Contributions of Basic Amino Acids in the Autolysis Loop of Factor XIa to Serpin Specificity†

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ABSTRACT: The autolysis loops (amino acids 143−154, chymotrypsinogen numbering) of plasma serine proteases play key roles in determining the specificity of protease inhibition by plasma serpins. We studied the importance of four basic residues (Arg-144, Lys-145, Arg-147, and Lys-149) in the autolysis loop of the coagulation protease factor XIa (fXIa) for inhibition by serpins. Recombinant fXIa mutants, in which these residues were replaced individually or in combination with alanine, were prepared. The proteases were compared to wild-type fXIa (fXIa-WT) with respect to their ability to activate factor IX in a plasma clotting assay, to hydrolyze the chromogenic substrate S2366, and to undergo inhibition by the C1-inhibitor (C1-INH), protein Z dependent protease inhibitor (ZPI), antithrombin (AT), and α_1-protease inhibitor (α_1-PI). All mutants exhibited normal activity in plasma and hydrolyzed S2366 with catalytic efficiencies similar to that of fXIa-WT. Inhibition of mutants by C1-INH was increased to varying degrees relative to that of fXIa-WT, with the mutant containing alanine replacements for all four basic residues (fXIa-144-149A) exhibiting an ∼15-fold higher rate of inhibition. In contrast, the inhibition by ZPI was impaired 2−3-fold for single amino acid substitutions, and fXIa-144-149A was essentially resistant to inhibition by ZPI. Alanine substitution for Arg-147 impaired inhibition by AT ∼7-fold; however, other substitutions did not affect it or slightly enhanced inhibition. Arg-147 was also required for inhibition by α_1-PI. Cumulatively, the results demonstrate that basic amino acids in the autolysis loop of fXIa are important determinants of serpin specificity.

Factor XI (fXI)[1] is the zymogen of a plasma trypsin-like serine protease (fXIa) that contributes to blood coagulation through the conversion of factor IX (fIX) to fIXa by limited proteolysis (*1*−*4*). FXIa is probably required to sustain thrombin generation with certain types of injuries, and hereditary fXI deficiency is associated with a mild to moderate bleeding diathesis (*5*, *6*). The protein circulates in plasma as a disulfide linked homodimer with a molecular mass of ∼160 kDa (*7*, *8*). The conversion of fXI to fXIa involves proteolytic cleavage at the peptide bond between Arg-369 and Ile-370 on each polypeptide of the homodimer by either thrombin or factor XIIa (fXIIa) (*3*, *7*−*9*). Autoactivation by fXIa on negatively charged surfaces has also been demonstrated (*4*, *10*). The conditions under which fXI is activated in vivo are not clearly understood; however, activation by thrombin on the surface of platelets or negatively charged surfaces such as glycosaminoglycans are possibilities (*1*, *4*, *10*, *11*). The *N*-terminal heavy chain of each fXIa monomer contains four 90−91 amino acid repeats called apple domains, which facilitate interactions between the protease and natural ligands, such as fIX, high molecular weight kininogen, glycosaminoglycans, and platelet glycoproteins (*7*, *9*, *11*−*13*). The *C*-terminal light chain of each fXIa monomer contains a trypsin-like catalytic domain (*3*, *9*).

Several serpin inhibitors, including C1 inhibitor (C1-INH) (*14*−*16*), antithrombin (AT) (*16*−*18*), α_1-protease inhibitor (α_1-PI) (*19*), α_2-antiplasmin (*17*), type 1 plasminogen activator inhibitor (*20*), protein C inhibitor (*21*), and protease nexin I (*22*), have been reported to inhibit the proteolytic activity of fXIa in plasma. All of these inhibitors react with fXIa by the suicide-substrate mechanism typical of serpin interactions with their target coagulation proteases (*15*, *23*). On the basis of the second-order association rate constants for fXIa, C1-INH ($\sim 2 \times 10^3$ M^{-1} s^{-1}) and protease nexin I ($\sim 8 \times 10^4$ M^{-1} s^{-1}) are the most potent inhibitors of fXIa (*14*, *21*), and fXIa inhibition by both serpins is enhanced by unfractionated heparin ∼50-fold and ∼20-fold, respectively (*22*). AT inhibition of fXIa is slower ($\sim 3 \times 10^2$ M^{-1} s^{-1}) than that for C1-INH and protease nexin I; nevertheless, unfractionated heparin enhances inhibition of fXIa by AT up to 500-fold (*16*, *18*). Therefore, AT may play an important role in the regulation of fXIa activity, particularly during therapeutic administration of heparin. More recently, it has been shown that protein Z dependent protease inhibitor (ZPI), another

