The glycosaminoglycan heparin enhances several reactions involving coagulation factor XI (FXI) including activation of FXI by factor XIIa, thrombin, and autoactivation; and inactivation of activated FXI (FXIa) by serine protease inhibitors. We examined the effect of heparin on inhibition of FXIa by the inhibitors C1-inhibitor (C1-INH) and antithrombin III (ATIII). Second order rate constants for inhibition in the absence of heparin were $1.57 \times 10^6$ and $0.91 \times 10^3$ s$^{-1}$ s$^{-1}$ for C1-INH and ATIII, respectively. Therapeutic heparin concentrations (0.1–1.0 units/ml) enhanced inhibition by ATIII 20–55-fold compared with 0.1–7.0-fold for C1-INH. For both inhibitors, the effect of heparin over a wide range of concentrations ($10^{-1}$ to $10^5$ units/ml) produced bell-shaped curves, demonstrating that inhibition occurs by a template mechanism requiring both inhibitor and protease to bind to heparin. This implies that FXI/XIa contains structural elements that interact with heparin. Human FXI contains a sequence of amino acids (R250-I-K-K-S-K) in the apple 3 domain of the heavy chain that binds heparin (Ho, D., Badellino, K., Baglia, F., and Walsh, P. (1998) J. Biol. Chem. 273, 16382–16390). To determine the importance of this sequence to heparin-mediated reactions, recombinant FXI molecules with alanine substitutions for basic amino acids were expressed in 293 fibroblasts, and tested in heparin-dependent assays. Inhibition of FXIa by ATIII in the presence of heparin was decreased 4-fold by alanine substitution at Lys$^{255}$ (A253), with smaller effects noted for mutants A255 and A252. FXI undergoes autoactivation to FXIa in the presence of heparin. The rate of autoactivation was decreased substantially for A253 with modest decreases for A255 and A252. Substituting all four charged residues in the sequence resulted in a profound decrease in autoactivation, significantly greater than for any single substitution. Relative affinity for heparin was tested by determining the concentration of NaCl required to elute FXIa from heparin-Sepharose. Wild type FXIa eluted from the column at 320 mM NaCl, whereas FXIa with multiple substitutions (A252–254 or A250–255) eluted at 230 mM NaCl. All proteins with single substitutions in charged amino acids eluted at intermediate NaCl concentrations. The data indicate that FXI/XIa must bind to heparin for optimal inhibition by ATIII and for autoactivation. Lys$^{253}$ is the most important amino acid involved in binding, and Lys$^{255}$ and Lys$^{252}$ also have roles in interactions with heparin.

**Characterization of a Heparin Binding Site on the Heavy Chain of Factor XI**

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Coagulation factor XI (FXI)$^1$ is the zymogen of a plasma serine protease that contributes to normal hemostasis by activating factor IX through limited proteolysis, in a calcium-dependent manner (1–3). The regulation of activated FXI (FXIa) appears to involve several plasma serine protease inhibitors (serpins). Initial work suggested that α1-antitrypsin (4–6) is the predominant plasma inhibitor of FXIa with some contribution from antithrombin III (ATIII) (5, 7, 8). Recent reports using monoclonal antibody-based enzyme-serpin complex capture techniques disagree with the earlier findings, and indicate a predominant role for C1-inhibitor (C1-INH) and a significant contribution from α2-anti-plasmin (9). The important anticoagulant drug heparin, a highly sulfated glycosaminoglycan isolated from bovine or porcine lung and gut, enhances the inhibition of FXIa by the inhibitors ATIII (8, 10), protease nexin II (11), and C1-INH (12). Although most studies indicate that saturating concentrations of heparin enhance ATIII-mediated inhibition 20–40-fold (8, 10, 13), a recent report indicates a considerably smaller effect and proposes that heparin’s primary anticoagulant effect on FXIa is through C1-INH (12).

There is evidence that FXIa inhibition by ATIII in the presence of heparin proceeds by a template mechanism in which both protease and serpin are approximated by binding to the glycosaminoglycan (13, 14). This process is exemplified by heparin-enhanced inhibition of the coagulation protease thrombin by ATIII (14, 15). A template mechanism has also been demonstrated for autoactivation of FXI in the presence of polyanions such as heparin and the synthetic polysaccharide dextran sulfate (16, 17). The template mechanism implies that FXIa contains structural elements involved in interactions with heparin. Numerous proteins have been described that interact with heparin and other glycosaminoglycans, many of which contain clusters of basic amino acids that form positively charged binding sites for the negatively charged glycosaminoglycan (18). The nature of these binding sites vary. Relatively short “heparin-binding consensus sequences” have been described in a few proteins (19), whereas other molecules such as thrombin have a more complex binding region involving multiple charged residues that are not necessarily adjacent to each other in the primary amino acid sequence, but are approximated in the properly folded protein (20, 21). The serpins ATIII, heparin cofactor II, protease nexin I, and plasminogen activator inhibitor type 1 also contain well conserved regions of basic amino acids required for interactions with heparin (22).

