDTRVV86. This is the most extended natural sequence introduced into a protein-based inhibitor to date. Since protease enzymes such as FXIa are highly selective for the sequences they cleave, EcotinD provides us with a model system to examine the details of the interactions between P5 and P2 of the natural substrate with the S5 to S2 subsites of FXIa. When reducing SDS-PAGE was performed on fractions from the gel filtration column used in

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**Table III**

| Number of residues in the loops of FXIa | FXIa | Trypsin | FXa | FIXa | FVIIa | Thrombin |
|----------------------------------------|------|---------|-----|------|-------|----------|
| 37a loop (34–39)                       | 10   | 7       | 8   | 7    | 7     | 8        |
| 60s loop (59–65)                       | 10   | 5       | 7   | 7    | 10    | 15       |
| S2 loop (94–100)                       | 7    | 7       | 7   | 8    | 7     | 8        |
| 146s loop (144–149)                    | 6    | 5       | 5   | 5    | 11    | 5        |

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**FIG. 5. Stereoview of the superposition of the ecotin 50s and 80s loops in the rhFXI370–607-ecotin mutant structures.** rhFXI370–607 of the rhFXI370–607-ecotinM84R structure is presented in gray surface representation. rhFXI370–607-ecotinD and rhFXI370–607-ecotinP structures were superimposed on rhFXI370–607-ecotinM84R by aligning the Cα atoms of the catalytic triads. Ecotin residues 76–85 and 50–56 for each ecotin mutant are presented in stick representation with ecotinM84R in green, ecotinD in red, and ecotinP in purple. The labels for the residues are the same color as the ecotin structure. A, superposition of rhFXI370–607-ecotinM84R and rhFXI370–607-ecotinD structures. B, superposition of rhFXI370–607-ecotinM84R, rhFXI370–607-ecotinD, and rhFXI370–607-ecotinP structures.
purifying the rhFXI_370–607–ecotinD complex, it was noted that the protein band for the full-length ecotinD was replaced by two lower molecular weight bands corresponding to cleavage fragments of ecotin (data not shown). The cleavage of an ecotin mutant by a serine protease was also reported for granzyme B-ecotin (61IEPD64) complex (21). In the crystal structure of granzyme B-ecotin (61IEPD64), the prime-side residues 85–96 of ecotin were not visible in the electron density maps. To our surprise, the rhFXI_370–607–ecotinD structure showed an intact peptide bond between residues 84 and 85 of ecotin. Ecotin and various ecotin mutants were reported to be cleaved by trypsin and/or bovine trypsin (45–48). To reduce the proteolytic activity of trypsin, trypsin DI02N was used to form complexes with ecotin mutants for crystallization studies (47). In our case, an intact ecotin mutant was present in the crystal structure, but showed evidence of being cleaved based on SDS-PAGE analysis. While this ecotin mutant is cleaved by rhFXI_370–607, the prime side of the ecotin molecule is still bound to rhFXI_370–607 by secondary binding sites under non-denaturing conditions.

We speculate that the leaving group (prime side residues) of ecotin were not visible in the electron density maps. To our surprise, the rhFXI_370–607–ecotinD structure showed an intact peptide bond forming, reaction. The overall structure of rhFXI_370–607–ecotinD is very similar to rhFXI_370–607–ecotinM84R. The peptide bonds between residues 84 and 85 in both copies of ecotinD are intact. In one ecotinD molecule (chain D), the prime side residues 88–91 were too flexible to be seen, but the same residues were visible in the other ecotinD molecule (chain C) due to a symmetry related molecule of rhFXI_370–607 packing against the area and stabilizing the loop. When the catalytic triads of rhFXI_370–607, rhFXI_370–607–ecotinM84R and rhFXI_370–607–ecotinD were superimposed (Fig. 5A), the main-chain conformations of residues 79 and 80 in ecotinD were different from those in ecotinM84R. There is a corresponding main-chain flip between Leu52 and His53 in the 50s loop of ecotinD. We suspected these changes might be caused by residue 80, Asn in ecotinD, and Pro in ecotin. This was confirmed in the rhFXI_370–607–ecotinP structure (see below). P1 and P2 of ecotinD are the same as those of ecotinM84R and orient similarly. P3 of ecotinD (Phe85) is packed against the side of P3 (Asn80). P4 (Asp81) is a negatively charged residue, and its Os forms a hydrogen bond with Nε of rhFXI_370–607–His74 in the S2 site.

**rhFXI_370–607–EcotinP Structure**—To confirm that the structural changes in the rhFXI_370–607–ecotinD structure were due to Asn80 of ecotinD, we obtained the crystal structure of rhFXI_370–607–ecotinP with residue 80 of ecotinD changed back to the native Pro of ecotin. EcotinP was also cleaved by rhFXI_370–607 as shown by SDS-PAGE analysis (data not shown). This suggested that P3 is not important in the cleavage of these ecotin mutants by rhFXI_370–607. The backbone conformations of ecotinP residues 79 and 80, as well as residues 52 and 53, are the same as ecotinM84R (Fig. 5B). This confirmed that the backbone changes in the rhFXI_370–607–ecotinD structure were indeed induced by the Pro-to-Asn mutation at residue 80. When the catalytic triads of rhFXI_370–607, were superimposed, rhFXI_370–607–ecotinD showed more deviation from rhFXI_370–607–ecotinM84R than did rhFXI_370–607–ecotinP. This result also demonstrated the adaptability of the ecotin molecule. Although the side-chain orientation of Asp81 (P3) in ecotinP is different from that in ecotinD, Asp81 still interacts with His74 in S2.

All three structures of rhFXI_370–607–ecotinD provided detailed information on rhFXI_370–607 and its substrate-binding interactions. This structural information is very useful for the development FXIa-selective inhibitors.

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