Localization of a Heparin Binding Site in the Catalytic Domain of Factor XIa†

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ABSTRACT: Inhibition of factor XIa by protease nexin II ($K_i \approx 450$ pM) is potentiated by heparin ($K_I \approx 30$ pM). The inhibition of the isolated catalytic domain of factor XIa demonstrates a similar potentiation by heparin (K_i decreasing from 436 ± 62 to 88 ± 10 pM) and also binds to heparin on surface plasmon resonance (K_d 11.2 ± 3.2 nM vs K_d 8.63 ± 1.06 nM for factor XIa). The factor XIa catalytic domain contains a cysteine-constrained α-helix-containing loop: ^{527}CQKRYRGHKITHKMIC^{542}, identified as a heparin-binding region in other coagulation proteins. Heparin-binding studies of coagulation proteases allowed a grouping of these proteins into three categories: group A (binding within a cysteine-constrained loop or a C-terminal heparin-binding region), factors XIa, IXa, Xa, and thrombin; group B (binding by a different mechanism), factor XIIa and activated protein C; and group C (no binding), factor VIIa and kallikrein. Synthesized peptides representative of the factor XIa catalytic domain loop were used as competitors in factor XIa binding and inhibition studies. A native sequence peptide binds to heparin with a $K_d = 86 \pm 15$ nM and competes with factor XIa in binding to heparin, $K_i = 241 \pm 37$ nM. A peptide with alanine substitutions at ^{534}H, ^{535}K, ^{538}H, and ^{539}K binds and competes with factor XIa for heparin-binding in a manner nearly identical to that of the native peptide, whereas a scrambled peptide is ∼10-fold less effective, and alanine substitutions at residues ^{529}K, ^{530}R, and ^{532}R result in loss of virtually all activity. We conclude that residues ^{529}K, ^{530}R, and ^{532}R comprise a high-affinity heparin-binding site in the factor XIa catalytic domain.

Factor XI (FXI)[1] is a homodimeric zymogen protein, composed of two 607 amino acid chains, which circulates in plasma at a concentration of ∼30 nM (*1*). Its importance to the process of blood coagulation is demonstrated by the hemostatic defects that occur in individuals with FXI deficiency (*2*−*4*). The natural substrate of enzyme FXIa is FIX, a protein essential for production of an adequate hemostatic response (*5*). It is, therefore, important to understand the processes by which FXI is activated and regulated.

FXI is activated in vitro to a trypsin-like serine protease by FXIIa (*6*), thrombin, or FXIa (*7*, *8*) in the presence of high molecular weight kininogen and negatively charged surfaces. One surface known to support this activation is the platelet surface (*9*). Additionally, FXI activation by all three proteases is enhanced in the presence of glycosaminoglycans (GAGs) (*7*, *10*). Whether GAGs represent a physiologic surface for FXI activation has not been definitively determined, however. Gailani and Broze (*11*) demonstrated that FXI activation, and subsequent FIX activation, occurred within 20 min in plasma which contained sulfatides and tissue factor. In contrast, Wuillemin et al. (*12*) found that FXI activation in plasma supplemented with various GAGs required high concentrations of added thrombin. Other studies have demonstrated that FXI is efficiently activated on immobilized dextran sulfate and heparin sulfate (*7*, *8*). The efficiency of activation positively correlates with the amount of sulfation, or negative charge, associated with the GAG. These studies suggest that immobilization of FXI may be important to the activation process. Cell-surface bound GAG may be one of the activating surfaces.

It is also possible that FXIa binding to GAGs represents a means of inhibiting its activity. The inhibition of FXIa by antithrombin III (ATIII) and protease nexin II (PNII) is potentiated in the presence of heparin and other GAGs (*13*−*16*). ATIII is a serine protease inhibitor present in plasma at a concentration of ∼2.7 μM. The second-order rate constant for FXIa inhibition by ATIII was reported as 3.2×10^4 M^{-1} s^{-1} in the absence of heparin with a 100-fold potentiation in the presence of heparin (*16*). One part of this increase in association rate is proposed to be a result of the co-localization of the proteins to the same heparin chain (*16*). Another part of this increase is a result of a conformational change in ATIII induced upon its binding to heparin (*14*). PNII is a kunitz-type protease inhibitor that inhibits FXIa with a second-order rate constant of 3×10^6 M^{-1} s^{-1}. This association rate constant increases ∼10-fold, to 3×10^7 M^{-1} s^{-1}, in the presence of heparin chain lengths of 64 disaccharide units, with a resultant inhibition constant (K_i) of

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[1] Abbreviations: FXI, Factor XI; GAGs, glycosaminoglycans; ATIII, antithrombin III; PNII, protease nexin II; K_i, inhibition constant; A3, Apple 3 domain; K_d, equilibrium constant; $K_{d,app}$, apparent K_d; FMGB, fluorescein mono-*p*-guanidinobenzoate hydrochloride; HABA, 4′-hydroxyazo-benzene-2-carboxylic acid; Mr, molecular weight; IC_{50}, the concentration at which 50% activity remained; APC, activated protein C; cpm, counts per minute; SPR, surface plasmon resonance.

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~30 pM (*17*). The proposed mechanism for this increase in association rate is that of a simple template, i.e., co-localizing both FXIa and PNII to the same heparin chain (*17*).

Amyloid precursor protein is a term applied to various isoforms of a protein encoded on human chromosome 11. Two isoforms, of 751 (*18*, *19*) and 770 (*20*) amino acids, contain a Kunitz-type inhibitor domain. PNII, the soluble form of amyloid precursor protein containing the Kunitz-type inhibitor domain, is an abundant platelet protein released from α-granules upon platelet activation (*21*−*24*). It has previously been shown to bind to heparin, heparan sulfate and other proteoglycans (*25*−*30*). The amino acid sequences in PNII mediating these interactions were mapped to residues 96−110 (*29*), 375−388 (*26*), 436−469, and 469−504 (APP_{751} numbering) by deletion mutagenesis and peptide mapping (*25*).

A known heparin-binding site in FXI is located within a heparin-binding consensus sequence, ^{250}R−I−K−K−S−K^{255}, in the Apple 3 (A3) domain (*31*, *32*). Residues ^{252}K, ^{253}K, and possibly ^{255}K, have been shown to be essential for binding of the zymogen FXI to heparin. The A3 domain site is a low affinity site, equilibrium constant (K_d) ≈ 110 nM, relative to the FXI plasma concentration of 30 nM. Zhao et al. (*32*) reported that a mutant FXIa with alanine substitutions of ^{252}K, ^{253}K, and ^{255}K in the A3 domain, lost only part of its heparin-binding activity, as determined by the NaCl concentration required to elute the protein from a heparin-agarose column, and its heparin-potentiated inhibition by ATIII (*32*). The heparin-binding affinity of the mutant FXIa (eluted by 230 mM NaCl) was decreased in comparison to wild-type FXIa (eluted by 320 mM NaCl). The degree of heparin-potentiation of mutant FXIa inhibition by ATIII, 22-fold, was decreased from that of wild-type FXIa, 55-fold. Since the conformational change induced in ATIII, upon binding to heparin, contributes to ATIII's effectiveness as an inhibitor, it is difficult to interpret the significance of this change relative to the affinity of FXIa for heparin. FXIa binds to heparin with relatively high affinity (K_d = 1.6−4.0 nM) compared with FXI (K_d ≈ 110 nM) (*31*). This finding suggests exposure of an additional heparin-binding site on FXIa that could be involved in co-localizing FXIa and PNII on full-length heparin.

The primary structure of FXIa was examined for amino acid sequences homologous to known heparin-binding sequences. Cardin et al. (*33*), Margalit et al. (*34*), and Fromm et al. (*35*) found that two amino acids, arginine and lysine, occur with great frequency in heparin/heparan sulfate binding sites, usually separated by one hydropathic amino acid. Leucine, tryptophan, and tyrosine are also found at a frequency higher than their natural occurrence in proteins (*34*). β-Sheet and α-helix are common structural motifs found in these sites (*33*). Two sequences in the catalytic domain of FXIa are consistent with these characteristics: ^{509}YRKLRDK^{515} and ^{527}CQKRYRGHKITHKMIC^{542}. The sequence ^{527}CQKRYRGHKITHKMIC^{542} is a carboxyterminal cysteine-constrained loop retained by all coagulation proteins. FIXa, FXIa, and thrombin contain heparin-binding consensus sequences within this loop. The arginine residues in this loop in thrombin have been identified as part of thrombin exosite 2, which binds heparin (*36*). The purpose of this study was to determine if either ^{509}YRKLRDK^{515}, ^{527}CQKRYRGHKITHKMIC^{542}, or both of these potential binding sites represent a high affinity heparin-binding site on FXIa.