FXI is a disulfide bond-linked homodimer of 80 kDa polypeptides, each containing a trypsin-like catalytic (light chain) domain and an amino-terminal non-catalytic heavy chain (23, 24). The heavy chain, in turn, comprises four in-tandem re-

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1 The abbreviations used are: FXI, factor XI; FXIa, activated FXI; ATIII, antithrombin III; C1-INH, C1-inhibitor; aPTT, activated partial thromboplastin time; BSA, bovine serum albumin; TBS, Tris-buffered saline; TBSA, TBS with BSA; ELISA, enzyme-linked immunosorbent assay; pNA, p-nitroaniline.
peats called apple domains that are required for interactions with other macromolecules (25). Cardin and Weintraub (19) reported that the amino acid sequence X-B-B-X-B-X, where X is a hydrophobic and B a basic amino acid, is present in several proteins that bind to heparin. Recently, Ho and co-workers (25–27), using binding assays, demonstrated that a cluster of amino acids in the third apple (A3) domain of the heavy chain starting at Arg250 (R-I-K-K-S-K) is such a heparin binding site. In this paper, we report on studies that demonstrate a template mechanism of inhibition of FXIa by ATIII and C1-INH in the presence of heparin. A panel of recombinant FXI molecules containing alanine or glutamic acid substitutions for the amino acids in the heparin binding site from Arg250 (R-I-K-K-S-K) to Lys256 were prepared. These molecules were used to determine the importance of this area to heparin-mediated inhibition of FXIa by ATIII, FXI autoactivation in the presence of heparin, and binding of FXIa to heparin-Sepharose.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**

**Molecular Biology**—The human FXI complementary DNA (cDNA) was a gift from Dr. Dominic Chung (University of Washington, Seattle). Chameleon site-directed mutagenesis kit (SK+ version) were from Stratagene (La Jolla, CA).

**Tissue Culture and Recombinant Protein Production**—The 293 human fetal kidney fibroblast cell line was from the American Type Culture Collection (Rockville, MD; ATCC CRL 1573). Dulbecco’s modified Eagle’s medium and G418 were from Life Technologies, Inc., and Cellgro Tissue Culture and Recombinant Protein Production—Characterization of Recombinant Factor XI—Chromogenic substrate S-2366 (t-pyroglutamyl-l-prolyl-l-arginine-p-nitroaniline hydrochloride) was from Diapharma (Malmo, Sweden). FXI-deficient and pooled normal human plasmas were from George King Biomedical (Overland Park, KS). Thrombosil activated partial thromboplastin time (aPTT) reagent was from Ortho Diagnostic (Raritan, NJ). C1-INH was from Alexis Biochemicals (San Diego, CA). ATIII and factor XIIa were from Enzyme Research Laboratories (South Bend, IN).

**Preparation of Recombinant FXI Expression Constructs and Transfection of 293 Cells**

Point mutations to produce alanine or glutamic acid substitutions were introduced into the wild type FXI cDNA in vector pBluescript (SK+ version) using a Chameleon site-directed mutagenesis kit. Proper introduction of the mutation was confirmed by dideoxy-chain termination DNA sequencing. The oligonucleotides used to introduce the mutations are shown in Table I. cDNAs were subsequently ligated into mammalian expression vector pJVCVM, which contains the cytomegalovirus promoter (28). A total of 5 × 10⁶ 293 fibroblasts were co-transfected with 40 μg of FXI cDNA/pJVCVM construct and 2 μg of a plasmid BSNeo, which contained a gene conferring resistance to neomycin (28). Transfection was by electroporation using an electrodcell manipulator 600 (BTX, San Diego, CA). Transfected cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum for 24 h and then switched to the same medium supplemented with the neomycin analog G418 at 500 μg/ml. Medium was exchanged every 48 h for 2 weeks. G418-resistant clones were transferred to 24-well tissue culture plates, and culture supernatants were tested for protein expression by ELISA. Clones expressing the highest levels of protein were expanded to confluence in 175-cm² tissue culture flasks. When the cells had reached >50% confluence, medium was replaced with 75 μl of serum-free Cellgro complete medium supplemented with 10 μg/ml soybean trypsin inhibitor, 10 μg/ml lima bean trypsin inhibitor, and 5 μg/ml aprotinin. Medium was exchanged every 48 h. After collection, conditioned medium was supplemented with benzamidine to a final concentration of 5 mM and stored at −20 °C pending purification.