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[1] Abbreviations: FXI, factor XI; fXIa, activated factor XI; fIX and fIXa, factor IX and activated fIXa, respectively; fX and fXa, factor X and activated factor X, respectively; fXIIa, activated factor XII; fXIa-WT, wild-type recombinant fXIa; fXIa-CD, catalytic domain of fXIa; C1-INH, C1-inhibitor; ZPI, protein Z dependent protease inhibitor; AT, antithrombin; α_1-PI, α_1-protease inhibitor; BSA, bovine serum albumin; PEG, poly(ethylene glycol).

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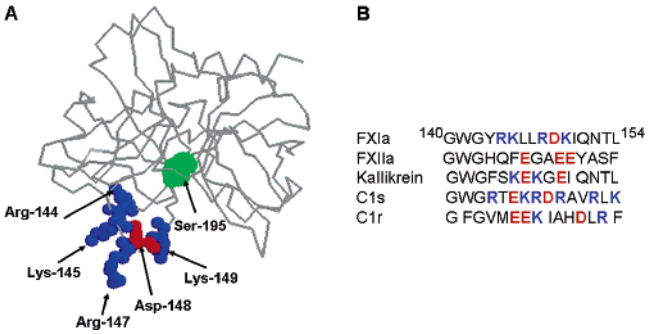

FIGURE 1: Crystal structure of the catalytic domain of fXIa in complex with *p*-aminobenzamidine. (A) Side chains of basic residues of the autolysis loop of fXIa shown in blue and the side chain of the single acidic residue Asp-148 (chymotrypsinogen numbering) shown in red. The serine residue of the catalytic active site (Ser-195) is shown in green. The coordinates (Protein Data Bank code 1ZHM) of the catalytic domain of fXIa were used to prepare the Figure (*25*). (B) Amino acid sequence of the autolysis loops of plasma serine proteases known to be regulated by C1-INH. The basic and acidic residues are shown in blue and red, respectively.

member of the serpin superfamily, also inhibits fXIa in a protein Z independent reaction (*24*).

All of the serpins mentioned above, with the exception of ZPI and α_1-PI, contain the typical Arg at the P1 site of their reactive center loops. However, the P1 residue is a Tyr in ZPI and a Met in α_1-PI, suggesting that the interaction of these serpins with the primary binding pocket of fXIa cannot solely determine their specificity. How fXIa specifically interacts with these serpins has not been studied in detail. Recently, three X-ray crystal structures have been solved for the fXIa catalytic domain in complex with the small active-site inhibitor benzamidine (*25*), the *E. coli* protease inhibitor, ecotin (*26*), and the Kunitz protease inhibitor domain of protease nexin II (*27*). These structural data show that the fXIa catalytic domain, like other coagulation proteases, has several solvent exposed surface loops that surround the enzyme substrate-binding pocket. In general, the loops are conserved at similar 3D locations on all coagulation proteases; however, the length and amino acid residues forming these loops differ among members of the family. The surface loop containing residues 143−154 (chymotrypsinogen numbering [28]) is referred to as the autolysis loop, and it plays a critical role in determining substrate and inhibitor specificities for several coagulation proteases (*29*, *30*). For example, in factors IXa (fIXa) and Xa (fXa), the basic residues of the autolysis loop are critical for interactions with AT (*29*, *30*). The autolysis loop of fXIa also contains basic residues (Arg-144, Lys-145, Arg-147, and Lys-149; chymotrypsinogen numbering) located at positions similar to those of the corresponding residues in fIXa and fXa, immediately below the active-site pocket (*25*) (Figure 1). To evaluate the contribution of these basic residues to substrate and serpin specificity, we expressed and purified fXI mutants in which these residues are changed to alanine, either individually or in combination (*12*). The catalytic properties of the proteases were characterized with respect to their ability to hydrolyze the tripeptide chromogenic substrate S2366, to activate fIX in a plasma-based clotting assay, and to undergo inhibition by C1-INH, ZPI, AT, and α_1-PI. The results show that basic residues of the fXIa autolysis loop play a pivotal role in determining the specificity of the fXIa interaction with plasma serpins.

