Zn$^{2+}$ Mediates High Affinity Binding of Heparin to the αC Domain of Fibrinogen*

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Background: The interaction of heparin with fibrinogen compromises its anticoagulant activity. Results: Zn$^{2+}$ promotes heparin binding to His-544–His-545 on the fibrinogen α-chain. Conclusion: We identified a novel Zn$^{2+}$-dependent heparin binding site on fibrinogen. Significance: Platelet release of Zn$^{2+}$ at sites of vascular injury may promote heparin binding to fibrinogen, thereby further attenuating the anticoagulant activity of heparin.

The nonspecific binding of heparin to plasma proteins compromises its anticoagulant activity by reducing the amount of heparin available to bind antithrombin. In addition, interaction of heparin with fibrin promotes formation of a ternary heparin-thrombin-fibrin complex that prevents fibrin-bound thrombin from inhibition by the heparin-antithrombin complex. Previous studies have shown that heparin binds the E domain of fibrinogen. The current investigation examines the role of Zn$^{2+}$ in this interaction because Zn$^{2+}$ is released locally by platelets and both heparin and fibrinogen bind the cation, resulting in greater protection from inhibition by antithrombin. Zn$^{2+}$ promotes heparin binding to fibrinogen, as determined by chromatography, fluorescence, and surface plasmon resonance. Compared with intact fibrinogen, there is reduced heparin binding to fragment X, a clottable plasmin degradation product of fibrinogen. A monoclonal antibody directed against a portion of the fibrinogen αC domain removed by plasmin attenuates binding of heparin to fibrinogen and a peptide analog of this region binds heparin in a Zn$^{2+}$-dependent fashion. These results indicate that the αC domain of fibrinogen harbors a Zn$^{2+}$-dependent heparin binding site. As a consequence, heparin-catalyzed inhibition of factor Xa by antithrombin is compromised by fibrinogen to a greater extent when Zn$^{2+}$ is present. These results reveal the mechanism by which Zn$^{2+}$ augments the capacity of fibrinogen to impair the anticoagulant activity of heparin.

Heparin and heparin derivatives are effective anticoagulants that target thrombin, factor Xa, and other upstream enzymes in the coagulation system. Heparin principally functions as a catalyst by binding antithrombin and promoting its interaction with coagulation proteases. Two distinct mechanisms comprise the catalytic role of heparin and both require heparin to bind antithrombin (1). For inhibition of factor Xa, the conformational change in antithrombin that accompanies heparin binding is essential. In contrast, because this conformational change is without catalytic effect for thrombin, heparin serves as a template onto which both the protease and inhibitor bind, thereby promoting bimolecular interaction.

Despite its efficacy, the anticoagulant response to therapeutic doses of heparin is unpredictable. Consequently, frequent coagulation monitoring is necessary to ensure that therapeutic levels of heparin are achieved (2). This variability is due in part to interaction of heparin with numerous plasma proteins, some of which are acute phase reactants whose levels vary in certain pathological states. Previously, we have shown that displacement of catalytic heparin from plasma proteins recovers the anticoagulant activity of heparin in vitro (3). Thus, interaction with plasma proteins reduces the bioavailability of heparin. Abundant plasma proteins that bind heparin include fibrinogen, histidine-rich glycoprotein (HRG), and high molecular weight kininogen (HK) (4–6).

In addition to reducing the concentration of heparin available to bind to antithrombin, there is another consequence of the high affinity of heparin for fibrinogen. Because both heparin and fibrinogen also bind thrombin, a ternary complex is formed that sequesters thrombin and restricts accessibility of antithrombin (6). Thus, thrombin bound to heparin and fibrin within this ternary complex retains its catalytic activity and is protected from inhibition (6, 7). This phenomenon likely contributes to the prothrombotic nature of thrombi and to the rethrombosis that can occur despite heparin therapy (2).