**Purification and Activation of Recombinant FXI**

Seven hundred ml of conditioned medium was passed over a 3-m1 column of murine anti-human FXI monoclonal antibody IgG5.12 linked to Affigel-10 as described previously (28). After loading, the columns were washed with 10 column volumes of 25 mM Tris-HCl, pH 7.4, 100 mM NaCl (TBS) supplemented with 5 mM benzamidine, followed by elution with 2 mM sodium thiocyanate in 25 mM Tris-HCl, pH 7.4, 5 mM benzamidine. Protein-containing fractions were pooled and concentrated in an ultra-filteration concentrator (Amicon, Inc., Beverly, MA), dialyzed against TBS, and stored at −70 °C. Recombinant proteins were analyzed by SDS-polyacrylamide gel electrophoresis for purity (29). Protein concentration was determined using a dye binding assay (Bio-Rad). Less than 0.1% of the purified protein was in the active form, as determined by chromogenic substrate assay (28). FXIa was prepared by diluting the zymogen preparation to 300 μg/ml in TBS supplemented with 5 μg/ml human factor XIa and incubating at 37 °C. Progress of activation was followed by measurement of absorbance at 405 nm on a ThermoMax microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). Michaelis-Menten constants (Km and Vmax) for cleavage of the chromogenic substrate were determined by standard methods. The value for Vmax was converted to nanomolar pNA generated per second using an extinction coefficient for pNA of 9,800 optical density (OD) units (405 nm)/μmol of pNA. The turnover number (kcat) was calculated from the ratio of Vmax to enzyme concentration.

**Activity of Recombinant Protein in an aPTT Assay**

The coagulant activity of recombinant protein in an aPTT assay was determined as follows: FXI was diluted to 5 μg/ml (30 nM) in TBS containing 0.1% BSA (TBSA), and serial 1:2 dilutions of these preparations were made in TBSA. Sixty microliters of each dilution was mixed

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**Table I**

Oligonucleotides used for site-directed mutagenesis to prepare recombinant FXI molecules containing alanine or glutamic acid substitutions in the putative heparin binding site

| Factor XI Species | Amino acid sequence (amino acids 249–251) | Oligonucleotide sequence (5’ to 3’) |
|------------------|------------------------------------------|----------------------------------|
| Wild type        | TRIKKSKAL                                 | GCTCTTTTAATGGCTGTACTGGGC         |
| A250             | TRIKKSKAL                                 | GCTCTTTGAAGCTGCGCTCTGCTGCAATGCTGTACTGGGC |
| A252             | TRIKKSKAL                                 | GCTCTTTGAAGCTGCGCTCCTGCTGCAATGCTGTACTGGGC |
| A253             | TRIKKSKAL                                 | GAGCTTTGCCCTGGCTTATTTGGCTGCGCGGTCGGTGG |
| A254             | TRIKKSKAL                                 | GAGACAGCTTGGCTTATTTTAATGCCGAAGAGCTGCGCTCTGCTGCAATGCTGTACTGGGC |
| A255             | TRIKKSKAL                                 | CCCAGAAAGAGCTGCGCTCCTGCTGCAATGCTGTACTGGGC |
| A252–254         | TRIKKSKAL                                 | GCTCTTTGAAGCTGCGCTCCTGCTGCAATGCTGTACTGGGC |
| A252–253         | TRIKKSKAL                                 | GCTCTTTGAAGCTGCGCTCCTGCTGCAATGCTGTACTGGGC |
| A250–255          | TRIKKSKAL                                 | GGCTTTGCCCTGGCTTATTTGGCTGCGCGGTCGGTGG |

* A250–255 designates a mutant in which only the basic amino acids at positions 250, 252, 253, and 254 are changed to alanine. The amino acids at positions 251 and 254 are the same as for wild type sequence.
with 60 μl of FXI-deficient plasma and 60 μl of Thrombolizol aPTT reagent. After incubation at 37 °C for 5 min, 60 μl of 25 mM CaCl₂ was added and the time to clot formation was determined on a Datatoc II fibrometer (Helena Laboratories, Beaumont, TX). The clotting time in seconds for each dilution of protein was plotted on log-log paper, and results were compared with a standard curve prepared with wild type recombinant FXI. We have previously demonstrated that recombinant wild type FXI has identical activity in an aPTT assay to FXI purified from human plasma (30).

