Structural Role of Gly$^{193}$ in Serine Proteases

INVESTIGATIONS OF A G555E (GLY$^{193}$ IN CHYMOTRYPSIN) MUTANT OF BLOOD COAGULATION FACTOR XI*

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INVESTIGATIONS OF A G555E (GLY$^{193}$ IN CHYMOTRYPSIN) MUTANT OF BLOOD COAGULATION FACTOR XI*

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The abbreviations used are: FXI, factor XI; FIX, factor IX; FXIaWT, wild-type factor XI; FXIaG193E, factor XI with Gly$^{193}$ → Glu mutation; FXIa, factor XIIa; APPI, protease nexin-2/α2-microglobulin β protein precursor Kunitz domain inhibitor; TBS, Tris-buffered saline; pNA, p-nitroaniline; S-2288, H-n-Ile-Pro-Arg-p-nitroanilide; S-2366, pyro-Glu-Pro-p-nitroanilide; BSA, bovine serum albumin; PEG, polyethylene glycol 8000; DFP, diisopropyl fluorophosphate; WT, wild type.

In serine proteases, Gly$^{193}$ is highly conserved with few exceptions. A patient with inherited deficiency of the coagulation serine protease factor XI (FXI) was reported to be homozygous for a Gly$^{555}$ → Glu substitution. Gly$^{555}$ in FXI corresponds to Gly 193 in chymotrypsin, which is the numbering system used subsequently. To investigate the abnormality in FXIaG193E we expressed and purified recombinant FXIaG193E and compared its activity to wild type-activated FXIa (FXIaWT). FXIaG193E activated FIX with ~30-fold reduced $K_{cat}$ and similar $K_{m}$, and hydrolyzed synthetic substrate with ~10-fold reduced $k_{cat}$ and modestly reduced $k_{cat}/K_{m}$. Binding of antithrombin and the amyloid β-precursor protein Kunitz domain inhibitor (APPI) to FXIaG193E was impaired ~8000- and ~100,000-fold, respectively. FXIaG193E inhibition by diisopropyl fluorophosphate was ~30-fold slower and affinity for p-amino-benzamidine (S1 site probe) was 6-fold weaker than for FXIaWT. The rate of carboxamidation of NH2-Ile$^{16}$, which forms a salt bridge with Asp$^{194}$ in active serine proteases, was 4-fold faster for FXIaG193E. These data indicate that the unoccupied active site of FXIaG193E is incompletely formed, and the amide N of Glu$^{193}$ may not point toward the oxyanion hole. Inclusion of saturating amounts of p-aminobenzamidine resulted in comparable rates of carboxamidation for FXIaGP and FXIaG193E suggesting that the occupied active site has near normal conformation. Thus, binding of small synthetic substrates or inhibitors provides sufficient energy to allow the amide N of Glu$^{193}$ to point correctly toward the oxyanion hole. Homology modeling also indicates that the inability of FXIaG193E to bind antithrombin/APPI or activate FIX is caused, in part, by impaired accessibility of the S2′ site because of a steric clash with Glu$^{193}$. Such arguments will apply to other serine proteases with substitutions of Gly$^{193}$ with a non-glycine residue.

Amino acids Ser$^{193}$, His$^{37}$, and Asp$^{102}$ (chymotrypsin numbering system) form a catalytic triad, which is integral to the catalytic activity of all serine proteases (1, 2). These residues are located at the entrance to the substrate binding pocket, and their geometry is stabilized by hydrogen bonds. Serine proteases hydrolyze peptide bonds via the formation of tetrahedral transition state intermediates. Stabilization of the transition state intermediate occurs through formation of hydrogen bonds between the oxyanion intermediate and the amido groups of residues Gly$^{193}$ and Ser$^{193}$. The substrate binding sites in the enzyme involved in precise interactions are referred to as Sn...S3, S2, S1, S1′, S2′, S3′, ... Sn′ sites, and the amino acid residues of the substrate or inhibitor that occupy these sites are referred to as Pn, ... P3, P2, P1′, P2′, P3′, ... Pn′, respectively. These complementary sites permit specific alignment of the substrate/inhibitor with the catalytic triad and the oxyanion hole for enzymatic specificity (3). In trypsin-like serine proteases, Asp$^{193}$ is at the bottom of the primary S1 substrate binding site, and forms a salt bridge with the guanidino group of P1 Arg residues in the substrate/inhibitor.