EXPERIMENTAL PROCEDURES

Materials. PNII was purified from stably transfected 293-human embryonic kidney cells (a gift from W. Van Nostrand). Recombinant Kunitz protease inhibitor domain was also a kind gift of W. VanNostrand. Human FXIa and zymogen FXI were purchased from Haemotologic Technologies, Inc. (Essex Junction, VT). Fractionated heparin of 64 disaccharide units was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). The chromogenic substrate, L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline hydrochloride (S2366), was purchased from Chromogenix (Mölndal, Sweden). The monoclonal antibody, 22C11, which recognizes PNII, was purchased from Boehringer Mannheim (Indianapolis, IN). The monoclonal antibody, 5F7, which binds with high affinity to the heavy chain region of FXI (*37*), was prepared in the hybridoma facility of the Temple University Thrombosis Center. Dulbecco's modified Eagle's medium (DMEM) was obtained from Mediatech (Herndon, VA). Newborn calf serum and Geneticin were obtained from Gibco (Grand Island, NY). Microtiter plates were purchased from Becton Dickinson Labware (Lincoln Park, NJ). Streptavidin coated microtiter plates, chemiluminescent substrate and Iodogen were purchased from Pierce (Rockford, IL). Na-^{125}I was obtained from New England Nuclear (Boston, MA). Avidin and biotin-XX-hydrazide were purchased from Calbiochem (San Diego, CA). Streptavidin-coated sensor chips and HBS buffer were obtained from Biacore Inc. (Uppsala, Sweden). Alkaline phosphatase conjugated anti-mouse IgG, horseradish peroxidase conjugated anti-mouse IgG, *p*-nitrophenyl phosphate tablets, fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB), fluorescein, Na *m*-periodate, 4′-hydroxyazo-benzene-2-carboxylic acid (HABA), dinitrothiobenzoate, glutamine, penicillin/streptomycin, Sepharose CL-6B, DEAE-Sepharose, Sephacryl S-400, dextran sulfate (Mr = 500 000), Sephadex G-25, dithiothreitol, iodoacetamide, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Centricon concentrators were from Millipore (Bedford, MA). All other reagents were analytical grade or best quality commercially available.

Methods. Preparation of Isolated FXIa Catalytic Domain. The catalytic domain, or light chain, of FXIa was isolated as described (*37*). FXIa, 200 μg, was placed in 0.5 mL of Tris/succinate buffer, pH 8.3 (40 mM Tris, 10 mM succinic acid, 1 mM benzamidine, 1 mM EDTA, 50 μg/mL Polybrene), reduced with dithiothreitol (0.1 mM, 1 h) at room temperature under nitrogen in the dark, and alkylated with iodoacetamide (0.5 mM,1 h). The reduced and alkylated material was dialyzed against phosphate-buffered saline, then applied to a heavy chain-specific monoclonal antibody (5F7) column. The flow through fraction was analyzed by 10% SDS−PAGE and the active site concentration determined using FMGB.

Purification of PNII. PNII was purified from transfected 293-human embryonic kidney cells as described (*38*). Briefly, serum-free medium from confluent cells was removed, filtered, and chilled to 4 °C, and 200 μM phenylmethanesulfonyl fluoride was added. The medium was applied to a dextran-sepharose column equilibrated in phosphate-buffered saline. Adsorbed protein was eluted with a linear gradient

of 0.15−1.5 M NaCl in phosphate buffer. Fractions containing PNII were detected by dot-blot on nitrocellulose (Bio-Rad Laboratories, Hercules, CA) using monoclonal antibody 22C11, horseradish peroxidase conjugated antimouse IgG and chemiluminescent substrate. Positive fractions were diluted with 20 mM potassium phosphate, pH 7.4, to reduce the conductivity, and applied to a DEAE-Sepharose column equilibrated in 20 mM K_2HPO_4, 0.2 M NaCl, pH 7.4. Bound protein was eluted with 0.75 M NaCl in 20 mM K_2HPO_4. Fractions were collected and analyzed by dot-blot as described above. PNII-positive fractions were concentrated to 1 mL and applied to a Sephacryl S-400 column equilibrated in 20 mM K_2HPO_4, 0.5 M NaCl. Fractions were collected and analyzed by SDS−PAGE. Positive fractions were combined and active site concentration determined using active site-titrated FXIa.

Determination of Protein Concentration by Active-Site Titration. FXIa or isolated catalytic domain was evaluated for active-site concentration using the method of Melhado et al. (*39*). The release of fluorescein from 10 μM FMGB was monitored in a Bowman Series 2 spectrofluorimeter (SLM Aminco, Urbana, IL) both before and after the addition of enzyme (excitation wavelength was 500 nm, emission 520 nm with 4 nm slit widths). After correction for spontaneous hydrolysis, the burst hydrolysis was converted to a molar concentration of enzyme by comparison to a standard curve. The active site concentration of FXIa was essentially identical to the protein concentration calculated using the published extinction coefficient of FXIa, 1.34 at 0.1%, absorbance at 280 nm. The active-site concentration of the isolated catalytic domain was determined by absorbance at 280 nm using a calculated extinction coefficient of ~0.6485 (*40*). The active-site concentration of purified PNII was determined by titration with active-site-titrated FXIa as described (*17*).

Biotin Labeling of Heparin. Heparin with 64 disaccharide subunits was biotin-labeled using the method of O'Shannessy (*41*). Fifty milligrams of heparin was dissolved in 2.5 mL of 0.1 M sodium acetate, 0.15 M NaCl, pH 5.5, and chilled on ice. Sodium periodate, 10 mM, was added for 30 min in the dark. The reaction was stopped with 20 mM sodium sulfite solution. The heparin solution was desalted using a PD-10 column then concentrated to 0.9 mL using Centricon-3. Biotin-X-X-hydrazide, 100 μL of an 8 mg/mL solution in dimethylformamide, was added, mixed, and incubated 24 h at room temperature. Excess biotin was separated by concentration × 2 using Centricon-3 after adding HBS. The degree of biotin incorporation was determined by measuring the absorbance at 500 nm of 0.9 mL avidin/HABA solution (6 μM avidin, 0.3 mM HABA) (A1) then determining the change in absorbance after addition of 0.1 mL biotin-labeled heparin (A2). The percent incorporation is then calculated:

$$D_{A500} = 0.9(A1 - A2)$$

$$\text{Biotin (M)} = D_{A500}/34\,000$$

$$\text{Heparin (M)} = [\text{heparin in mg/mL}] \times 0.1/\text{MW}$$

$$\text{Molar degree of biotin incorporation} = \frac{[\text{biotin}]}{[\text{heparin}]}$$

^{125}I-Labeling and Activation of FXIa. FXI was ^{125}I-labeled according to the method of Tuszynski et al. (*42*). One microcurie of ^{125}I was combined with 20 μg of Iodogen in an Eppendorf tube and vortexed for 5 min. The [^{125}I]Iodogen was transferred to an Eppendorf tube containing FXI and incubated ~20 min, then the reaction stopped using 50 μg/mL sodium metabisulfite. Labeled FXI was removed from free ^{125}I by centrifugation through a Sephadex G-50 column equilibrated in HBS, 0.1% BSA. The percentage of free ^{125}I was determined by trichloroacetic acid precipitation. Labeled FXI was mixed with 1 μg of unlabeled FXIa and applied to a 1 mL of dextran-Sepharose column. After 2 h at 22 °C, the column was washed with HBS until no radioactivity was measured in the flow-through. The column was then eluted using 0.01 M Hepes, 1 M NaCl. Fractions with amidolytic activity were assessed by SDS−PAGE and protein concentration determined by A_{280}.

Peptide Folding and Characterization. Peptides were synthesized on an Applied Biosystems 430A Peptide Synthesizer (Foster City, CA) using a modification of the procedure of Kent and Clark-Lewis (*43*). All peptides displayed a single peak on reverse phase HPLC, using a Waters C8 μBondapak column on a Waters HPLC system (Milford, MA). The procedure for folding of cysteine-containing peptides has been described previously (*44*). Each peptide was diluted to <0.1 mg/mL in dH_2O, pH adjusted to 8.5 using 1 M NH_4OH, and gently stirred at 4 °C for 3 days. The peptides were then separated from any aggregates using Centricon-3. The flow-through fractions from the Centricon-3 filters were concentrated to greater than 1 mg/mL using 1000 MWCO dialysis tubing and PEG 10000. Samples of each peptide were then analyzed on 20% SDS−PAGE. The concentrations of free thiols were measured using an Ellman assay.

Ellman Assay. Free thiols in the peptides were determined by Ellman assay as described (*45*). The 412 nm absorbance of 0.1 mg/mL peptide in 0.1 M phosphate, 1 mM EDTA, pH 7.3, buffer was measured before and after the addition of 10 μL of 3 mM dinitrothiobenzoate. The molar concentration of free thiols was determined by the extinction coefficient at 412 nm for the TNB anion: 14150/M cm.