**Binding of Recombinant FXIa and FXI to Heparin-Sepharose**

A 1-ml heparin-Sepharose column attached to a BioLogic FPLC Workstation (Bio-Rad) was equilibrated with 20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM EDTA. Seven micrograms of FXIa or FXI in buffer A was applied to the column, and the column was washed with an additional 10 column volumes of the above buffer. Protein was eluted with a 15-mI linear sodium chloride gradient (50–1000 mM), and 0.5-ml fractions were collected. For experiments with FXIa, the amount of enzyme in each fraction (0.5 ml) was determined by measuring the cleavage of chromogenic substrate S-2366 using the microtiter plate assay described above. Experiments examining zymogen FXI binding to heparin-Sepharose were carried out in buffers supplemented with the protease inhibitor benzamidine (10 mM) to prevent FXI autoactivation. Fractions were collected as above and the presence of factor XI was determined by ELISA. The conductivity of fractions was measured with a meter conductivity meter (Amber Science, Inc., San Diego, CA) and compared with known standards to determine the sodium chloride concentration of the elution fractions.

**Inhibition of FXIa by ATIII and C1-INH**

All inhibition reactions were carried out in polypropylene tubes in TBS containing 0.1% Tween 20 (TBS/Tween) (12). FXIa (6 nM) was incubated at 37 °C with ATIII (0.35–1.40 μM) or C1-INH (0.38–1.54 μM) in the presence or absence of unfractionated heparin. One unit/ml heparin = 425 mM heparin, assuming an average molecular weight for heparin of 15,000 daltons; 6.4 μg of heparin is equivalent to 1 unit. At various time points, 20-μl samples from the reactions were mixed with 80 μl of TBS/Tween containing 300 μl S-2366 and 5 μg/ml Polybrene, in a 96-well microtiter plate, and the change in OD at 405 nm was followed on the ThermoMax plate reader. The change in absorbance per minute (ΔA) was constant during the course of the measurement. Inhibition was studied under pseudo-first order conditions with inhibitor in 25–250-fold molar excess of FXIa. Pseudo-first order rate constants for each reaction were determined using the equation ln(ΔA/ΔA₀) = -k × t, where ΔA₀ is the rate of cleavage of chromogenic substrate/min of 6 nM FXIa in the absence of inhibitor at time t, ΔA is the rate of cleavage of chromogenic substrate in the presence of inhibitor at time t, and k is the pseudo-first order rate constant. The pseudo-first order rate constants were plotted against the inhibitor concentration using linear regression analysis (Prism version 2.0 software, Graphpad Software, San Diego, CA) to obtain the second order rate constant for each inhibitor. Consistent with previously published results (12), heparin did not affect the cleavage of S-2366 by FXIa in the absence of ATIII or C1-INH.

**Autoactivation of FXI in the Presence of Heparin**

FXI at 50 nM in TBSA containing 25 μM ZnCl₂ was supplemented with unfractionated heparin (1 unit/ml final concentration). At various time points, 10-μl samples were removed and mixed with 85 μl of TBSA containing 6 μg/ml Polybrene to terminate the autoactivation reaction (17). Five microliters of 6 mM S-2366 (300 μM final concentration) was added, and the change in absorbance at 405 nm was followed on the ThermoMax plate reader. The cleavage rate of chromogenic substrate was used to determine the amount of FXIa generated by comparison to a control curve constructed with known amounts of purified recombinant FXIa.