MATERIALS AND METHODS

Proteins and Reagents. Human plasma factor Xa (fXa), protein Z, and AT were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). C1-INH was purchased from Sigma (St. Louis, MO), and α_1-PI was from Athens Research and Technology, Inc. (Athens, GA). ZPI was expressed in 293 human embryonic kidney fibroblasts (ATCC CRL 1573) and purified to homogeneity as described (*31*). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (*32*). The AT-binding pentasaccharide fondaparinux sodium (Organon Sanofi-Synthelabo) was purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The chromogenic substrate S2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide) was from Diapharma (West Chester, OH), and Spectrozyme FXa (SpFXa, MeO-CO-Dcyclohexylglycyl-Gly-Arg-*p*-nitroaniline) was from American Diagnostica (Greenwich, CT).

Mutagenesis and Expression of Recombinant Proteins. The fXI autolysis loop amino acids Arg-504, Lys-505, Arg-507, Asp-508, and Arg-509 (fXI numbering system (*9*)) correspond to residues 144, 145, 147, 148, and 149, respectively in the chymotrypsinogen numbering system (*28*), which will be used hereafter. Point mutations were introduced into the human fXI cDNA (*9*) to change each of these residues to alanine, using a Quick Change mutagenesis kit (Stratagene, La Jolla, CA) as previously described (*12*). The corresponding recombinant fXI proteins are designated fXI-R144A, K145A, R147A, D148A, and K149A, and wild-type fXI is designated fXI-WT. In addition, a single cDNA was created in which the four basic residues at positions 144, 145, 147, and 149 were changed to alanine (fXI-144-149A). After the accuracy of mutagenesis was confirmed by DNA sequencing, the cDNAs were ligated into the mammalian expression vector pJVCMV (*12*, *13*), and 5×10^7 293 fibroblasts were cotransfected with 40 μg of fXI/pJVCMV construct and 2 μg of pRSVneo by electroporation (Electrocell Manipulator 600 BTX, San Diego) (*12*, *13*). Cells were grown in DMEM, 5% fetal bovine serum, and 500 μg/mL of G418. The expression of human fXI by G418-resistant clones was determined by ELISA using goat anti-human fXI antibodies (Affinity Biologicals, Hamilton, Ontario). Expressing clones were expanded in 175 cm^2 flasks and switched to Cellgro Complete serum free media (Mediatech, Herndon, VA). Media were collected every 48 h, supplemented with benzamidine (5 mM), and stored at −20 °C pending purification. The expression and purification of the fXI mutant fXI-Ser-362,482 containing serine substitutions for both Cys-362 and Cys-482 (fXI numbering) has been described (*33*). This protein was used to isolate the fXIa catalytic domain (fXIa-CD) following activation by fXIIa as described below.

All recombinant human fXI proteins were purified from media on an anti-fXI IgG 1G5.12 affinity column (*12*, *13*). Then 4 L of conditioned media were dialyzed against 50 mM sodium acetate at pH 5.2, 250 mM NaCl, and 1 mM EDTA and loaded onto a 100 mL S-Sepharose fast-flow cation exchange column equilibrated with the same buffer.

Protein was eluted with a one liter linear NaCl gradient (250 to 1000 mM). FXI containing fractions (determined by aPTT assay, see below) were pooled and dialyzed against 25 mM Tris-HCl at pH 7.4 and 100 mM NaCl (TBS) and loaded onto a 10 mL heparin−agarose column equilibrated with TBS. The column was eluted with a 100 mL linear NaCl gradient (100−1000 mM). Fractions containing pure fXI were identified on a 10% SDS−polyacrylamide gel, pooled, concentrated, and dialyzed against TBS. The concentrations of all recombinant proteins were determined using a dye-binding assay (Bio-Rad). FXI (∼100−300 μg/mL) was activated with 5 μg/mL of fXIIa (Enzyme Research Laboratories, South Bend, IN) at 37 °C, and complete activation was confirmed by SDS−PAGE. The catalytic domain of human fXIa was isolated from activated fXI-Ser-362,482 using the 1G5.12 affinity column as described (*33*).