Computer Modeling of the FXIa Catalytic Domain. The catalytic domain of FXIa was modeled using the crystal structure of bovine pancreatic trypsinogen, the structure of which has been refined to 1.9 angstroms (*46*) and with which the catalytic domain is ~49% homologous. Amino acids for the FXIa catalytic domain were substituted, as necessary, in the trypsinogen sequence. Only one gap occurred, one amino acid in size, which was closed by joining of amino acids in the FXIa sequence. Using Sybyl 6.4 computer modeling software (Tripos, Inc., St. Louis, MO), cysteine bonds were established, charges assigned using the Gasteiger-Hückel method (*47*) and the energy minimized using a combination of the Simplex method and MAXIMIN2 procedure.

Inhibition Kinetics. Either FXIa or isolated catalytic domain were diluted in 0.05 M HEPES, 0.15 M NaCl, 0.1% BSA to a final concentration of 0.1 nM, then incubated with increasing concentrations of PNII or recombinant KPI, in the presence or absence of 100 nM full-length fractionated (64 subunits) heparin sulfate, for 1 h at 37 °C. Residual amidolytic activity was measured under pseudo-first-order kinetic conditions for 15 min on a Hewlett-Packard diode array spectrophotometer. The substrate cleavage rate in the presence of inhibitor was divided by the uninhibited rate and

the concentration at which 50% activity remained (IC_{50}) calculated by Kaleidograph v3.05 (Abelbeck Software) nonlinear least squares regression software. The IC_{50} was converted to K_i using the equation:

$$K_i = IC_{50}/1 + S/K_m \quad (1)$$

where S is the substrate concentration and K_m is the K_m of FXIa for S2366, determined to be $\sim 250 \pm 20$ μM in previous work in this laboratory (*22*).

Competition with Heparin-Binding Peptides in Heparin-Potentiated PNII Inhibition of FXIa. In experiments examining the effects of different peptides, samples of 0.1 nM FXIa were incubated in the presence of one peptide concentration and increasing concentrations of PNII. The K_i was determined as described. Results were then graphed as K_i versus peptide concentration and the IC_{50} determined.

Surface Plasmon Resonance Studies of FXIa, FXIa Catalytic Domain, FXIa Heparin-Binding Peptides, FVIIa, FIXa, FXa, FXIIa, Kallikrein and Activated Protein C Binding to Heparin. Biotin-heparin was immobilized on a streptavidin-coated sensor chip to a response unit value of 100−150. A reference cell for nonspecific binding was treated with biotin-hydrazide/Tris. Various concentrations of FXIa, FXIa catalytic domain, FXIa-derived peptides, FXIIa, or kallikrein were diluted in HBS, 3.4 mM EDTA, 0.005% Surfactant P20. FVIIa, FIXa, FXa, thrombin, and activated protein C (APC) were diluted in HBS buffer containing 2 mM calcium. Each protein was injected with 5−6 min association and dissociation times to allow for direct comparison. Conditions were not optimized for each protein. After subtraction of nonspecific binding curves, the association and dissociation rate constants were determined using a global fit to a Langmuir one to one model using Biaevaluation software (Biacore, Inc.). The best fit had a χ^2 <10 or <5% of the equilibrium response unit value for the highest concentration. The χ^2 value is the square of the differences between the theoretical ideal curve and the actual curve and was calculated according to the equation:

$$\chi^2 = \frac{\sum (r_f - r_x)^2}{n - p}$$

where r_f indicates the fitted value at a given point, r_x indicates the experimental value at that point, n is the number of data points, and p indicates the number of fitted parameters.

Equilibrium Binding Studies of FXIa and Heparin. To 48 wells of a streptavidin-coated polystyrene removable well plate was added 100 μL of a biotin-heparin-containing solution was added and incubated for ≥2 h at room temperature. After washing three times with TBST, increasing concentrations of FXIa were added, 100 μL/well, in triplicate and incubated for 2 h at 37 °C. A second set of blocked wells was used to determine nonspecific binding. Then, 100 μL of a 1 μg/mL solution of 5F7 was added to each well and incubated 1 h at 37 °C, followed by 2 μg/mL alkaline phosphatase conjugated anti-mouse antibody for 1 h. To detect bound protein, 100 μL/well of a 1 mg/mL *p*-nitrophenyl phosphate was added and color developed for 20−30 min. The reaction was stopped with 100 μL/well of 2 N NaOH. Absorbance at 405 nm was measured on a ThermoMax microtiter plate reader (Molecular Devices, Palo Alto, CA). Specific binding was the amount of protein bound after subtracting binding to blocked wells. For comparison, varying concentrations of ^{125}I-labeled FXIa were added in 100 μL volumes, in triplicate, to wells containing biotin-heparin. After washing, the wells were removed, placed in individual tubes, and the amount of bound FXIa determined by measuring counts per minute (cpm) for 60 s in a γ counter. Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled FXIa. The cpm bound were converted to amount of protein bound and nonspecific binding subtracted.

Competition Binding Studies. [^{125}I]FXIa, 2 nM, was combined with increasing concentrations of a synthetic peptide, 100 μL total volume, to biotin-heparin-containing wells in triplicate. A second set of wells, with blocker only, was used to measure nonspecific binding. The number of cpm bound were measured and converted to a fraction of total binding by dividing by the cpm bound in the absence of peptide competitor. The IC_{50} was then determined by curve fit using Kaleidograph software, as described previously.

RESULTS

Isolation and Characterization of FXIa Catalytic Domain. After FXIa was gently reduced with dithiothreitol and alkylated with iodoacetamide, the FXIa catalytic domain was separated by immunoaffinity chromatography on 5F7, a heavy chain-specific, monoclonal antibody column. On 10% SDS−PAGE, the isolated catalytic domain migrated as a single band at the same Mr as the 34 kDa FXIa catalytic domain obtained by reduction with β-mercaptoethanol. The isolated catalytic domain was tested for active-site concentration by comparison of its hydrolysis of FMGB to a fluorescein standard curve. The active-site concentration was ~580 nM, as determined by $E^{1\%}_{A280,1cm} = 0.65$. The concentration of ~0.020 mg/mL gave a specific activity of 0.87 mol of active site/mol of monomer.

Binding of FXIa and FXIa Catalytic Domain to Biotin-Heparin. The affinity of FXIa for heparin was examined under equilibrium binding conditions. The binding of ^{125}I-labeled FXIa was compared to the affinity of unlabeled FXIa (using antibody 5F7 and ELISA) to biotin-heparin in streptavidin-coated microtiter wells. The apparent K_d ($K_{d,app}$) for FXIa binding to biotin-heparin in the microtiter well is 1.45 ± 0.26 nM using the ELISA method (Figure 1, panel A) and is 2.14 ± 0.45 nM using [^{125}I]FXIa (panel B). This is in good agreement with the results reported previously (*31*).

The binding of FXIa to biotin-heparin was then studied using surface plasmon resonance (SPR). Binding specificity was confirmed by the loss of FXIa binding to immobilized heparin in the presence of a 10-fold molar excess of heparin in solution (Figure 2, panel A). FXIa concentrations between 7.5 and 20 nM were infused across a streptavidin sensor chip with biotin-heparin bound (Figure 2, panel B).Under kinetic conditions, the rate constants and resultant K_d were determined. The association rate constant is $\sim 5.2 \pm 2 \times 10^5$ M^{-1} sc^{-1}, dissociation rate constant $4.5 \pm 0.7 \times 10^{-3}$ s^{-1}, with a $K_{d,app}$ of 8.6 ± 1.1 nM (Table 1). The catalytic domain, at concentrations between 10 and 50 nM, was injected using the same conditions as for FXIa (panel C). The association rate constant is $6.6 \pm 1.9 \times 10^5$ M^{-1} s^{-1}, dissociation rate