**RESULTS**

**Recombinant FXI Expression, Activity, and Binding to Heparin-Sepharose**—Recombinant FXI proteins were expressed in 293 cells, an adenovirus-transformed human fetal kidney fibroblast line, using an expression vector containing the cytomegalovirus promoter. This system has been used to produce recombinant wild type FXI with properties identical to those of protein derived from human plasma (28, 30). All proteins were expressed at concentrations comparable to wild type protein, and run similarly to wild type protein on SDS-polyacrylamide gels (Fig. 1). Zymogen proteins were converted to FXIa by incubation with human factor XIIa. The activated proteins cleave the chromogenic substrate S-2366 with similar kinetic parameters (Table II), indicating that amino acid substitutions in the A3 domain do not disturb the catalytic function of the light chain. Areas of the A3 domain near the heparin binding site (but distinct from it) have been implicated in binding interactions with the FXIa substrate factor IX (31). To determine if interactions with factor IX have been compromised by the alanine substitutions, an aPTT clotting assay was performed that requires FXIa to activate factor IX in the presence of calcium ions. The results (Table II) show that all proteins retain capacity to activate factor IX. These data indicate that the amino acid substitutions in the A3 domain do not cause severe structural abnormalities in the recombinant proteins.

Relative affinities of the recombinant proteins for heparin were studied by determining the concentration of sodium chloride required to elute them from a heparin-Sepharose column. This technique has been used to study thrombin anion binding exosite interactions with heparin (21). Data are shown in Table II for FXIa molecules. The assay was repeated with zymogen proteins (data not shown), and the results were identical to those obtained with activated protein. Peak wild type FXIa activity eluted from the column at 320 mM NaCl, whereas proteins with multiple alanine substitutions (A250–255 and A252–254) demonstrated the weakest binding, eluting at approximately 230 mM NaCl. All single alanine substitution mutants showed some degree of decreased affinity compared with wild type with the exception of A254. The wild type residue at position 254 is a serine residue that would not be expected to be as critical for heparin binding as a charged residue. The glutamic acid mutant E252–253 showed slightly poorer binding to heparin-Sepharose than A250–255 or A252–254. Glutamic acid carries a negative charge at neutral pH and would likely repel the heparin if the substitution was in the vicinity of the heparin binding site. This may amplify the effect of the amino acid change compared with a substitution with a neutral amino acid with a small side chain such as alanine. This strategy has been used previously when analyzing heparin binding to thrombin (20, 21).

**Inhibition of Recombinant Wild Type FXIa by ATIII and C1-INH, and the Effects of Heparin on Inhibition**—The inhibition of wild type recombinant FXIa by ATIII and C1-INH was tested under pseudo-first order conditions using a chromogenic substrate assay (Fig. 2). The calculated second order rate constants for the inhibition of wild type FXIa are 0.91 × 10³ M⁻¹ s⁻¹ and 1.51 × 10⁴ M⁻¹ s⁻¹ for ATIII and C1-INH, respectively (Table III). These values are in close agreement with previously published data (8, 12), and indicate that C1-INH is a slightly
The activity of recombinant FXIa in a chromogenic substrate (S-2366) cleavage assay, and ofzymogen recombinant FXI in an aPTT clotting assay were determined as described under “Materials and Methods.” For the heparin-Sepharose binding experiment, 7 μg of recombinant FXIa was applied to a 1-ml heparin-Sepharose column and then eluted with a linear sodium chloride gradient. The sodium chloride concentration of the fractions containing peak FXIa activity was determined by measuring fraction conductivity and comparing the result to a control curve prepared with known concentrations of sodium chloride. All results are means of duplicate experiments.

### TABLE II

| Factor XI species | Cleavage of S2366 | Clotting activity | Heparin-Sepharose
|-------------------|-------------------|-------------------|-------------------|
|                   | K_m (μM) | k_cat (s^{-1}) | % of wild type | μM |
| Wild type         | 375     | 540              | 100*             | 320 |
| A250              | 325     | 425              | 108              | 270 |
| A252              | 320     | 400              | 80               | 270 |
| A253              | 290     | 390              | 100              | 250 |
| A254              | 355     | 410              | 120              | 330 |
| A255              | 345     | 440              | 96               | 275 |
| A252-254          | 375     | 450              | 110              | 230 |
| A254-253          | 350     | 470              | 136              | 215 |
| A250-255          | 350     | 470              | 94               | 230 |

* Value for wild type FXI was arbitrarily set at 100%.

**FIG. 2.** Kinetics of wild type FXIa inhibition by ATIII and C1-INH in the absence of heparin. Recombinant FXIa (6 nM) was incubated at 37 °C with varying concentrations of ATIII (A) or C1-INH (B) in 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20. At various time points, samples were removed from the reaction and the residual FXIa activity was measured by chromogenic substrate assay. The concentrations of serine protease inhibitor tested were: A, 0 μM (○), 0.35 μM (●), 0.70 μM (○), 1.05 μM (●), and 1.4 μM (○) for ATIII; and B, 0 μM (○), 0.38 μM (●), 0.77 μM (○), 1.15 μM (●), and 1.54 μM (○) for C1-INH. The natural logarithm of the ratio of the residual FXIa activity to the starting FXIa activity was plotted against time. Pseudo-first order rate constants (the slopes of the individual lines from panels A and B) were plotted against inhibitor concentration for ATIII (C) and C1-INH (D) and subjected to linear regression analysis. The slopes of the plots in panels C and D are the second order rate constants for inhibition.