FXIa Hydrolysis of S2366. FXIa (3 nM protein = 6 nM active sites,) was diluted in TBS containing 0.1 mg/mL bovine serum albumin (TBSA) and 50−2000 μM S2366 in a microtiter plate. Rates of generation of free *p*-nitroaniline (*p*NA) in 100 μL reaction volumes (3 mm path length) were measured by continuous monitoring of absorbance at 405 nm on a SpectraMax 340 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA) (*33*). Assays were performed in triplicate. Peptide *p*NA substrate concentrations were determined by absorbance at 342 nm using an absorption coefficient of 8266 M^{-1} cm^{-1}, and product concentrations were calculated using an absorption coefficient of 9933 M^{-1} cm^{-1} at 405 nm (*34*). K_m and k_{cat} values for the hydrolysis of S2366 by fXIa derivatives were obtained by initial rate analysis of *p*NA generation as a function of S2366 concentration. Nonlinear regression was performed with Scientist Software (MicroMath Scientific Software, Salt Lake City, UT), and estimates of error represent ± 2 SD.

FXI Specific Activity in a Plasma Clotting Assay. A standard curve for fXI activity in plasma was constructed using an aPTT clotting assay as follows. Serial dilutions of plasma fXI (specific activity ∼200 U/mg, Enzyme Research Laboratories), starting at 5 μg/mL, were prepared in TBSA. Dilutions of fXI (65 μL) were mixed with 65 μL of fXI deficient plasma (George King, Overland Park, KS) and 65 μL of PTT A reagent (Diagnostica Stago, Asnieres-sur-Seine, France) at 37 °C on a Dataclot II fibrometer (Helena Laboratories, Beaumont, TX). After 5 min of incubation, 65 μL of 25 mM $CaCl_2$ was added and the time taken for clot formation determined. A standard curve was prepared by creating a log−log plot of fXI concentration versus aPTT clotting time. Serial dilutions of each recombinant protein were prepared in TBSA starting at 5 μg/mL and tested in the aPTT assay in triplicate, and the results were compared to the standard curve.

Inhibition of FXIa by Serpins. The rate of inactivation of fXIa by serpins were measured under pseudo-first-order rate conditions by a discontinuous assay (*31*). Briefly, fXIa (0.5 nM) was incubated with serpin (50−400 nM for C1-INH, 10−50 nM for ZPI, 200−1000 nM for AT, and 2.5 μM for α_1-PI) in TBSA containing 0.1% poly(ethylene glycol) (PEG) 8000 for 3−140 min. All reactions were carried out in 50 μL volumes in 96-well polystyrene assay plates at room temperature. Additional reactions under the same conditions were carried out with ZPI and AT in the presence of saturating concentrations of protein Z (100 nM) or fondaparinux pentasaccharide (2 μM), respectively. Residual protease activity was determined by the addition of 50 μL of S2366 to a final concentration of 0.5 mM and monitoring the change in OD 405 nm on the microtiter plate reader. The observed pseudo-first-order rate constants (k_{obs}) were determined by fitting the time-dependent change of protease activity to the first-order rate equation. Second-order rate constants (k_2) were obtained from the slopes of linear plots of k_{obs} versus serpin concentration as described (*31*).

Table 1: Amidolytic and Plasma Clotting Activities of Recombinant Factor XI/XIa Derivatives[a]

	S2366 cleavage		plasma coagulation
protein	K_m (μM)	k_{cat} (s^{-1})	specific activity (U/mg)
FXI-WT	437 ± 52	114 ± 4	200
FXI-R144A	380 ± 42	116 ± 4	195
FXI-K145A	443 ± 28	103 ± 2	215
FXI-R147A	527 ± 38	103 ± 3	195
FXI-D148A	1365 ± 164	47 ± 3	130
FXI-R149A	531 ± 32	103 ± 3	200
FXI-144−149A	476 ± 102	124 ± 10	230

[a] The kinetics of S-2366 cleavage by fXIa derivatives were determined in TBSA at room temperature using 3 nM fXIa as described in Materials and Methods. All proteases were tested in triplicate, and the values are ±2 SD. The specific activities of fXI zymogens were determined in an aPTT assay using human fXI deficient plasma. The means of results from two separate experiments were compared to those for a preparation of plasma derived fXI with a specific activity of 200 U/mg of protein. One unit of fXI activity is defined as the amount of activity in 1 mL of pooled normal plasma.

RESULTS

Expression, Purification, and Activation of Recombinant Proteins. The SDS−PAGE of purified recombinant fXI proteins suggested that both fXI derived from human plasma and recombinant human fXI run as ∼160 kDa homodimeric proteins under nonreducing conditions and ∼80 kDa monomers under reducing conditions (data not shown). Recombinant fXI autolysis loop mutants also migrated as ∼160 kDa proteins indicating that the introduction of mutations did not interfere with dimer assembly and secretion (data not shown). After activation with fXIIa, a 50 kDa band representing the noncatalytic heavy chain and a 30 kDa band representing the catalytic domain are observed for all proteins, indicating normal activation for all mutant zymogens (data not shown).