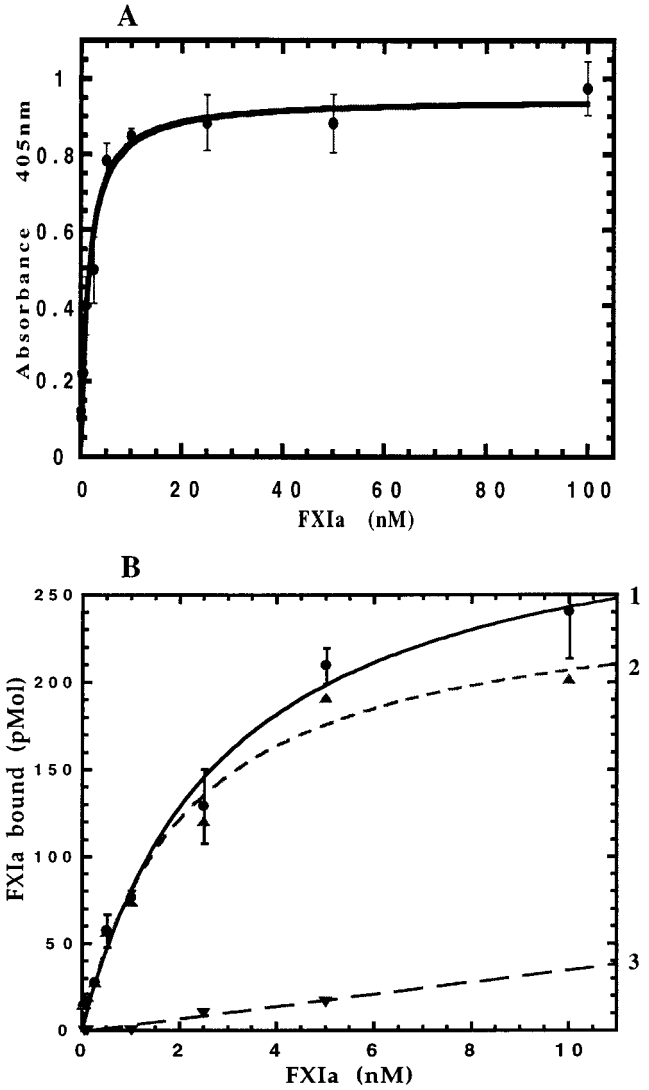

FIGURE 1: FXIa binding to immobilized heparin in microtiter wells. The affinity of FXIa for full-length heparin was determined in streptavidin-coated wells containing biotin-labeled heparin. (A) FXIa was incubated for 1 h. Bound FXIa was detected using 5F7, a monoclonal antibody to FXI. Results are graphed with nonspecific binding subtracted. (B) [^{125}I]FXIa was added to wells under the same conditions as in panel A. 1 = total binding. 2 = specific binding, i.e., total binding minus nonspecific binding. 3 = nonspecific binding, i.e., blocker only. Results represent the means (± SEM) of four determinations, each carried out in triplicate.

constant $7.4 \pm 0.9 \times 10^{-3}$ s^{-1}, and $K_{d,app}$ of 11.2 ± 3.2 nM (Table 1).

Inhibition of FXIa and FXIa Catalytic Domain in the Presence and Absence of Heparin. Since the FXIa catalytic domain bound to heparin on SPR, the effect of heparin on catalytic domain inhibition by PNII was compared to the heparin-potentiation of FXIa inhibition by PNII, using the equilibrium inhibition method. In the absence of heparin, the inhibition of the FXIa catalytic domain by PNII is characterized by a K_i of 436 ± 62 pM, compared with a K_i of 229 ± 40 pM for intact FXIa (Figure 3). In the presence of 100 nM 64-disaccharide heparin and otherwise identical methods, the inhibition of the catalytic domain by PNII was potentiated ~5-fold, with a resultant $K_i = 88 \pm 10$ pM. This was compared to the inhibition of FXIa by PNII in the presence of heparin (K_i = 30 pM). This result further supports the possibility that there is a heparin-binding site

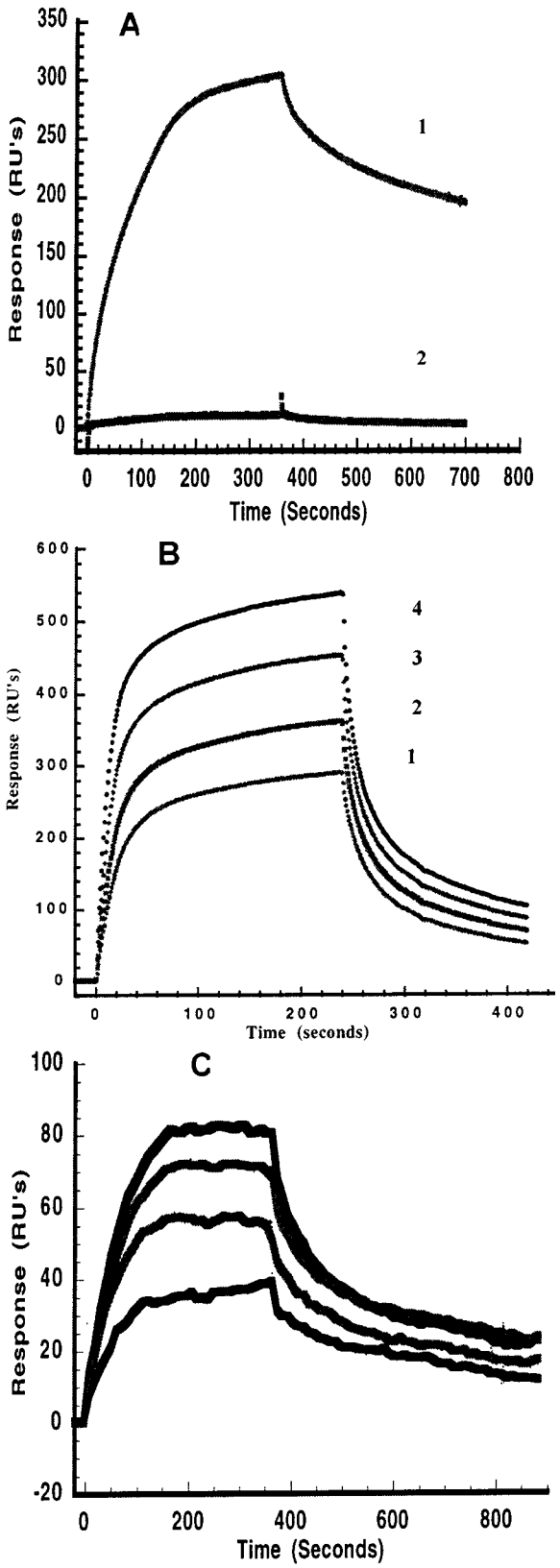

FIGURE 2: FXIa and catalytic domain binding to heparin detected by surface plasmon resonance. (A) 10 nM FXIa without (1) and with (2) preincubation with 100 nM heparin was infused across a biotin-labeled heparin surface immobilized on a streptavidin sensor chip. (B) 7.5 (curve 1), 10 (2), 15 (3), and 20 nM (4) concentrations of FXIa were infused for 4 min across the same surface. Dissociation was followed for four minutes. (C) Isolated FXIa catalytic domain, at concentrations between 10 and 40 nM, were infused for 6 min with a 6 min dissociation time. Results are representative of five determinations.

Table 1: Coagulation Proteins Binding to Heparin Using Surface Plasmon Resonance

protein/peptide	association rate constant (M^{-1} s^{-1})	dissociation rate constant (s^{-1})	K_d ($\times 10^{-9}$ M)
FXIa	$5.2 \pm 2 \times 10^5$	$4.5 \pm 0.68 \times 10^{-3}$	8.6 ± 1.1
FXIa catalytic domain	$6.6 \pm 1.9 \times 10^5$	$7.4 \pm 0.9 \times 10^{-3}$	11.2 ± 3.2
thrombin, <20 nM	$4.7 \pm 0.95 \times 10^4$	$2.15 \pm 0.2 \times 10^{-2}$	420 ± 140
>30 nM	4.25×10^5	$2.12 \pm 0.3 \times 10^{-2}$	52 ± 17
FIXa	$3.9 \pm 0.95 \times 10^5$	$3.8 \pm 1.2 \times 10^{-3}$	17.8 ± 2.7
FXa	$2.0 \pm 0.3 \times 10^4$	$5.9 \pm 1.25 \times 10^{-3}$	331 ± 43
FXIIa	$6.2 \pm 1 \times 10^5$	$6.3 \pm 0.7 \times 10^{-3}$	12 ± 1.35
activated protein C	$5.8 \pm 0.7 \times 10^4$	$5.3 \pm 0.65 \times 10^{-3}$	66 ± 18
FXIa native peptide	$1.1 \pm 0.2 \times 10^4$	$8.5 \pm 1.7 \times 10^{-3}$	86 ± 14.8
FXIa $G^{533}-C^{542}$ alanine peptide	$2.4 \pm 0.6 \times 10^4$	$3.7 \pm 0.9 \times 10^{-3}$	154 ± 31
FXIa $C^{527}-R^{532}$ alanine peptide	no binding		
FXIa scrambled peptide	$5.4 \pm 0.7 \times 10^3$	$1.9 \pm 0.6 \times 10^{-2}$	2600 ± 580

[a] All proteins were injected across a flow cell containing immobilized heparin, as described in the Experimental Procedures. Sensorgrams were analyzed using Biaevaluation 3.02 and fit to a Langmuir one-to-one model. Results represent means ± SEM of at least four determinations at different concentrations.