**TABLE III**

| Factor XI species | Heparin concentration (units/ml) | k2 10^{3} μM^{-1}s^{-1} |
|-------------------|---------------------------------|--------------------------|
|                   | 0                               | 0.125                     | 1.0 |
| ATIII             | Wild type 0.91 20.2 51.3         |                          |     |
| A250              | 0.64 14.3 52.3                   |                          |     |
| A252              | 0.52 10.1 38.1                   |                          |     |
| A253              | 0.56 4.6 24.2                    |                          |     |
| A254              | 0.49 13.8 36.7                   |                          |     |
| A255              | 0.79 8.1 39.7                    |                          |     |
| A252-254          | 0.75 4.4 16.9                    |                          |     |
| E252-253          | 0.79 2.5 14.0                    |                          |     |
| A250-255          | 0.70 3.6 18.9                    |                          |     |
| C1-INH Wild type  | 1.5 1.7 11.0                     |                          |     |

**FIG. 3.** Effect of heparin on FXIa inhibition by serine protease inhibitors. Recombinant wild type FXIa (6 nM) was incubated at 37 °C in 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20, with 130 nM ATIII (○) or C1-INH (●) in the presence of varying concentrations of heparin for 2 min. The amount of residual uninhibited FXIa was determined by chromogenic substrate assay of heparin selected. At heparin concentrations typically achieved during therapeutic anticoagulation (0.1–1.0 unit/ml) (32–34), the second order rate constant for AT III increases 20–55-fold (Table III), whereas the increase for C1-INH is only up to 7.0-fold. Greater rates of inhibition may be achieved with C1-INH with larger amounts of heparin (for example the second order rate constant of inhibition is 45.5 × 10^{3} μM^{-1}s^{-1} at 10
units/ml heparin); however, these concentrations are substantially in excess of those achieved during therapy. As therapeutic concentrations of heparin enhance inhibition of FXIa by ATIII to a greater degree than by C1-INH, we used ATIII to test FXI molecules with amino acid substitutions in the putative heparin binding region.

The Effect of Heparin on ATIII Inhibition of Recombinant FXIa Molecules with Alanine or Glutamic Acid Substitutions from Amino Acid Arg250 to Lys255—The inhibition of FXIa molecules containing amino acid substitutions was performed in a manner identical to that for wild type protein. The second order rate constants for inhibition by ATIII were reasonably similar for all proteins tested (0.49–0.91 × 10^3 M^-1 s^-1) in the absence of heparin (Table III), indicating that the Arg250 to Lys255 region of the A3 domain is not required for interactions with ATIII in the absence of heparin. Inhibition in the presence of heparin was initially tested under conditions in which heparin would be limiting (130 nM ATIII and 0.125 units/ml (45 nM) heparin). Results are shown in Table III. Of the single amino acid substitutions, A253 had the largest effect, decreasing inhibition about 4-fold compared with wild type protein. Combining A253 with an alanine substitution at Lys252 (A252-254) did not increase the effect caused by the A253 substitution alone. The second order rate constants for proteins with substitutions A255 and A252 were decreased approximately 2.5- and 2.0-fold, respectively, compared with wild type. Finally, the protein with all four basic residues changed to alanine (A250-255) was inhibited with a second order rate constant of 3.6 × 10³ M^-1 s^-1 (Table III). These data indicate that the lysine residue at position 253 is of primary importance in FXIa interactions with heparin during ATIII inhibition. Inhibition of mutant (E252–253), containing glutamic acid substitutions for Lys252 and Lys253 was reduced about 8-fold compared with wild type. In studies using heparin at 1.0 units/ml (the upper end of the therapeutic heparin range), the results with single amino acid substitutions were not as large; however, proteins with multiple amino acid substitutions, A252–254 and A250–255, demonstrated 3-fold lower rates of inhibition.