Amidolytic Activity. The capacity of autolysis loop mutants to cleave the chromogenic substrate S2366 was determined to assess the integrity of the catalytic domain active site. The Glu-Pro-Arg tripeptide of S2366 binds to the S1, S2, and S3 substrate-binding subsites of the catalytic active site, and the distortion of these binding sites will alter S2366 cleavage. Kinetic parameters for S2366 cleavage are shown in Table 1. With the exception of fXIa-D148A, which exhibited an ∼3-fold increase in K_m and a comparable reduction in k_{cat}, all autolysis loop mutants hydrolyzed the S2366 with a catalytic efficiency similar to that observed with fXIa-WT. The results indicate that the mutagenesis of basic residues in the autolysis loop have no adverse effect on the conformation of the fXIa S1−S3 substrate-binding sites. These results were expected because these residues are solvent exposed in the crystal structure of human fXIa

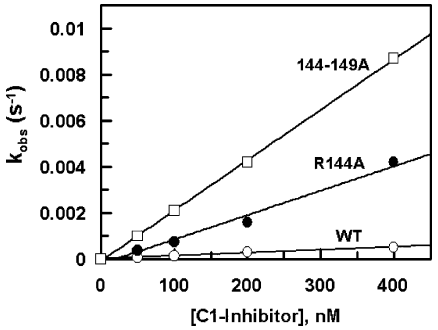

FIGURE 2: Inhibition of fXIa by C1-INH. The dependence of k_{obs} for the inhibition of fXIa-WT (○), fXIa-R144A (●), and fXIa-144−149A (□) on C1-INH concentration is shown. The k_{obs} values were determined from residual fXIa activity (starting concentration 1 nM) following incubation with C1-INH (50−400 nM) for 3−140 min at room temperature as described in Materials and Methods. The solid lines are the best fit to a linear equation.

(Figure 1). The activity of fXIa-D148A was impaired in all assays, including the clotting and inhibition assays described below; therefore, no specific role can be assigned to this acidic residue in the interaction between fXIa and its substrates and inhibitors. The second-order association rate constants (k_2) for fXIa-D148A interactions with serpin inhibitors will be presented without further discussion of results.

Specific Activity in a Plasma Clotting Activity. The specific activities of recombinant proteins were assessed in an aPTT assay that requires fXI to be activated to fXIa by fXIIa and for fXIa to subsequently activate fIX. The results are shown in Table 1. FXI-WT has a specific activity identical to that of the plasma fXI (200 units/mg). All autolysis loop mutants had activity comparable to that of fXI-WT, with the exception of fXI-D148A, which exhibited a modest reduction in specific activity. Thus, the substitution of basic amino acids in the fXIa autolysis loop does not alter catalytic activity toward the physiologic macromolecular substrate (fIX), supporting data from the chromogenic substrate assay and indicating that these residues are not required for the proper recognition of fIX.

Inhibition of fXIa by C1-INH. The k_2 values for the inhibition of fXIa by C1-INH, as determined from the slope of the linear dependence between k_{obs} and serpin concentration (Figure 2), are presented in column 1 of Table 2. Relative to that of fXIa-WT, the inhibition of all mutants was increased from a minimum of 1.5-fold for fXIa-K145A up to ~17-fold for the multiple mutant fXIa-144-149A. These results suggest that basic residues in the autolysis loop inhibit the interaction with C1-INH, preventing the rapid inactivation of fXIa by this serpin. Comparisons of the reactivities of the single mutants indicate that Arg-144 has the greatest effect on restricting the interaction between fXIa and C1-INH because its replacement with alanine increased the rate of inhibition by C1-INH ~8-fold.