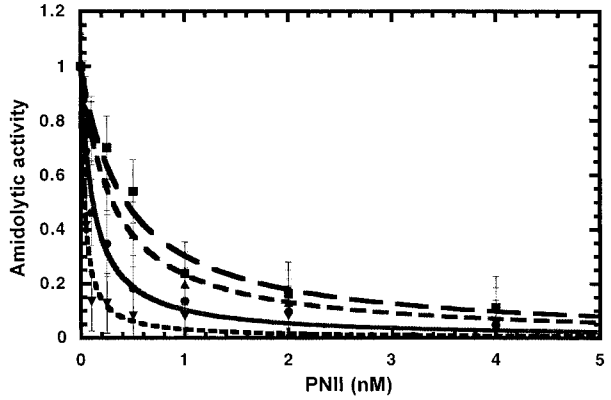

FIGURE 3: The inhibition of the isolated catalytic domain and FXIa by PNII in the presence and absence of heparin. After 1 h incubation with PNII at 37 °C, the residual amidolytic activity was determined using S2366 and compared to the uninhibited amidolytic activity. Results shown are the means (± SEM) of 10 determinations, expressed as a fraction of the uninhibited amidolytic activity. (▲) FXIa; (▼) FXIa + 100 nM heparin; (■) FXIa catalytic domain; (●) FXIa catalytic domain + 100 nM heparin.

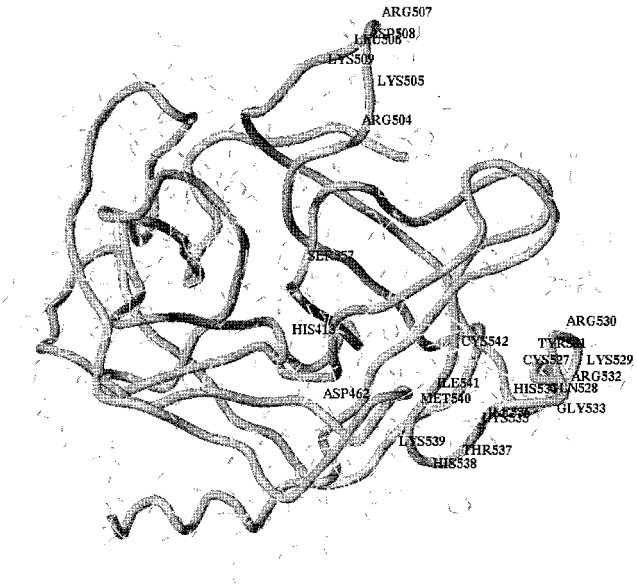

FIGURE 4: Computer generated molecular model of the FXI catalytic domain based on trypsinogen. Two regions are identified: ^{504}RKLRDK^{509} and ^{527}CQKRYRGHKITHKMIC^{542}, with a high positive charge density. These regions, located on either side of the catalytic binding pocket, may bind to heparin and other glycosaminoglycans. The cysteine-constrained loop is a retained feature in all coagulation proteases.

in the FXIa catalytic domain that forms a ternary complex between heparin, PNII, and FXIa.

Binding of FVIIa, FIXa, FXa, FXIIa, Thrombin, Kallikrein, and Activated Protein C to Heparin on SPR. The significance of the FXIa catalytic domain binding to heparin was further examined by comparison to other coagulation proteins with reported heparin-binding activity. Each of these proteases was injected at various concentrations between 7.5 and 50 nM across a heparin-containing sensor chip. As has been reported for FXa (*48*), FIXa, FXa, and APC did not bind to heparin in EDTA-containing buffer. Kallikrein also did not bind, even in the presence of 25 μM zinc. FXIIa demonstrated specific binding, with a K_d of ~12 ± 1.35 nM. In the presence of 2.5 mM calcium, FIXa, APC, and FXa did bind specifically, with $K_{d,app}$ values of 17.8 ± 2.7, 66 ± 18, and 331 + 43 nM, respectively. At lower concentrations, thrombin bound with a $K_{d,app}$ of 420 ± 140 nM. At higher concentrations, the association phase of the sensorgram consistently appeared biphasic. The global fit was, therefore, less than optimal but did show a 5−10-fold increase in association rate constant. Kinetic constants are summarized in Table 1. FVIIa still did not bind, even in the presence of calcium.

Synthetic Peptides Binding to Heparin on SPR. Since the FXIa catalytic domain binds to heparin, the primary structure was examined for amino acid sequences that might represent heparin-binding sites. Two sites were identified: ^{509}YRKLRDK^{515} and ^{527}CQKRYRGHKITHKMIC^{542} , which is a cysteine-constrained loop in the FXI catalytic domain structure, as seen in Figure 4. Peptides were synthesized which contained these two sequences of amino acids. The loop peptide, ^{527}CQKRYRGHKITHKMIC^{542}, was cysteine-constrained. The ability of these peptides to bind to heparin on SPR was examined. The peptide representing the site ^{509}YRKLRDK^{515} did not bind to heparin in this system (data not shown). The cysteine-constrained loop peptide did bind specifically, shown in Figure 5, panel A.

To begin to examine which amino acids are important for FXIa binding to heparin, two peptides were constructed with alanine substitutions. In the first peptide, the basic residues in the α-helix region were substituted with alanines to produce the peptide, ^{527}CQ**AA**Y**A**GHKITHKMIC^{542}, in

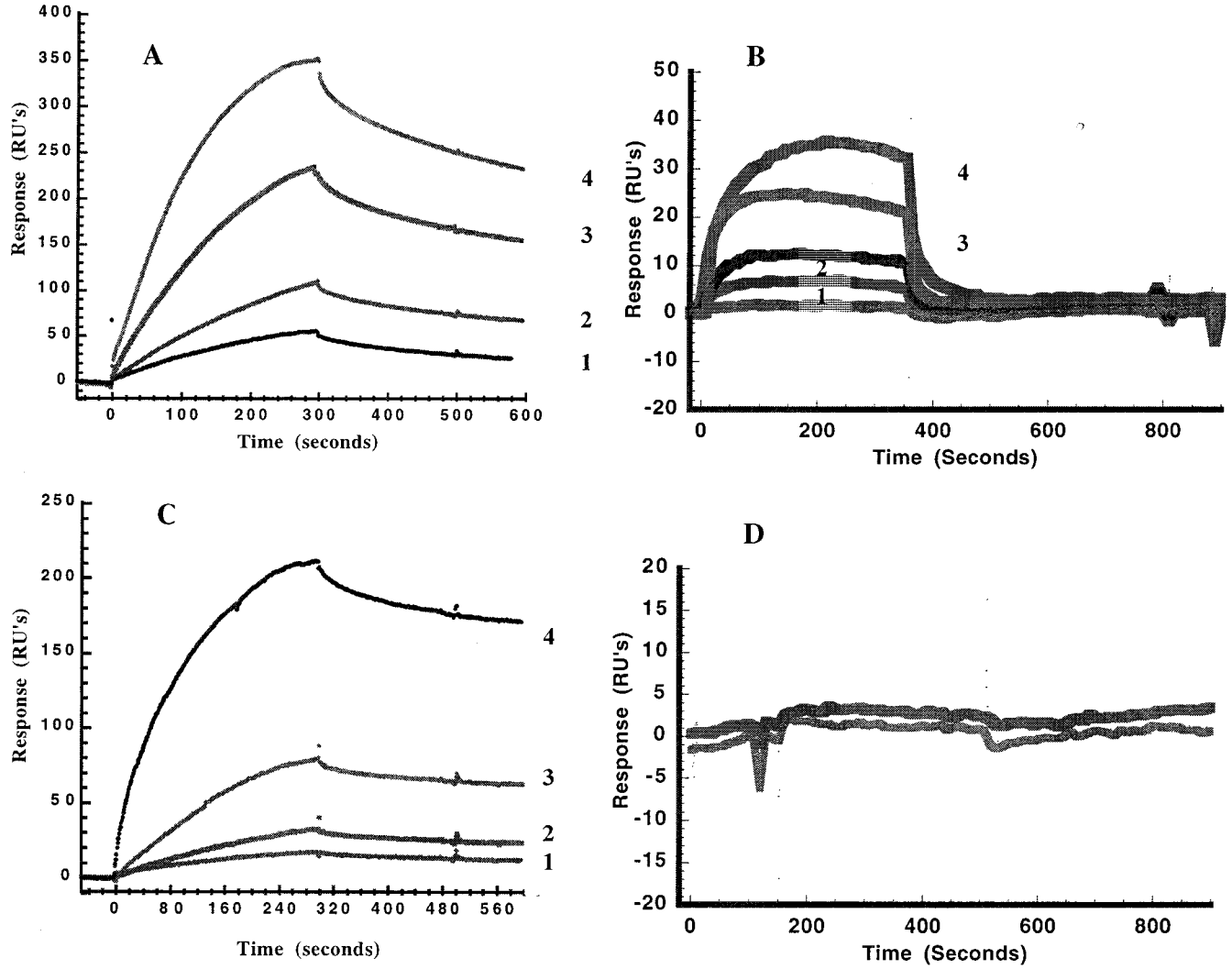

FIGURE 5: Binding of FXIa peptides to heparin detected by surface plasmon resonance. Peptides at 250 nM (1), 500 nM (2), 1 μM (3), and 2 μM (4) were infused across a sensor chip containing biotin-heparin immobilized on streptavidin. Association time = 5 min; dissociation time = 5 min. (A) Native peptide; (B) scrambled peptide; (C) peptide with alanine substitutions on carboxyterminal side of loop (G^{533}–C^{542}); (D) peptide with alanine substitutions within the α helix (C^{527}–R^{532}).