Gly$^{193}$, which is part of the oxyanion hole structural unit, is highly conserved in serine proteases with only a few exceptions (see ‘Discussion’). Several blood coagulation proteins with mutations at Gly$^{193}$ (chymotrypsin equivalent) in their protease domains have been reported. These include FXI (Gly$^{297}$ → Glu), FIX (Gly$^{363}$ → Ala, Arg, Glu, or Val), and FVII (Gly$^{412}$ → Glu or Arg) (4–11). Each patient had normal plasma antigen levels associated with very low coagulant activity. In two cases (FXIaG193V [555] and FXIaG193R [665]) the protein was activated normally (4, 9). These data indicate that the functional abnormalities in these proteins stem from their inability to interact with their biological macromolecular substrate/inhibitors. This has been demonstrated for FIXaG193Y (9).

FXI is a disulfide-linked homodimer with a molecular weight of ~160,000 (12). Deficiency of FXI results in a bleeding diathesis sometimes referred to as hemophilia C, and is most common in the Ashkenazi Jewish population (13, 14). FXI can be activated to FXIa by FXIIs, thrombin, or by autoactivation (15, 16). Upon cleavage of the Arg$^{15}$-Ile$^{16}$ peptide bond, a heavy chain and a light chain are formed that are held

1 The abbreviations used are: FXI, factor XI; FIX, factor IX; FXIaWT, wild-type factor XI; FXIaG193E, factor XI with Gly$^{193}$ → Glu mutation; FXIa, factor XIIa; APPI, protease nexin-2/α2-microglobulin β protein precursor Kunitz domain inhibitor; TBS, Tris-buffered saline; pNA, p-nitroaniline; S-2288, H-n-Ile-Pro-Arg-p-nitroanilide; S-2366, pyro-Glu-Pro-Arg-p-nitroanilide; pAB, p-aminobenzamidine; AT, antithrombin; BSA, bovine serum albumin; PEG, polyethylene glycol 8000; DFP, diisopropyl fluorophosphate; WT, wild type.

2 For comparison, the chymotrypsin amino acid numbering system is used throughout. Residue 555 in FXIa, 363 in FIXa, and 342 in FVIIa corresponds or inhibitors provides sufficient energy to allow the amide N of Glu$^{193}$ to point correctly toward the oxyanion hole. Homology modeling also indicates that the inability of FXIaG193E to bind antithrombin/APPI or activate FIX is caused, in part, by impaired accessibility of the S2′ site because of a steric clash with Glu$^{193}$. Such arguments will apply to other serine proteases with substitutions of Gly$^{193}$ with a non-glycine residue.

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together by a disulfide bond (12, 17). Thus, FXIa contains two heavy chains and two light chains. Each heavy chain contains four Apple domains, and each light chain a serine protease domain containing the catalytic triad His57, Asp102, and Ser195 (18). The new NH2-terminus of the light chain contains the sequence Ile16-Val1-Gly18 (370–372). The NH2-terminal Ile16 (370), which is characteristic of serine proteases, inserts into the protease domain of FXIa, and its NH2 group forms a salt bridge with the COOH group of Asp556 (558). This salt bridge is a defining feature of active serine protease structure (19). FXIa contributes to conversion by activating FIX to FIXa in a Ca2+-dependent manner (20, 21). The activation of FIX by FXIa has been shown to involve the Apple 2, Apple 3, and protease domains in FXIa and the activation peptide region and γ-carboxyglutamic acid domains of FIX (22–25).

In this report, we describe a series of experiments using physiologic macromolecular and small synthetic substrate/inhibitors to discern the nature of the proteolytic defect in a naturally occurring FXI mutant with a Glu substitution for Gly193 (555). The data on the rate of carbamylation of the NH2 group of Ile16, which serves as an index of salt bridge formation with Asp556, as well as p-aminobenzamidine (pAB) binding and the rate of diisopropyl fluorophosphate (DFP) incorporation, strongly indicate that the S1 binding site and oxyanion hole are incompletely formed in the mutant enzyme. Binding of small synthetic substrates, however weak, provides sufficient energy to reorient the amido group of Glu193 and restore the proper conformation of the oxyanion hole. Modeling efforts indicate that impairment of the interaction with physiologic macromolecular substrate/inhibitors is, in part, because of inaccessibility of the S2 site, which is attributable to a steric clash with Glu193 (555).