Inhibition of fXIa by ZPI. The k_2 values for the inhibition of fXIa mutants by ZPI are presented in the second column of Table 2. FXIa-WT was inhibited by ZPI with a k_2 value of 3.4×10^5 M^{-1} s^{-1}. In contrast to C1-INH, the inhibition of fXIa by ZPI was impaired ~2-fold for each mutant with a single basic amino acid substitution. The effect of these substitutions on the inhibition of fXIa by ZPI was synergistic because fXIa-144-149A exhibited a dramatic defect in

Table 2: Second-Order Rate Constants for the Inhibition of FXIa by Serpins[a]

	C1-INH 10^3 (M^{-1} s^{-1})	ZPI 10^3 (M^{-1} s^{-1})	AT 10^3 (M^{-1} s^{-1})	AT+ H_5 10^3 (M^{-1} s^{-1})
plasma fXIa	1.6 ± 0.1	440 ± 20	0.3 ± 0.04	1.1 ± 0.1
FXIa-WT	1.3 ± 0.1	340 ± 30	0.3 ± 0.05	0.9 ± 0.1
FXIa-R144A	10.1 ± 0.8	170 ± 10	0.4 ± 0.02	1.5 ± 0.1
FXIa-K145A	2.0 ± 0.3	250 ± 20	0.4 ± 0.02	1.1 ± 0.1
FXIa-R147A	2.5 ± 0.1	130 ± 10	0.04 ± 0.004	0.4 ± 0.1
FXIa-D148A	1.1 ± 0.1	70 ± 10	0.03 ± 0.01	0.1 ± 0.01
FXIa-K149A	2.4 ± 0.4	220 ± 20	0.3 ± 0.01	0.8 ± 0.1
FXIa-144-149A[b]	21.8 ± 0.3	<0.2	0.09 ± 0.01	1.3 ± 0.1

[a] All second-order inhibition rate constants were determined by the incubation of fXIa (0.5 nM) with each serpin (50−400 nM C1-INH, 10−50 nM ZPI, and 200−1000 nM AT) in TBSA containing 0.1% PEG 8000 for 3−140 min. In AT reactions in the presence of pentasaccharide (H_5), 2 μM pentasaccharide was included in each reaction. The values for k_2 were determined by measuring residual enzyme activity in an amidolytic activity assay as described in Materials and Methods. All values are the average of three measurements ± SD. [b] The incubation of 1 nM fXIa-144−149A with 100 nM ZPI for 1 h did not result in any decline in the amidolytic activity of the mutant.

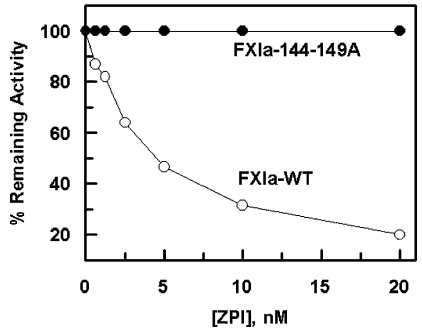

FIGURE 3: Inhibition of fXIa by ZPI. The inhibition of human fXIa-WT (○) and fXIa-144-149A (●) by ZPI is shown. FXIa (0.5 nM each) was incubated with different concentrations of ZPI for 10 min at room temperature. Residual fXIa activity was determined using the chromogenic substrate S2366 (0.5 mM) as described in Materials and Methods.

interactions with this serpin, and the incubation of this mutant with 100 nM ZPI for 1 h did not produce any decline in amidolytic activity (Figure 3). The results indicate that the basic residues of the fXIa autolysis loop are essential for a productive interaction with ZPI.

ZPI inhibits fXa on negatively charged phospholipid vesicles in the presence of Ca^{2+} in a reaction that is dependent on the phospholipid-binding cofactor protein Z (*35*, *36*). We compared the inhibition of fXa and fXIa by ZPI in the presence of PC/PS vesicles and in the presence or absence of a saturating concentration of protein Z (Figure 4). Consistent with published results (*24*, *35*, *36*), the inhibition of fXa by ZPI required protein Z and PC/PS vesicles, whereas the inhibition of fXIa by ZPI was not enhanced by either protein Z or PC/PS vesicles. In fact, protein Z appeared to interfere slightly with the inhibition of fXIa by ZPI (*24*). The rates of inhibition of fXa in the presence of protein Z and fXIa in the absence of protein Z by ZPI are comparable, suggesting that ZPI may play an important physiological role in regulating the activity of both proteases in plasma.