Table 2: FXIa-Derived Synthetic Peptides Competing with FXIa Binding to Heparin in Binding and Kinetic Studies[a]

Peptide	Amino Acid Sequence	K_i – Binding Studies	K_i –Kinetic Studies
Native	CQKRYRGHKITHKMIC	241 ± 24.7 nM	149 ± 37 nM
Scrambled	CKQRYHMKGHIRTIKC	1.42 ± 0.36 μM	1.12 ± 0.12 μM
G^{533}-C^{542} Alanine	CQKRYRGAAITAAMIC	316.7 ± 48 nM	115 ± 26.5 nM
C^{527}-R^{532} Alanine	CQAAYAGHKITHKMIC	> 10 μM	No effect

[a] Peptides were based on the amino acid sequence of the carboxyterminal cysteine-constrained loop in FXIa. Results are summarized from data in Figures 6 and 7. Peptides were added in increasing concentrations to a single concentration of FXIa in both studies, as described in the Materials and Methods. Inhibition constants were calculated based on measured IC_{50}.

which the alanine substitutions are in bold. In the second peptide, the basic residues in the carboxyterminal end of the loop were replaced with alanines (in bold), to produce the peptide ^{527}CQKRYRG**AA**IT**AA**MIC^{542}. A peptide comprising a scrambled sequence was synthesized as a control. The peptide sequences are presented in Table 2. The cysteine-containing peptides were characterized by reversed-phase HPLC, folded, as described in the Experimental Procedures, and further characterized by SDS−PAGE and Ellman assay. All peptides migrated on SDS−PAGE as discreet bands (data

Table 3: Carboxyterminal Heparin-Binding Regions in Coagulation Proteins[a]

GROUP	Protein	Cysteine-Constrained Loops	C-Terminal Regions
A	FXIa	C^{158} - Q **K** - **R Y R** G H K - - - I T H K M I C^{177}	E^{226} Y V D W I L E K T Q^{236}
	Thrombin	C^{173} K D S T **R I R** - - - - - - - I T I N M F C^{187}	**R^{245}** L **K K** W I Q **K** V I D^{255}
	FIXa	C^{177} L - - - **R S T K** -- - - F T I Y N N M F C^{191}	R^{244} Y V N W I **K** E K T **K^{254}**
	FXa	C^{156} K L S S S F I - - - - - - - I T Q N M F C^{170}	A^{223} F L **K** W I D **R** S M **K^{233}**
B	APC	C^{162} - - S E V M S N M V S E - - - - N M L C^{176}	R^{229} Y L D W I H G H I R^{239}
	FXIIa	C^{160} S A P D V H - G - S S - - I L P G M L C^{176}	Y^{232} Y L A W I R E H T V^{242}
C	Kallikrein	C^{168} - Q K - R Y Q - D Y K - - I T Q R M V C^{183}	E^{236} Y M D W I L E K T Q^{246}
	FVIIa	C^{168} L Q Q S R K V G D - S P N I T Y - M F C^{187}	Q^{240} Y I E W L Q K L M R^{250}

[a] Two regions have been identified in thrombin, FIXa and FXa as important for heparin binding. These regions are compared to FXIa and to other proteins that lack heparin-binding affinity. Amino acids identified as part of heparin-binding regions in thrombin, FIXa, and FXa are represented in bold. The potentially important residues in FXIa are also in bold.

not shown). Each contained less than 0.03 mol of free thiol/mol of peptide. All peptides were then injected at concentrations of 0.25, 0.5, 1, and 2 μM across a streptavidin-coated sensor chip with ~100 response units of immobilized biotin-heparin. As summarized in Table 1, the cysteine-constrained loop peptide bound specifically, with a $K_{d,app} = 86 \pm 14.8$ nM (Figure 5, panel A). The scrambled peptide also bound specifically, but with lower affinity, $K_{d,app} = 2.6 \pm 0.58$ μM, due to a 10-fold slower association rate constant and faster dissociation rate constant (Figure 5, panel B). This result is consistent with previous reports (*33*, *43*) that conformation is important, as well as charge density, in heparin interactions. To test this hypothesis, the peptides with alanine substitutions of basic residues either within the amino-terminal α-helix or within the carboxy-terminal sequence of the loop were also examined for heparin-binding ability. Substitution of basic residues in the carboxy-terminal sequence of the loop had little effect on heparin-binding, since the G^{533}-C^{542} alanine peptide had a $K_{d,app} = 154 \pm 31$ nM, which is nearly identical to the native peptide (Figure 5, panel C, and Table 1). When the basic residues within the α-helix were substituted with alanines, the C^{527}-R^{532} alanine peptide failed to bind to heparin (Figure 5, panel D).

Competition Binding Studies. To further confirm that the cysteine-constrained loop in the FXIa catalytic domain contains a heparin-binding site, the same peptides were used as competitors for [^{125}I]FXIa binding to biotin-heparin in streptavidin-coated microtiter wells. The peptides as competitors were compared to unlabeled FXIa, which had K_i = 1.8 nM) in this system, as shown in Figure 6, panel A. The peptides as competitors are shown in Figure 6, panel B, with K_i summarized in Table 2. The native peptide competed effectively with FXIa, with a $K_i = 241 \pm 24.7$ nM, very similar to the K_d on SPR (86 nM). Similarly, the G^{533}-C^{542} alanine peptide was also an effective competitor, $K_i = 316.7 \pm 48$ n. The scrambled peptide was much less effective as a competitor, with a $K_i = 1.42 \pm 0.36$ μM. Consistent with a heparin-binding site being located in the α-helix of the cysteine-constrained loop, the C^{527}-R^{532} alanine peptide showed little ability to compete with FXIa for binding to heparin (IC_{50} >10 μM).

Competition Kinetics. The ability of the synthetic peptides to reverse the heparin-potentiation of FXIa inhibition by PNII was studied using the equilibrium inhibition method. FXIa (0.1 nM), full-length heparin (10 nM), and one concentration of peptide were combined with increasing concentrations of PNII. In Figure 7, panel A, the equilibrium inhibition curves obtained with increasing concentrations of native peptide are shown. The inhibition constant was calculated as described, then divided into the K_i in the absence of heparin (~300 pM), to determine the amount of potentiation. This figure was divided by 10, the appropriate amount of potentiation in the presence of heparin and plotted versus the concentration of peptide (Figure 7, panel B, with results summarized in Table 3). The native peptide effectively reversed the potentiation, with a $K_i = 149 \pm 37$ nM. Consistent with both SPR binding and competition binding studies, the scrambled peptide had significant loss of activity, with a $K_i = 1.12 \pm 0.12$ μM. The G^{533}-C^{542} alanine peptide retained activity, $K_i = 115 \pm 26.5$ nM, similar to the native peptide. The C^{527}-R^{532} alanine peptide had no significant effect at concentrations up to 8 μM.