The Effect of Alanine or Glutamic Acid Substitutions between Arg250 and Lys255 on Autoactivation of FXI in the Presence of Heparin—Autoactivation of FXI occurs in the presence of several glycosaminoglycans including heparin (17), as well as the synthetic polysaccharide dextran sulfate (16, 35). This phenomenon involves a template mechanism (17, 19). Recombinant zymogen proteins were incubated with heparin, and FXIa generation was followed over time by chromogenic substrate assay. A representative example of such an experiment is shown in Fig. 4. The typical sigmoidal curve characteristic of an autoactivation reaction is demonstrated for wild type FXI, and the time course is consistent with previously published results (17). This assay may be influenced by the presence of FXIa in the zymogen preparation; therefore, serum-free medium was supplemented with protease inhibitors known to inhibit FXIa and its activation prior to adding the medium to cell cultures. In addition, all purification steps were carried out in the presence of the reversible serine protease inhibitor benzamidine (28). Although it is unlikely that any of the preparations are completely devoid of small amounts of active enzyme, these precautions kept the level of FXIa below detectable limits by chromogenic substrate assay (<0.1% of the total protein). Of the proteins with single amino acid substitutions, A253 was most severely affected (Fig. 4B), consistent with the results from the ATIII inhibition assay. A more modest effect was noted for A255, and autoactivation of A252 was slightly retarded compared with wild type FXI. Autoactivation of A252–254 was slightly slower compared with A253, and A250–255 was markedly slower, failing to achieve complete activation after 350 min (Fig. 4B). These data suggest that there are amino acids other than Lys255 that are involved in interactions with heparin, with Lys255 being the next most important and some contribution from Lys252. The effect of glutamic acid substitutions at amino acids 252 and 253 on autoactivation were greater than alanine substitutions at these two positions, as might be expected from a negatively charged amino acid repelling the negatively charged heparin template.

DISCUSSION

A substantial amount of literature is now available regarding the mechanisms by which heparin and related glycosaminoglycans enhance the inhibition of blood coagulation proteases by plasma serine protease inhibitors (serpins) (36). Two major mechanisms have been observed. Optimal enhancement of inhibition of thrombin and activated factor IX by ATIII requires that both serpin and protease bind to heparin in proximity to each other (14, 15, 22). This so-called template mechanism requires the heparin to be of sufficient length (18–20 saccharide residues) to bind both proteins and is characterized by a decrease in rate of inhibition as heparin concentration is increased above an optimum concentration. This is presumably due to the increased likelihood that serpin and enzyme will bind to different heparin molecules, rather than to the same molecule. In contrast, heparin’s effect on inhibition of activated factor X requires only that the serpin bind to the heparin for optimal inhibition (14, 37). Once the serpin is saturated with heparin, maximum rate of inhibition is achieved and no further change (increase or decrease) in inhibition occurs at higher heparin concentration. Heparin and similar polymers have been demonstrated to enhance several biochemical reactions involving FXI and FXIIa. These include the activation of FXI by factor XIIa, thrombin, and autoactivation (16, 17, 35); and the inhibition of FXIa by the serpins ATIII (7, 8, 13), protease nexin II (11, 38), and C1-INH (12). In the cases of autoactivation and protease nexin II-mediated inhibi-
Heparin Binding Site on Factor XI

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The regulation of FXIa appears to be complex, involving several plasma serpins. In addition, platelets release a Kunitz-type FXIa inhibitor from α-granules that is a form of the β-amyloid precursor protein, also referred to as protease nexin-II (11, 38, 39). There is conflicting data concerning the major protease inhibitor of FXIa in plasma. Initial studies using kinetics and plasma-based assays suggested that α2-antitrypsin (4–6) is the predominant plasma inhibitor of FXIa with some contribution from antithrombin III (ATIII) (7, 8, 13). C1-inhibitor (C1-INH) (40, 41), α2-antiplasmin (42), and protein C inhibitor (43) were also shown to inhibit FXIa. Recent reports using monoclonal antibody based enzyme-serpin complex capture techniques disagree with the earlier findings and indicate that C1-INH is the major regulator of FXIa in plasma (9). Several groups have reported a 20–40-fold increase in ATIII-mediated inhibition of FXIa by heparin; however, Wullemien and co-workers recently reported a considerably smaller effect, while observing a nearly 50-fold increase in C1-INH-mediated inhibition of FXIa (12). A template mechanism for FXIa inhibition by these serpins could explain the discrepancies in the literature. The optimal concentration of heparin for enhancement of ATIII- or C1-INH-mediated inhibition could be substantially different, and the protease that predominates in a particular study would depend on the concentration of heparin selected.