**EXPERIMENTAL PROCEDURES**

**Reagents—**H-1-O-Ile-Pro-Arg-p-nitroanilide (S-2288) and pyro-Glu-Pro-Arg-p-nitroanilide (S-2366) were purchased from DiaPharma (West Chester, OH). Sodium borohydride was obtained from PerkinElmer (Boston, MA). S-2288 or S-2366. The rate of release of tritiated peptide was measured by quantifying the amount of radioactive peptide released at various times of incubation with FXIa. The procedure used was that described previously (28). Each reaction was carried out in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.5 mg/ml BSA and 5 mM CaCl2 at 37 °C. The concentration of FXIa was varied from 2.0 to 25 μg/ml. The concentrations of FXIaG193E and FXIaG193E were used as controls.

**Kinetics of Factor IX Activation—**The rate of FIX activation was measured by quantifying the amount of radioactive peptide released at various times of incubation with FXIa. The procedure used was that described previously (28). Each reaction was carried out in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.5 mg/ml BSA and 5 mM CaCl2 at 37 °C. The concentration of FIX was varied from 2.0 to 25 μg/ml. The concentrations of FXIaG193E and FXIaG193E were used as controls.

**Measurement of S-2288 and S-2366 Amidolytic Activity of FXIa and FXIaG193E—**Each reaction contained TBS with 5 mM CaCl2, 100 μg/ml BSA, 0.5–1 μM FXIa, and 2 nM FXIaG193E (the results were normalized to 2 nM FXIa protein), and increasing amounts of either S-2288 or S-2366. The rate of release of pNA was measured using a Beckman DU800 spectrophotometer with a Kinetics module at 405 nm for 15 min. An extinction coefficient of 9.9 mM−1 cm−1 at 405 nm was used in calculating the amount of pNA released (29). The initial rate, which is linear, was then converted to micromolar substrate hydrolyzed per min. The program GraFit was used to determine the K0 and kcat values using the Enzyme Kinetics program from Erithacus Software.

In this report, we describe a series of experiments using physiologic macromolecular and small synthetic substrate/inhibitors to discern the nature of the proteolytic defect in a naturally occurring FXI mutant with a Glu substitution for Gly193 (555). The data on the rate of carbamylation of the NH2 group of Ile16, which serves as an index of salt bridge formation with Asp556, as well as p-aminobenzamidine (pAB) binding and the rate of diisopropyl fluorophosphate (DFP) incorporation, strongly indicate that the S1 binding site and oxyanion hole are incompletely formed in the mutant enzyme. Binding of small synthetic substrates, however weak, provides sufficient energy to reorient the amido group of Glu193 and restore the proper conformation of the oxyanion hole. Modeling efforts indicate that impairment of the interaction with physiologic macromolecular substrate/inhibitors is, in part, because of inaccessibility of the S2 site, which is attributable to a steric clash with Glu193 (555).
where A1 and A2 are the percent of FXIa activity at time t and 0 s, respectively. The values of kobs were then plotted against the AT concentration to obtain second-order rate constants.

Interaction of FXIaG193E and FXIaWT with APPI—These reactions were carried out using microtiter well plates from Dynatech. For experiments with FXIaWT, each reaction (150 μl) contained 0.5 mM FXIaWT, 100 μM S-2288, and increasing amounts of APPI in TBS with 5 mM CaCl2 containing 100 μg/ml BSA. For experiments with FXIG193E, each reaction (150 μl) contained 1 mM FXIG193E, 500 μM S-2288, and increasing amounts of APPI in TBS with 5 mM CaCl2 containing 100 μg/ml BSA. Absorbance at 405 nm (pNA release) was measured for up to 4 h in a Bio-Rad model 550 microtiter plate reader (Molecular Devices) and the residual FXIa activity was determined at each time point. The residual activity was then plotted as a percent of initial activity and the first-order rate constants, kobs, for each concentration of DFP used were obtained using Equation 1, where A1 and A2 are the percent FXIa activity at time t and 0 s, respectively. The values of kobs were then plotted against the DFP concentration to obtain second-order rate constants.

Kd determination of Ile26-APPI183 and FXIaG193E by Reaction with NaNCO—These experiments were performed as described by Camire (34). Briefly, each reaction mixture contained 1 μM FXIa mutant or normal protein in 20 mM Hapes, 0.15 mM NaCl, 0.1% PEG 8000, 2 mM CaCl2, pH 7.5 (HBSP). Each experiment was performed in the absence and presence of pAB (10× the Kp of pAB) and each reaction was started by the addition of 0.2 mM NaNCO. The final pH after the addition of NaNCO was 7.5. Every 30 min, a 5-μl aliquot was removed and added to 145 μl of HBSP containing 500 μM S-2288. The residual activity was determined from the initial linear rates of hydrolysis using a Beckman DU 800 spectrophotometer. The residual activity was plotted as a percent of initial activity and kobs for carbanucleation were determined using Equation 1.