Another factor that may compromise the availability of heparin is Zn$^{2+}$, an essential metal ion that has numerous effects in

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‡§ These abbreviations used are: HRG, histidine-rich glycoprotein; 5-IAF, 5-iodoacetamido-fluorescein; αC-20, peptide corresponding to fibrinogen αC chain residues 529–548; αC-20AA, a variant of αC-20 with Ala residues in place of the two His residues; d, deaminated; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl; f, fluorescein; HBS, HEPES-buffered saline; HK, high molecular weight kininogen; l, fluorescence intensity; IAF, iodoacetamidofluorescein; R$_{eq}$, resonance units at equilibrium; RU, resonance units; SPR, surface plasmon resonance.
hemostasis and is present in plasma at 10–20 μM (8, 9). Recently, we demonstrated that Zn²⁺ augments ternary heparin-thrombin-fibrin complex formation and increases the protection of thrombin from inhibition by antithrombin in the presence of fibrin (10). The 4-fold increase in the affinity of heparin for fibrin in the presence of Zn²⁺ leads to a similar elevation in the apparent affinity of thrombin for fibrin. Zn²⁺ also has been shown to increase the affinity of heparin for HRG and HK; interactions that reduce the catalytic activity of heparin. HK and HRG bind Zn²⁺ through their histidine-rich regions and the negatively charged sulfate groups of heparin then bind Zn²⁺ (11). Although it is known that fibrinogen binds Zn²⁺ (12), the effect of Zn²⁺ on heparin binding has not been investigated. The current study reveals a novel Zn²⁺-dependent heparin binding site localized to the αC domains of fibrinogen that is distinct from the previously reported heparin binding site on the β-chain.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human thrombin, plasminogen-free fibrinogen, and factor Xa were from Enzyme Research Labs, Inc. (South Bend, IN). Fibrinogen and fibrinogen fragment X were prepared and characterized as described (13–15). Human antithrombin was from Affinity Biologicals, Inc. (Ancaster, ON, Canada). Unfractionated heparin (heparin), deaminated heparin (catalog no. H7405), and 5-iodoacetamidofluorescein (5-IAF) were from Sigma. Similar to unfractionated heparin, which has equivalent inhibitory activity against factor Xa and thrombin, deaminated heparin has anti-factor Xa and anti-thrombin activities of 101 and 104 units/mg, respectively. α-C-20, a peptide analog of fibrinogen Asp529–548 containing an added NH₂-terminal Cys residue (CGSEGIFTNTKESSSHHPGI) and α-C-20AA, a variant with Ala residues in place of the two His residues, were synthesized by GenScript Corp. (Scotch Plains, NJ). A polyclonal antibody directed against α-C-20 was raised in sheep by Affinity Biologicals, Inc., and the IgG fraction was then affinity purified using immobilized α-C-20. Thrombin was radiolabeled at its active site by reaction with [125I]-Tyr-Pro-Arg-chloromethyl ketone (13). Monoclonal antibodies directed against various epitopes on the αC domain, designated F-102 and F-103, 134-B29, and TF-359 were generous gifts from Drs. Joan Sobel (16), Zaverio Ruggeri (17), and Bohdan Kudryk (18), respectively. Chromogenic substrate for factor Xa, BIOPHEN CS-11(65), was from Aniara (Mason, OH), whereas FluoZin-1, a fluorescent indicator for Zn²⁺, was from Invitrogen.

**Heparin-Sepharose Chromatography**

Fibrinogen and fragment X were subjected to chromatography on a 1-ml Hi-Trap heparin-Sepharose column (GE Healthcare) using a Beckman System Gold chromatography system. The column was equilibrated with 10 mM Tris-HCl, pH 7.4, 0.005% Tween 20, in the absence or presence of 12.5 μM ZnCl₂, 2 mM CaCl₂, or 2 mM EDTA. CaCl₂ and ZnCl₂ concentrations were chosen to approximate those in plasma. Protein (1 mg in 1 ml) was applied to the column in equilibrium buffer at a flow rate of 1 ml/min. After washing, a 40-ml linear gradient from 0 to 1 M NaCl in equilibration buffer was applied, fractions were collected, and absorbance was monitored at 280 nm.