The interaction of fXIa with its macromolecular substrate fIX requires the initial binding of fIX to an exosite on the

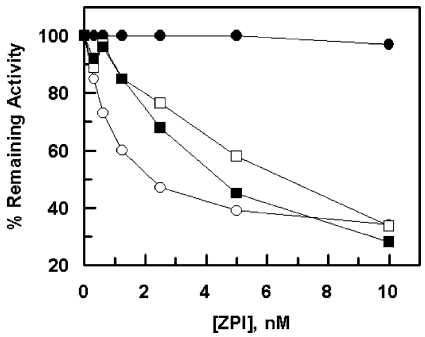

FIGURE 4: Effect of protein Z on ZPI-mediated inhibition. The inhibition of fXIa and fXa by ZPI in the presence or absence of protein Z and PC/PS vesicles is shown. FXa (○,●) or fXIa (□,■) (1 nM) was incubated with varying concentrations of ZPI in either the presence (○,□) or the absence (●,■) of protein Z (100 nM) and PC/PS (25 μM) vesicles in TBSA containing 2.5 mM $CaCl_2$. After 10 min of incubation at room temperature, S2366 was added to a final concentration of 0.5 mM, and residual fXIa activity was measured as described in Materials and Methods.

noncatalytic heavy chain of fXIa (*33*). To investigate the possibility that the fXIa heavy chain provides a binding exosite for ZPI that obviates the requirement for protein Z, we compared the inhibition of fXIa-WT by ZPI to the inhibition of the isolated fXIa catalytic domain (fXIa-CD). Similar k_2 values for the inhibition of fXIa-WT and fXIa-CD were observed (data not shown), demonstrating that ZPI does not require sites outside of the fXIa catalytic domain for normal inhibition of the protease.

Inhibition of fXIa by AT and α_1-PI. The k_2 values for the inhibition of fXIa derivatives by AT are presented in the third column of Table 2. FXIa-WT was inhibited by AT with a k_2 of $\sim 0.3 \times 10^3$ M^{-1} s^{-1} in the absence of heparin, comparable to our previous results with recombinant fXIa (*16*). With the exception of fXIa-R147A, where inhibition was impaired by an order of magnitude, all other fXIa mutants were inhibited with similar rate constants to fXIa-WT. This suggests that Arg-147 is important for a productive interaction between fXIa and AT. Previous work has shown that a unique pentasaccharide sequence in heparin binds to a basic region on AT, allosterically enhancing the inhibition of fIXa and fXa more than 300-fold (*37*−*40*). Both fIXa and fXa have three or four basic amino acids in their autolysis loops, and mutagenesis studies identified Arg-150 in both proteases as critical for the interaction with the heparin-activated conformation of AT (*29*, *30*). To determine whether the basic residues of the fXIa autolysis loop influence inhibition by heparin-bound AT, the inhibition of fXIa by AT was studied in the presence of the therapeutic pentasaccharide fondaparinux. Fondaparinux enhanced AT inhibition of fXIa only ~3-fold (Table 2), consistent with published studies showing that heparin enhancement of fXIa inhibition by AT involves a template mechanism requiring longer heparin chains (*16*, *18*) and that therapeutic low molecular weight heparins have a limited effect on fXIa (*41*). Inhibition was impaired ~3-fold for fXIa-R147A, and other mutants exhibited similar inhibition to fXIa-WT, again indicating that Arg-147 is required for the fXIa-AT interaction with both the native and activated conformation of the serpin.

Finally, the value for k_2 for the inhibition of fXIa-144-149A (20 M^{-1} s^{-1}) by α_1-PI was ~ 3.5-fold reduced compared to that of fXIa-WT (70 M^{-1} s^{-1}). Similar to the inhibition of fXIa by AT, studies with single alanine mutants revealed that the replacement of Arg-147 is primarily responsible for the defect in inhibition of fXIa-144-149A by α_1-PI (data not shown). It is worth noting that titration studies yielded an inhibition stoichiometry of 2:1 for the AT interaction with fXIa (data not shown). We assume that both catalytic sites of the fXIa homodimer have a similar reactivity with AT and other serpins; however, this hypothesis requires further investigation.