Molecular Modeling—The three-dimensional structure information of the zymogen and active serine protease domains of FXIa and FXIaG193E as well as the complexes of APPI and the serine protease domain of FXIa were derived using software from Biosym/MSI (San Diego, CA) and the Swiss-Model server using the optimize mode (36-38). The crystallographically determined structures of chymotrypsinogen (Ref 39, Protein Data Bank code 2gta, and Ref 40, Protein Data Bank code 1ex3) and trypsinogen (Ref 41, Protein Data Bank codes 2tga, 1tgc, and 1tgt) were used to model the serine protease domain of zymogen FXIa and FXIaG193E. Although FXI resembles trypsinogen, trypsinogen displays considerable disorder in several parts of the polypeptide chain including the region that contains residue 184-195 (55). Instead, this region was modeled based upon chymotrypsinogen where this region around Gly183 is well ordered. The crystallographically determined structures of enteropeptidase (Ref 42, Protein Data Bank code 1iek), α1-antitrypsin (Ref 43, Protein Data Bank code 1hio), α-chymotrypsin (Ref 44, Protein Data Bank code 6cha), and β-trypsin (Ref 45, Protein Data Bank code 3bth) were used to model the serine protease domains of FXIaWT and FXIG193E. APPI alone (Ref 46, Protein Data Bank code 1aap), bovine chymotrypsin inhibited with APPI (Ref 47; Protein Data Bank code 1ca0), and bovine trypsin inhibited with APPI (Ref 47; Protein Data Bank code 1taw) served as templates in building models of the serine protease domain of FXIaWT and FXIG193E with APPI. Bulk solvent is excluded from the protease-inhibitor complex, thus, it is anticipated that hydrogen bonds and ionic interactions can be accurately evaluated and play an important role in specificity. The relative positions of the inhibitor and protease domains were maintained, and adjustments were only made to the side chains. Hydrophobic/α-der Waals, hydrogen bonds, and ionic interactions were observed between each protease-inhibitor complex. These interactions were taken into consideration in evaluating each protease-inhibitor complex, and it was assumed that all potential hydrogen bond donors and acceptors would participate in these interactions.

RESULTS

FXIaWT and FXIG193E Amidolytic Activity toward Small Synthetic Substrates—We initially studied the effects of the Glu193 substitution on protease catalytic activity using the tripeptide chromogenic substrates S-2366 and S-2288. These data are presented in Table I. The catalytic efficiency of FXIaG193E was ~18.5-fold lower (~12-fold increase in Kcat and ~1.5-fold decrease in Kcatd) for S-2366 and ~11.5-fold lower (~7-fold increase in Km and ~1.7-fold decrease in Kcatd) for S-2288. These data indicate that the active site is impaired in FXIaG193E.

**FIX Activation by FXIaWT and FXIaG193E**—During physiologic coagulation, the macromolecular substrate for FXIa is FIX. We examined activation of FIX by FXIaWT and FXIaG193E. These data are presented in Fig. 1. The values for Km were similar for the two proteases (FXIaWT, 145 μM; and FXIaG193E, 145 μM).
The data presented are the averages of three experiments. The data in the previous two sections indicate that the S1 site of serine proteases, and the activation peptide cleavage sites of FIX with the active site of FXIaG193E (see “Discussion”).

**FXIa<sub>WT</sub> and FXIa<sub>G193E</sub> Inhibition by AT and APPI—AT and APPI are two known physiologic inhibitors of FXIa. AT belongs to the serpin family of inhibitors and APPI to the Kunitz family of inhibitors (similar to bovine pancreatic trypsin inhibitor). In contrast to small synthetic substrates and the activation peptide cleavage sites in FIX, AT and APPI are likely to make extensive contacts with the protease domain of FXIa. For this reason, we hypothesized that binding of these inhibitors to FXIa<sub>G193V</sub> may be severely impaired in comparison to synthetic substrate hydrolysis and FIX activation. The second-order rate constant, \( k \), for binding of AT to FXIa<sub>WT</sub> was 6.1 \( \mu M^{-1} s^{-1} \) and to FXIa<sub>G193V</sub> was \( 8 \times 10^{-4} \mu M^{-1} s^{-1} \). These data indicate that binding of AT to FXIa<sub>G193V</sub> is ~8,000-fold weaker than binding to FXIa<sub>WT</sub>. Binding data for APPI inhibition of FXIa<sub>WT</sub> and FXIa<sub>G193E</sub> are presented in Table II. The initial rapid equilibrium binding (\( K_i \)) of APPI to the mutant enzyme was impaired ~6000-fold. Furthermore, the isomerization step (\( k_{cat} \)) that leads to the tight binding complex was also impaired ~20-fold. However, dissociation of the tightly bound complex was equivalent for both enzymes. Thus, from the AT and APPI binding data, one may conclude that FXIa<sub>G193V</sub> may not be locked into an active enzyme conformation. Instead it may exist in an equilibrium state that fluctuates between the active and zymogen forms of the enzyme. The experiments described in the following sections were designed to test this concept.