DISCUSSION

We have previously reported that the affinity of FXIa for heparin is 50−100-fold tighter ($K_d \approx 2$ nM) than zymogen FXI ($K_d \approx 110$ nM) (*31*). Although a heparin-binding consensus sequence (^{250}RIKKSK^{255}) in the A3 domain mediates the binding of zymogen FXI to heparin (*31*), the residues mediating high affinity heparin-binding of FXIa

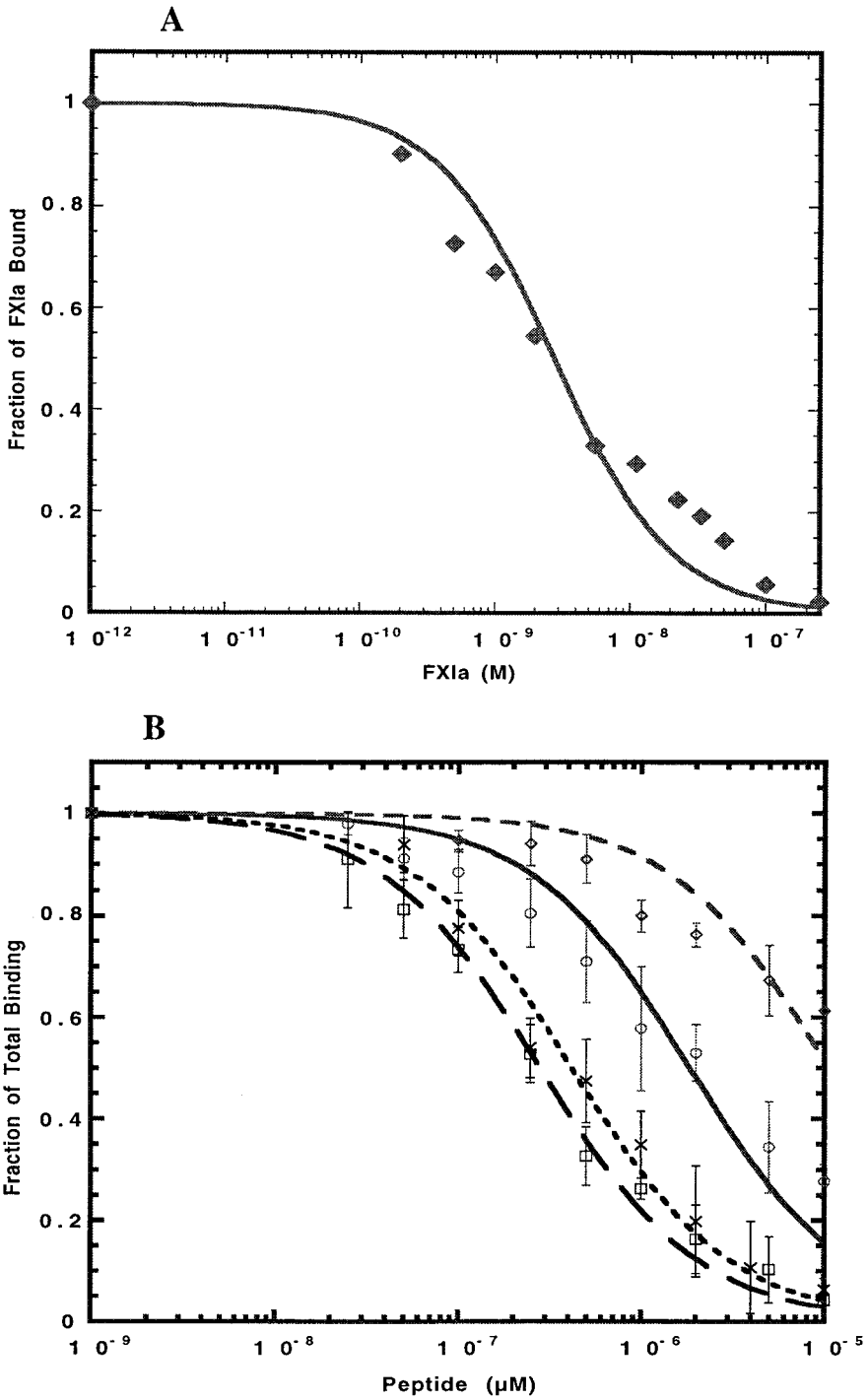

FIGURE 6: Biotin-labeled heparin was immobilized by incubation in streptavidin-coated microtiter wells. (A) Unlabeled FXIa, 0.1−250 nM, was combined with 2 nM ^{125}I-labeled FXIa and added to the wells. After 1 h of incubation, the wells were washed, separated, and radioactivity measured in a gamma counter. (B) Peptide concentrations between 10 nM and 10 μM were combined with 2 nM [^{125}I]FXIa then added to microtiter wells containing heparin. (□) Native peptide; (×) = G^{533}−C^{542} alanine peptide, (○) scrambled peptide; (◇) C^{527}−R^{532} alanine peptide.

were not previously identified. The possibility that additional heparin-binding sites mediate the high-affinity interaction of the enzyme FXIa with heparin (*31*) is consistent with the results of Zhao et al. (*32*), who reported a decrease in the heparin-induced potentiation of ATIII inhibition of FXIa from 55-fold to ~22-fold when the lysine residues, K^{252} and K^{253}, in the A3 domain were mutated to alanines. If the A3 domain heparin-binding site is the only heparin-binding site on FXIa, alanine substitutions would be expected to produce a mutant with no heparin-binding capacity. This suggests that only a part of the binding energy has been lost by replacing the A3 domain heparin-binding site with alanines.

Initially, we studied the binding of FXIa to heparin to confirm the affinity of the enzyme FXIa for heparin. As shown in Figure 1, using either [^{125}I]FXIa or detection of bound FXIa with antibody, the affinity of FXIa for heparin is much higher (K_d =1.45−2.14 nM) than that of FXI (K_d ≈ 110 nM) (*31*). The affinity of FXIa for heparin was also compared to that of the catalytic domain using SPR (Figure 2). The association rate constants were essentially the same

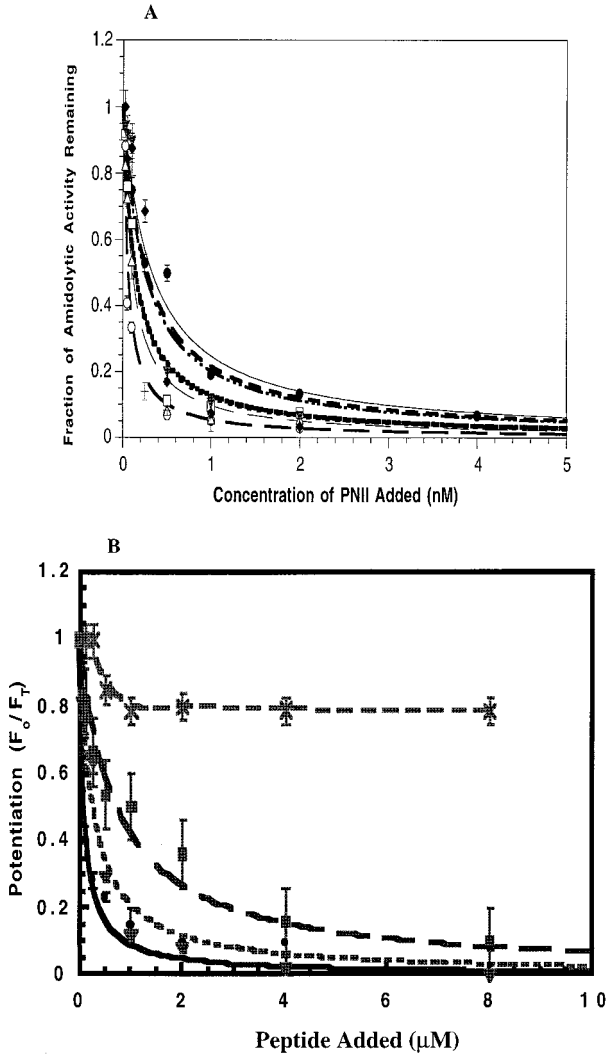

FIGURE 7: FXIa, 0.1 nM, was incubated with PNII, 10 nM full-length heparin and varying concentrations of each synthetic peptide. The inhibition constant was determined as described under equilibrium inhibition studies and the IC_{50} determined as described under competition kinetics. (A) Inhibition curves obtained by increasing concentrations of the homologous peptide. Each curve represents the mean of six separate repetitions. (●) PNII only; (○) PNII + heparin; (□) 500 nM peptide; (△) 1 μM; (+) 2 μM; (▼) 4 μM; (◆) 8 μM. (B) The inhibition constants obtained were calculated as fraction of potentiation remaining, as described under competition kinetics. (●) native peptide; (■) scrambled peptide; (▼) $G^{533}-C^{542}$ alanine peptide; (×) $C^{527}-R^{532}$ alanine peptide.

for FXIa and catalytic domain. The dissociation rate constant of the catalytic domain was slightly faster, $7.4 \pm 0.9 \times 10^{-3}$ s^{-1}, compared to $4.5 \pm 0.7 \times 10^{-3}$ s^{-1} for FXIa. Since the catalytic domain is smaller, 34 kDa, as compared to intact FXIa, 160 kDa, the faster dissociation rate would be an expected result of the larger diffusion coefficient of the catalytic domain. Although a possible reason for this difference in dissociation rate constant is that the A3 domain heparin-binding site further stabilizes the FXIa interaction, we conclude that the catalytic domain of FXIa contains a heparin-binding site.