DISCUSSION

In this study, we demonstrate that the basic residues of the autolysis loop of fXIa play critical roles in determining the specificity of interactions between the protease and the plasma serine protease inhibitors C1-INH, ZPI, AT, and α_1-PI. In the case of C1-INH, the side chains of the basic residues of fXIa serve an inhibitory function because replacing them with alanine enhanced inhibition ~15-fold. Replacing Arg-144 had the largest effect (enhanced inhibition by C1-INH ~8-fold). Other physiologic targets of C1-INH include contact proteases fXIIa and plasma kallikrein and complement proteases C1r and C1s. All are inhibited by C1-INH with higher second-order association rate constants than that of fXIa, ranging from 3.7×10^3 M^{-1} s^{-1} for fXIIa (*42*) to 1.7×10^4 M^{-1} s^{-1} for kallikrein (*43*). A comparison of the amino acid sequences of the autolysis loops of these proteases (Figure 1B) reveals that fXIa is the only protease with a basic residue at position 144, suggesting that Arg-144 is responsible for the slower rate of inhibition of fXIa by C1-INH. The inhibition of fXIa by C1-INH is accelerated more than 100-fold by heparin or dextran sulfate (*22*, *44*), in contrast to the inhibition of fXIIa or kallikrein, which is not influenced by glycosaminoglycans. Therefore, the rate of inhibition of fXIa by C1-INH on endothelial cell surfaces containing glycosaminoglycans may exceed the rate of inhibition for other contact proteases in vivo. Similarly, given the relatively high plasma concentration of C1-INH (~2.2 μM), heparin-mediated enhancement of fXIa inhibition by C1-INH likely contributes to some extent to therapeutic anticoagulation by heparin.

Unlike the case for fXIa inhibition by C1-INH, the basic residues of the fXIa autolysis loop are critical for the inhibition by ZPI. Replacing the four basic amino acids in the fXIa autolysis loop with alanine residues (fXIa-144-149Ala) essentially prevents inhibition by ZPI. The autolysis loop of fXa also has four basic residues, which are essential for normal interactions with ZPI, suggesting that the autolysis loop plays a similar role in the inhibition of both fXa and fXIa by this serpin (*31*). However, the maximum inhibition of fXa by ZPI requires phospholipid, calcium ions, and the cofactor protein Z (*35*), whereas the inhibition of fXIa by ZPI is independent of these factors. The second-order rate constants for the inhibition of fXIa by ZPI is more than 2 orders of magnitude greater than that for C1-INH, making ZPI the fastest known inhibitor of fXIa in plasma, in the absence of glycosaminoglycans. Protein Z impaired the inhibition of fXIa by ZPI ~2-fold by an unknown mechanism, as noted previously (*24*). It is not clear how ZPI effectively inhibits fXIa in the absence of protein Z; however, it is unlikely that an exosite interaction between ZPI and the heavy chain is involved because the isolated fXIa

catalytic domain (fXIa-CD) is inhibited by ZPI in a manner similar to that in fXIa-WT.

Among the basic residues of the autolysis loop, Arg-147 appeared to make the greatest contribution to the interaction with AT, and its replacement with alanine resulted in 1 order of magnitude reduction in the rate of inhibition. In contrast, the inhibition of mutants with substitutions for Arg-144 or Lys-145 slightly enhanced inhibition by AT. The opposing effects of the Arg-147 and the Arg-144 and Lys-145 substitutions may account for the relatively small change in the rate of inhibition for fXIa144-149A, which contains multiple substitutions. Heparin markedly accelerates the reactivity of AT with coagulation proteases by a template mechanism and/or a conformational activation of the serpin (*16*, *18*). In the cases of fIXa and fXa, the heparin activation of AT enhances the reactivity of the serpin more than 300-fold (*37*−*39*). Unfractionated heparin enhances fXIa inhibition by AT up to 500-fold, primarily through a template mechanism with conformational activation contributing only ∼3-fold to the promotion of inhibition (*16*, *18*). Consistent with previous results, fondaparinux, the AT-binding heparin pentasaccharide that allosterically activates the serpin but that cannot support a template mechanism, accelerates AT inhibition of fXIa only ∼3-fold. The autolysis loop residue Arg-150 serves a critical role in the recognition of the AT−pentasaccharide complex in fIXa and fXa (*29*, *30*). The recent X-ray crystal structure of a catalytically inactive mutant of fXa in complex with AT suggested that Arg-150 makes extensive contacts with an exosite of the heparin-activated conformation of AT, thereby stabilizing the Michaelis complex of the protease with the serpin (*45*). The inability of fXIa to effectively interact with AT in the presence of a pentasaccharide suggests that neither one of the basic residues of fXIa is a specific recognition site for this exosite of the activated serpin.

In summary, we have demonstrated that basic amino acids in the autolysis loop of fXIa are important determinants of serpin specificity for this protease. For inhibition by C1-INH, these residues inhibit the protease-serpin interaction, whereas for inhibition by ZPI, they are critical for protease inhibition. In the cases of AT and α_1-PI, fXIa Arg-147 contributes to the serpin recognition mechanism; however, other basic residues in the autolysis loop do not contribute to the interactions.

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