**Binding of pAB to FXIa<sub>G193E</sub> and FXIa<sub>WT</sub>—**The inhibitor pAB is known to bind to the S1 site of serine proteases, and was employed to investigate the integrity of the S1 site in FXIa<sub>G193E</sub>. The data are presented in Fig. 2. Notably, FXIa<sub>G193E</sub> bound to pAB with 6-fold weaker affinity (\( K_i = 126 \mu M \) versus 21 \( \mu M \)) than to FXIa<sub>WT</sub>. Thus, the S1 site is impaired in FXIa<sub>G193E</sub>, in agreement with the synthetic substrate hydrolysis data presented in Table I.

**Inhibition of FXIa<sub>G193E</sub> and FXIa<sub>WT</sub> by DFP—**One of the underlying features of serine proteases is the presence of the oxyanion hole, which develops upon conversion of the zymogen to the enzyme form. DFP specifically reacts with Ser<sup>195</sup> and contains an oxyanion that enables it to be used as a probe to test the integrity of the oxyanion hole (48). As shown in Fig. 3, FXIa<sub>G193E</sub> inhibition by DFP was ~30-fold slower when compared with inhibition of FXIa<sub>WT</sub> (20 \( \text{m}^{-1} \text{min}^{-1} \) versus 610 \( \text{m}^{-1} \text{min}^{-1} \), respectively). This demonstrates that the oxyanion hole in the mutant enzyme is not properly formed, and that the amide N of Glu<sup>193</sup> [555] in FXIa<sub>G193E</sub> is not pointing precisely toward the oxyanion hole.

**Carbamylation of Hle<sup>58</sup> Using NaNCO—**The data in the previous two sections indicate that the S1 site and the oxyanion hole in the mutant enzyme are not properly formed. The development of the S1 site and oxyanion hole in serine proteases requires formation of a salt bridge between the amino group of Hle<sup>58</sup> [370] and the carboxylate group of Asp<sup>194</sup> [556]. Covalent modification of the amino group of Hle<sup>58</sup> [370] by carbamylmethylation results in an inactive enzyme, and a faster rate of carbamylation is indicative of impaired and destabilized salt bridge for-
Fig. 2. Binding of pAB to FXIaWT and FXIaG193E. Each reaction was carried out in TBS with 5 mM Ca\textsuperscript{2+}, 100 µg/ml BSA, and either 100 µM S-2288 (for FXIaWT) or 500 µM S-2288 (for FXIaG193E). Increasing amounts of pAB were added to each mixture, and the reactions were initiated by addition of either 1 nM FXIaWT or 2 nM FXIaG193E. The initial rates of pNA release were measured, converted to micromolar substrate hydrolyzed per min, and the percent activity plotted as a function of pAB concentration. Open circles, FXIaWT; closed circles, FXIaG193E.

Fig. 3. Inhibition of FXIaWT and FXIaG193E by DFP. A, FXIaWT; B, FXIaG193E. Each reaction was carried out in TBS/BSA, 5 mM CaCl\textsubscript{2} at room temperature. The enzyme concentration for FXIaWT or FXIaG193E was 250 nM, and the concentration of DFP was varied from 2 µM to 4 nM. At various times, 5-µl aliquots were removed and added to 155 µl of TBS/BSA containing 625 µM S-2288. The percent residual FXIa activity at each point was plotted as a function of time to obtain the values of \( k_{obs} \). The first-order rate constants, \( k_{obs} \), are plotted against the DFP concentration.

As shown in Table III, the rate of carbamylation of FXIaG193E was ~4-fold faster than for FXIaWT. From these data, one can conclude that the main chain conformation involving residues 189–194 [551–556], which lines one side of the S1 pocket, is altered in the mutant enzyme. Because residue GLY193 [555] is a part of this peptide backbone, the amide N of Glu193 [555] may not be pointing toward the oxyanion hole in the mutant enzyme.