The inhibition of the FXIa catalytic domain by PNII was then compared to that of FXIa in the presence and absence of heparin. Heparin produced a 10-fold potentiation of FXIa inhibition by PNII (a decrease in K_i from 306 ± 13.4 to 33 ± 4.5 pM), whereas the inhibition of the catalytic domain was potentiated nearly 5-fold, from a K_i of 436 ± 62 to 88 ± 10 pM. This result strongly confirms the presence of a heparin-binding site on the catalytic domain, since most of the potentiation by heparin remains when the catalytic domain is compared to full-length FXIa.

Since heparin-binding regions have been identified in a number of coagulation proteins, the relative affinities of kallikrein, thrombin, FVIIa, FIXa, FXa, FXIIa, and APC for heparin were determined using SPR. Kallikrein and FVIIa showed no specific binding in this system. FIXa, FXa, and APC, all γ-carboxyglutamic acid-containing proteins, required the presence of calcium to allow specific heparin-binding to occur. The rate constants and calculated K_d values are summarized in Table 1. The $K_{d,app}$, 17.8 ± 2.7 nM, of FIXa binding to heparin is 14-fold tighter than the K_d reported by Jordan et al. (*49*), 258 nM, using fluorescamine-labeled heparin and fluorescence polarized spectroscopy. A more recent report by Chang et al. (*50*) characterized the FIXa-heparin interaction, by determination of a K_i for heparin inhibition of the tenase complex, as 12 nM. APC also bound with a $K_{d,app}$ of 66 ± 18 nM. Thrombin bound with a $K_{d,app}$ of 420 ± 140 nM at concentrations below 20 nM, a value more than 10 times tighter than the K_d of 6 μM reported by Olson et al. (*51*). At higher thrombin concentrations, the $K_{d,app}$ decreased to ~50 nM with a biphasic aspect to the association curve of the sensorgram. This is also consistent with the increase in affinity described by Olson et al. (*51*) as a result of more than one thrombin molecule binding to the same heparin strand. These data were further analyzed by Munro et al. (*52*) and found to fit to an overlap model in which thrombin demonstrates positive cooperativity in binding to 3, while occupying 6, disaccharide units. FXa bound to heparin with a $K_{d,app}$ of 331 ± 43 nM, a value nearly 10-fold tighter than that estimated by Rezaie, 2.3 μM (*48*). FXIIa, consistent with its known cell surface-binding activity, bound to heparin, using SPR, with a $K_{d,app}$ of 12 ± 1.35 nM. The residues in FXIIa mediating this tight association have not been identified, however.

The amino acids mediating the interactions of various coagulation proteins with heparin have been identified, and we have utilized this information to categorize these proteins into three groups (Table 3). Group A consists of four enzymes (FXIa, FIXa, FXa, and thrombin) postulated to bind heparin via residues within a cysteine-constrained loop (residues 168−179, trypsin numbering) and/or a C-terminal domain (residues 217−227). Group B consists of two proteins (APC and FXIIa) which bind to heparin by alternative mechanisms. Group C consists of two proteins (FVIIa and kallikrein) which do not bind to heparin. In FIXa, FXa, and thrombin, basic residues near the carboxyterminus of each protein have been mapped. A retained feature of all coagulation proteins is a cysteine-constrained loop of 15−20 amino acids near the carboxyterminus. FIXa, FXIa, and thrombin contain heparin-binding consensus sequences within this loop (displayed in Table 3). The identified heparin-binding residues of FIXa (*53*) include R^{338} (R^{182}, trypsin numbering) contained within this loop region. Thrombin exosite 2, a surface region of basic residues that binds prothrombin fragment 2 and heparin (*54*), includes R^{177} and R^{179} within this loop. Another area described as a heparin-binding region is a short carboxyterminal α-helix that has been identified as part of the FIXa heparin-binding site (*50*)

and of thrombin exosite 2 (*54*). The FXa heparin-binding site has been mapped to the basic residues in the carboxy-terminus, in particular K^{226} and R^{230} (*55*). The two proteins postulated to bind to heparin by mechanisms involving residues outside the cysteine-constrained loop and the C-terminal region are APC and FXIIa (Tables 1 and 3). The residues mediating the interaction of APC with heparin were identified by Neese et al. (*56*) as K^{37}, K^{38}, and K^{39}, and as K^{62}, K^{63} by Shen et al. (*57*), and are contained in two adjacent surface accessible loops. These residues are part of a predominant patch of basic residues reported in the crystal structure of Gla-domainless APC (*58*), near the amino-terminus. The FXIIa heparin-binding site has not been identified.

On the basis of the results of these heparin-binding studies, the primary sequence of the catalytic domain was scrutinized for areas rich in basic amino acids and containing potential heparin-binding consensus sequences. Two such sequences were noted, between ^{503}YRKLRDK^{509} and ^{527}CQKRYRGH-KITHKMIC^{542}. Peptides were synthesized containing these amino acid sequences and studied for binding to heparin on SPR. These studies showed specific, high-affinity binding of the cysteine-constrained peptide, ^{527}CQKRYRGHKITH-KMIC^{542} (Figure 5, panel A), whereas the ^{503}YRKLRDK^{509} peptide did not bind. The essential amino acids involved in this binding site are contained in the α-helix since the native peptide and the peptide with alanine substitutions of basic residues in the carboxyterminal half of the cysteine-constrained loop (G^{533}-C^{542}) bound with an almost identical affinity, i.e., $K_{d,app} = 86 \pm 14.8$ and 154 ± 31 nM, respectively. This is further confirmed by the nearly complete loss of heparin-binding capacity of the peptide with alanines substituting for the basic residues within the α-helix (C^{527}-R^{532}). The α-helical conformation is important in facilitating heparin-binding, since a peptide with all the same basic amino acids arranged in a scrambled sequence lost almost all of its binding affinity.

These findings are consistent with the characteristics of heparin-binding sites reported by Cardin and Weintraub (*33*) and others (*34*, *35*). Since the conformation of a synthetic peptide may not exactly mimic the native site, the heparin-binding activity of these peptides was confirmed in competition binding and kinetic studies. As heparin:protein interactions are based on charge interactions, any peptide or protein that has heparin-binding capacity will compete with any other heparin-binding protein depending only upon their relative affinities. If the peptide has significant affinity for heparin, it should compete with FXIa in binding studies and should reverse the heparin-potentiated inhibition by PNII.

Results of these studies are summarized in Table 2. The competition binding studies, shown in Figure 6, are consistent with the results of the SPR heparin-binding studies. The native peptide and G^{533}-C^{542} alanine peptide had the greatest ability to compete with FXIa for binding to heparin, with K_i of 241 ± 24.7 and 316.7 ± 48 nM, respectively. The scrambled peptide again showed significant loss of activity. The C^{527}-R^{532} alanine peptide did not compete. The same peptides also showed the same pattern of ability to reverse the heparin-potentiation of FXIa inhibition by PNII as demonstrated by competition kinetic studies (Figure 7). It can be concluded from all of these studies that K^{529}, R^{530}, and R^{532} are likely to be the important residues for heparin-binding within the catalytic domain loop peptide sequence, ^{527}CQKRYRGHKITHKMIC^{542}.

Since heparin is not normally present in plasma, the high affinity of its binding interaction with FXIa and PNII must be considered as a model for another similar physiologic interaction. Potential sources of GAG which could support formation of a ternary complex with FXIa and PNII include cell-surface proteoglycans, solution-phase GAGs released from cell surfaces and cell-surface PNII. PNII has been reported to bind to heparan sulfate proteoglycan (*27*, *59*). Narindrasorasak et al. (*27*) found that KPI-containing forms of APP bound with a K_d of 9−10 nM and continued to bind to the core protein after heparitinase treatment.

It is possible that the FXIa:PNII complex is removed from the blood stream and degraded through binding to a cell surface proteoglycan. An example of a Kunitz inhibitor:serine protease complex uptake and degradation by heparan sulfate proteoglycans is the tissue factor pathway inhibitor:FXa complex (*60*). In this study, treatment of the cells with heparinase, but not chondroitinase, markedly reduced uptake of the complex. The binding of FXa stimulated uptake and degradation of the inhibitor. Similarly, Johnson-Wood et al. (*61*) found that PNII complexed with trypsin bound to human fibroblasts (~50 000 sites/cell, K_d 3.5 nM). Additionally, Knauer et al. (*62*) found that when PNII was bound to one of its ligands, EGF binding protein, it was internalized by the cultured cells, which were transfected to overexpress it. The receptor they identified was the low-density lipoprotein receptor-related protein, also known as the α-2-macroglobulin receptor.

The results of the studies presented here support a model whereby FXIa binds to a cell surface GAGs via residues K^{529}, R^{530}, and R^{532}, contained in an α-helix within a carboxyterminal cysteine-constrained loop. The functional significance of this binding may be to promote FXIa inhibition by PNII and clearance via a cell-surface receptor.

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