The results reported here demonstrate a template mechanism is involved in FXIa inhibition by ATIII in the presence of heparin. A similar mechanism operates for inhibition by C1-INH but with a substantially greater optimal concentrations of heparin than for ATIII. When heparin is used as a therapeutic anticoagulant, plasma levels of 0.1–1.0 unit/ml are typically achieved (32–34). We observed an increase in rate of inhibition in excess of 50-fold for ATIII in the presence of 1 unit/ml heparin, whereas inhibition by C1-INH was increased about 7-fold under the same conditions. The discrepancy between our results and those of Wullemien and co-workers probably are due to the very high concentration of heparin used in the later study (50 units/ml) (12), which is considerably closer to the optimal concentration for C1-INH than for ATIII. Considering the second order rate constants for inhibition by ATIII and C1-INH in the absence of heparin, the similar plasma concentrations of the two proteins (approximately 2 μM), and the therapeutic range of plasma heparin concentrations, it is predicted that ATIII would be at least as potent, or perhaps a stronger inhibitor of FXIa compared with C1-INH in a patient receiving heparin.

The template mechanism implies that FXI/FXIa contains one or more sites for interactions with heparin. An examination of the FXI primary amino acid sequence reveals a cluster of amino acids starting at Arg250 with the sequence Arg-Ile-Lys-Lys253 and Lys252 within this sequence are required for binding of FXI to heparin. To determine the importance of the charged residues in this sequence to heparin-mediated activities, we prepared a series of recombinant FXI mutants containing alanine substitutions for the amino acids of interest, and tested them in heparin-dependent functional assays. We selected ATIII over C1-INH to test the putative heparin binding site mutants because of the larger degree of enhancement by heparin between 0.1 and 1.0 units/ml. The data indicate that Lys253 is of primary importance in interactions with heparin during ATIII-mediated inhibition, although smaller contributions from other charged amino acids (Lys255 and Lys252) in this area are probable. This finding is in agreement with the previously published work of Ho et al. (26). The second order rate constants for inhibition by ATIII and heparin of all mutants, including A250–255, which removes all charged residues in the binding site, were greater than the values in the absence of heparin. It is possible that other sequences in FXI are involved in heparin binding that were not affected by the alanine substitutions. Alternatively, heparin binding to ATIII may have potentiated its inhibitory effect even in the absence of FXIa binding to heparin. In support of this is a recent abstract describing enhancement of C1-INH inhibition of FXIa by low molecular weight heparins that are of insufficient length to support a template mechanism (44).

Factor XI undergoes autoactivation in the presence of dextran sulfate and several glycosaminoglycans, including heparin (16, 17, 35). As FXI is enzyme and substrate in this system, and the template mechanism requires both to bind to heparin, it was anticipated that this assay may be more sensitive to abnormalities in heparin binding than the ATIII inhibition assay. This turns out to be the case, with the protein containing alanine substitutions in all four charged residues in the putative binding site exhibiting a greatly reduced rate of activation in this assay. The results supported the importance of Lys253 in FXIa interactions, but indicate a significant contribution from Lys255, and that Lys252 is also involved. This is supported by the results of the heparin-Sepharose binding study, which demonstrated that all proteins with a substituted basic amino acid bound to heparin more weakly than wild type protein. Previously published work on the importance of the R250-I-K-S-K to heparin binding did not identify Lys255 as a critical residue. It is possible that functional assays are more sensitive to perturbations in heparin binding than direct binding assays. Such a phenomenon was previously observed for certain heparin binding site mutants of thrombin, which have modest defects in direct binding to heparin, but give substantially more abnormal results when tested in an ATIII/heparin inhibition assay (21).

In summary, our data demonstrate that FXIa must bind to heparin for optimal inhibition by ATIII and C1-INH. At concentrations of heparin typically achieved during therapy, inhibition by ATIII would be enhanced to a greater degree than inhibition by C1-INH. These data in conjunction with published results suggest that both serpins would be important regulators of FXI during heparin therapy. The R250-I-K-S-K sequence in the third apple domain of the heavy chain is critical for interactions with heparin. Lys253 is the single most important amino acid residue for heparin binding; however, other amino acids, most importantly Lys255 and to a lesser extent Lys252 also appear to play a role in heparin binding. The A3 domain appears to be a major area of interaction between FXI and other macromolecules, as amino acid sequences within A3 required for binding to factor IX (31) and to platelets (45, 46) have recently been reported.

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