Occupancy of the S1 site is known to order the binding pocket and stabilize the salt bridge between Ile\textsuperscript{16} [370] and Asp\textsuperscript{194} [556] in serine proteases (35). We therefore examined the effect of pAB of the S1 site on the stability of the salt bridge between Ile\textsuperscript{16} [370] and Asp\textsuperscript{194} [556] in FXIaG193E. Notably, pAB occupancy of the S1 site in FXIaG193E led to an ~8-fold decrease in the rate of carbamylation (Table III), indicating substantial stabilization of the salt bridge. Interestingly, occupancy of the S1 site of FXIaWT by pAB also resulted in an ~2-fold slower rate of carbamylation (Table III), suggesting that pAB further stabilizes the wild type enzyme in a more active conformation. Remarkably, the presence of pAB corrected the rate of carbamylation of FXIaG193E to that of FXIaWT. Thus, the presence of pAB in the S1 site appears to largely correct the abnormality in the main chain conformation involving residues 189–194 [551–556] in the mutant enzyme.

DISCUSSION

We have investigated the structural role of Gly\textsuperscript{193} [555] in the proper conformation and activity of the mutant coagulation serine protease FXIaG193E. The substitution of Glu for Gly\textsuperscript{193} [555] was based upon a naturally occurring mutation found in a patient with a history of excessive bleeding (4). In preliminary work reported in abstract form (4), zymogen FXIaG193E could be cleaved normally between Arg\textsuperscript{15} [369] and Ile\textsuperscript{16} [370] to generate the protease FXIaG193E. Thus, bleeding associated with FXIaG193E is likely because of the inability of FXIaG193E to function as an enzyme. FXIa was chosen for the present study because it has significant amidolytic activity and a well characterized physiologic macromolecular substrate/inhibitor profile.

Replacement of Gly\textsuperscript{193} [555] by Glu in FXIa may have two consequences: 1) it may change the conformation around the latent active site in the zymogen, and 2) it may not allow the specific conformational changes that accompany conversion of the zymogen to the enzyme to occur. Analysis of a model for the human FXI protease domain suggests that Glu at position 193 [555] can be easily accommodated in the zymogen form of the protein (Fig. 4A). When Gly\textsuperscript{193} [555] is replaced with Glu, close contacts with the side chains of Val\textsuperscript{162} [498] and Thr\textsuperscript{173} [575] are observed. This steric conflict is easily relieved by a slight rotation around the N-Cα (13°) and Cα-C' (32°) bonds resulting in movement of the Glu\textsuperscript{193} [555] side chain away from these
FIG. 4. Model of the zymogen and activated serine protease domain of FXI<sub>G193E</sub>. FXI<sub>WT</sub>, FXI<sub>G193E</sub>, FXI<sub>aWT</sub>, and FXI<sub>aG193E</sub> were modeled as described under “Experimental Procedures.” In all structures, oxygen atoms are red, nitrogens are blue, carbons are green, and sulfurs are yellow. The chymotrypsin numbering system is used to identify the residues. A, zymogen FXI<sub>WT</sub> and FXI<sub>G193E</sub>. In this figure, the backbone of residues 192–194 [554–556] from FXI<sub>WT</sub> is shown in cyan superimposed on FXI<sub>G193E</sub>. Glu<sub>193</sub> [555] is easily accommodated in FXI<sub>G193E</sub> and may make a H-bond with the hydroxyl of Tyr<sub>228</sub> [590]. Glu<sub>217</sub> [579] in both FXI<sub>WT</sub> and FXI<sub>G193E</sub> makes a H-bond with the backbone amide of residue 191 [553]. Notably, Asp<sub>194</sub> [556], which in the activated serine proteases makes a salt bridge with Ile<sub>16</sub> [370], makes a H-bond with His<sub>40</sub> [396] in the zymogen and Ile<sub>16</sub> [370] is located distant from the active site. Such is the case in other zymogens in this family as well. B, active serine protease FXI<sub>aG193E</sub>. In the activated enzyme, Asp<sub>194</sub> [556] makes H-bonds with the amide N of Ile<sub>16</sub> [370] instead of with His<sub>40</sub> [396] as shown in A for the zymogen. The backbone of residues 16–18 [370–372] is shown as a magenta ribbon. Glu<sub>193</sub> [555] is shown stabilized by making H-bonds with Arg<sub>39</sub> [395]. C of Glu<sub>193</sub> [555] sterically conflicts with the carbonyl O of residue 192 [554], which will not be the case for FXI<sub>aWT</sub>. To relieve the steric conflict in FXI<sub>aG193E</sub>, the 192–193 peptide bond may flip or reorient. The backbone of residues 192–194 [554–556] with this peptide bond flipped is shown in purple. Note that the amide N of 193 [555] is now pointing away from the oxyanion hole. The catalytic triad residues consisting of His<sub>57</sub> [413], Asp<sub>102</sub> [464], and Ser<sub>195</sub> [557] and residues 214–217 [576–579], which line one side of the S1 binding pocket are also shown. C, model of FXI<sub>aG193E</sub> in complex with APPI. The protease domain backbone of FXI<sub>aG193E</sub> is shown in magenta and that of APPI in cyan. Helices are depicted as cylinders, β-sheets as long thick arrows, and turns in the loops with short thin arrows. The NH<sub>2</sub> and COOH termini are labeled N and C, respectively. The interacting residues in FXI<sub>aG193E</sub> and APPI are colored by atom type. Residues from FXI<sub>a</sub> are labeled in white and residues from...
residues. The Glu\textsuperscript{193} [555] side chain would then point into a cavity that is filled with solvent in most serine proteases. The Glu\textsuperscript{193} side chain may also be able to make a hydrogen bond with the hydroxyl group of Tyr\textsuperscript{238} [590]. Thus, it is expected that Glu substitution at position 193 [555] can be easily accommodated in FXI without significant structural consequences.

In contrast, in the active form of the enzyme, the polypeptide backbone at Gly\textsuperscript{193} [555] assumes a conformation compatible only with a Gly residue. Gly\textsuperscript{193} [555] is located in position 3 of a type II hairpin loop, and has \( \phi-\psi \) conformation parameters of (105, –10) in the modeled FXI\textsubscript{appt} structure. These parameters are compatible with those reported in crystal structures of many serine proteases, and places this residue in a region of the Ramachandran plot that is compatible only with a Gly residue. Replacement of Gly\textsuperscript{193} [555] with any other residue will introduce a \( \beta \)-carbon that will have a steric conflict with the carbonyl O of residue 192 [554] (Fig. 4B). Relief of this conflict can only be achieved by a change in the conformation around the 192–193 [554–555] peptide bond. One possible way to achieve this is to flip the bond, thereby converting the type II turn into a type I turn. In this process, rotation (\( \phi \)) around the "CA-C\textsubscript{\alpha}" bond and rotation (\( \psi \)) around the "CA-C\textsubscript{\epsilon}" changes the orientation of the peptide bond without affecting side chain positions or the main chain conformation (49). The maximum activation barrier for a concerted flip of this type is \(<3 \text{ kcal/mol} \) (49). In the case of FXI\textsubscript{G193E}, this results in \( \phi-\psi \) conformation parameters of (–61, –45) in the modeled structure (Fig. 4B). Support for the premise that the 192–193 [554–555] bond can be readily flipped in FXI\textsubscript{G193E} comes from structures of the serine protease factor VIIa (bound to tissue factor) in complex with the inhibitor (Ref. 52, Protein Data Bank code 1bgy). Furthermore, a flip of the 192–193 peptide bond upon substrate binding is also supported by crystal structure and biochemical data for Staphylococcus aureus exfoliative toxins A (53–55) and B (55).

The crystal structure of mouse glandular kallikrein-13 with Asp at position 193 and no inhibitor is also known (Ref. 56, Protein Data Bank code 1ao5). Some residues lining both sides of the S1 site have very high B factors, suggesting that this region is somewhat mobile in the absence of inhibitor occupancy. In this structure, although the amide N of residue 193 is pointing toward the oxyanion hole to some extent, this region appears to be quite mobile. Thus, the S1 site may not be completely formed in the absence of occupancy of the S1 site.

The hydrolysis of the macromolecular substrate, FIX, is significantly more impaired than is hydrolysis of synthetic substrates by FXI\textsubscript{G193E}. This could be because of the side chain of Glu\textsuperscript{193} occupying part of the S2 site where the P2 residue of FIX is expected to reside (see below), in addition to the abnormal conformation of the S1 site and oxyanion hole. The sequence in FIX at its NH\textsubscript{2}-terminal most cleavage site (Asp\textsuperscript{145}, Ala\textsuperscript{146}) from P2 to P2' is Thr-Arg-Ala-Glu, and at the COOH-terminal cleavage site is Arg\textsuperscript{160} Val\textsuperscript{161} Thr-Arg-Val-Val. Considering the P2' residues it appears that the steric clash would be larger at the Arg\textsuperscript{145}-Ala\textsuperscript{146} cleavage site for two reasons: 1) the Glu at this site is larger than Val, and 2) Glu at the P2' position would produce a charge repulsion. Thus, we expect that the Arg\textsuperscript{145}-Ala\textsuperscript{146} peptide bond in FIX would be cleaved very slowly in comparison to cleavage of the Arg\textsuperscript{160} Ala\textsuperscript{161} peptide bond by mutant FXI\textsubscript{G193E}.

The inhibition of FXI\textsubscript{G193E} by AT and APPI was even more impaired than was activation of FIX. This may be due primarily to the larger contact regions between the protease domain of FXIa and the two inhibitors when compared with the limited contact region with the FIX activation peptide. A model of APPI in complex with FXI\textsubscript{G193E} is shown in Fig. 4C. The electrostatic and hydrophobic interactions are described in the figure legend. The indicated interactions are in agreement with data presented in abstract form by Navaneetham et al. (27). Clearly, APPI are labeled in cyan. As in A and B, the protease domain numbering is based upon chymotrypsin and the APPI numbering is based upon that of bovine pancreatic trypsin inhibitor. Specifically, Tyr\textsuperscript{69} [416] from FXIa makes H-bonds with Tyr\textsuperscript{35} and Arg\textsuperscript{20} from APPI. Arg\textsuperscript{39} [395] from FXIa makes a H-bond with Ser\textsuperscript{34} from APPI. This is important as Arg\textsuperscript{39} [395] in the absence of APPI may help to stabilize Glu\textsuperscript{193} [555] through a hydrogen bond (see B), which will not exist in the complex because of the role of Arg\textsuperscript{39} [395] in binding to APPI. Tyr\textsuperscript{69} [503] and Ile\textsuperscript{111} [510] of FXIa are expected to have van der Waals interactions with Phe\textsuperscript{34} of APPI. Arg\textsuperscript{39} of FXIa interacts with the S1 site residue Asp\textsuperscript{199} [551] in FXIa, and the carbonyl of Pro\textsuperscript{13} in APPI forms a H-bond with the N of Gly\textsuperscript{116} [578] in FXIa. These last two interactions are present between all bovine pancreatic trypsin-like inhibitors and their respective associated enzymes. Note that Glu\textsuperscript{193} [555] protrudes into the S2' site and sterically conflicts with Met\textsuperscript{12}, the P2' residue of APPI. This steric conflict is highlighted with a yellow circle. This conflict will not exist in the FXI\textsubscript{appt}-APPI complex where residue 193 [555] is Gly.
the presence of Glu at position 193 [555] will change the electrostatic potential around the active site and interfere with the binary collision with AT or APPI. Moreover, Glu193 [555] will have a steric clash with the P2′ Met residue of APPI as shown in Fig. 4C. Data presented in Table II indicate that the isomerization step that leads to the formation of the tight complex after the initial binary collision with FXIaG193E is also severely affected. This is expected because minor rearrangements of Glu193 [555] in FXIaG193E and the P2′ residue Met17 in APPI must take place for a stable complex to form. However, once a complex is formed, dissociation of the complex does not appear to be affected.

CONCLUSIONS

The Gly193 [555] residue of FXIa, a serine protease involved in blood coagulation, was changed to Glu to study the role of Gly193 [555] in serine proteases. The experimental and modeling data presented in this paper indicate that the S1 site, oxyanion hole, and salt bridge formation between Ile370 [370] and Asp194 [555] are impaired in the mutant enzyme. However, occupancy of the active site by substrate/inhibitors can correct these defects. The hydrolysis of macromolecular substrates and binding of macromolecular inhibitors can be further impaired by a steric clash between the S2′ site of the enzyme and P2′ residue of the substrate/inhibitor. Enzymes with residues other than glycine at position 193, such as human brain trypsin (Arg193 [555]) (51), T. stejnegeri plasminogen activator (Phe193 [555]) (52), and mouse glandular kallikrein-13 (Asp193 [555]) (56) would be compatible with substrate/inhibitors that have small side chains at the P2′ position. This will introduce a new level of structural plasticity and selectivity in serine proteases. Thus, it would appear that enzymes with Glu at position 193 will be more active but not have selectivity at the S2′ site; whereas, enzymes with non-Gly residues at position 193 will be an order of magnitude less active but have a restricted S2′ site and selectivity for their substrate/inhibitors. Bode and co-workers (52) have previously elaborated such plasticity and substrate/inhibitor selectivity in this class of proteases.

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Structural Role of Gly\textsuperscript{193} in Serine Proteases: INVESTIGATIONS OF A G555E (GLY193 IN CHYMOTRYPSIN) MUTANT OF BLOOD COAGULATION FACTOR XI

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