**Fluorescence Studies**

**Interaction of Fluorescein-Heparin with Fibrinogen**—For optimal labeling with fluorescein or biotin, deaminated heparin (d-heparin) was used in some studies. This heparin derivative was chosen to directly modify to the reducing end of the heparin molecule, thereby avoiding random labeling of internal residues, which may compromise the integrity of binding sites (19). d-heparin (20 mg) was fluorescein-labeled by reaction with 3 mg of fluorescein-5-thiosemicarbazide (Invitrogen) in 400 μl of phosphate buffer, pH 7.5. After mixing for 1 h in the dark, the sample was passed over a PD-10 column (GE Healthcare) equilibrated with water, and 0.5-ml fractions were collected. The heparin-containing fraction was lyophilized and resuspended in 500 μl of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS). The concentration of fluorescein-d-heparin (f-d-heparin) was determined using the extinction coefficient for fluorescein (68,000 M⁻¹ cm⁻¹ at 492 nm). For fluorescence studies, 900 μl of TBS containing 150 mM f-d-heparin and 2 mM CaCl₂, 2 mM EDTA, or 12.5 μM ZnCl₂ was placed in a quartz cuvette, stirred with a micro stir bar, and maintained at 23 °C with a circulating water bath. Using a LS50B luminescence spectrophotometer (PerkinElmer Life Science) with excitation and emission wavelengths of 492 and 535 nm, respectively, slit widths of 5 and 20 nm, respectively, and with a 515-nm cutoff filter, fluorescence was monitored before and after titration with 1- to 10-μl aliquots of 25 μM fibrinogen or 20 μM fragment X containing the appropriate concentration of CaCl₂, ZnCl₂, or EDTA. Fluorescence was allowed to stabilize before each addition and after the run, fluorescence intensity values (I) obtained from the time drive profile were corrected for dilution and calculated as a ratio of the initial fluorescence (Iₒ). Plots of I/Iₒ versus protein concentration were analyzed by nonlinear regression analysis of the binding isotherm equation to determine Kd values, which are reported as mean ± S.D. from three determinations (20).

**Interaction of Fluorescein-αC-20 and αC-20AA with Heparin**—Fluorescein-labeled αC-20 and αC-20AA were prepared by incubating 1 mg of peptide in 1 ml of 0.1 M sodium phosphate, pH 7.5, with 50 μl of 56 mg/ml 5-IAF in dimethyl sulfoxide for 3 h at 23 °C. The labeled peptides were separated from unincorporated 5-IAF by chromatography on a Sephadex G-10 column (GE Healthcare) equilibrated with water and the concentration of f-αC-20 or f-αC-20AA was quantified by measuring absorbance at 492 nm. HPLC analyses revealed >70% incorporation of 5-IAF into the peptides (data not shown). To quantify their interaction with heparin, 900 μl of 100 nm f-αC-20 or f-αC-20AA in TBS containing 12.5 μM ZnCl₂ was placed in a quartz cuvette, stirred with a micro stir bar and maintained at 23 °C using a circulating water bath. Fluorescence intensity was monitored as the sample was titrated with aliquots of 66 μM heparin containing 100 nm f-peptide, as described above. Intensity values were normalized relative to those determined in the absence of heparin and plotted versus heparin concentration. Data were analyzed by nonlinear regression of the binding iso-
therm to obtain $K_d$ and values are reported as mean ± S.D. of three determinations.

**Interaction of Fibrinogen, Fragment X, αC-20, and αC-20AA with Zn$^{2+}$**—A cuvette containing 1.0 ml of 0.5 μM FluoZin-1 and 15 μM ZnCl$_2$ was monitored at excitation and emission wavelengths of 495 and 515 nm and slit widths of 10 and 6 nm, respectively. Fluorescence was monitored continuously as the sample was titrated with aliquots of fibrinogen, fragment X, αC-20, or αC-20AA to 110 μM. Plots of I/I$_0$ values versus peptide concentration were analyzed by nonlinear regression of a binding isotherm equation to determine the IC$_{50}$ values. These were converted to $K_d$ values using the Cheng-Prusoff equation and the affinity of Zn$^{2+}$ for FluoZin-1, which was determined in a separate experiment. Values are reported as mean ± S.D. of three determinations.

**Binding of Zn$^{2+}$ to Dansyl-fibrinogen**—Dansyl-fibrinogen was prepared by incubating 3 mg of fibrinogen with 0.1 mg of dansyl chloride (Sigma) in the dark for 30 min at 23 °C. The reaction mixture was then passed over a PD-10 column equilibrated in TBS, and the concentration of dansyl-fibrinogen was determined by iteratively correcting the A$_{280}$ value with readings determined at A$_{340}$ (21). The stoichiometry of labeling was 15 dansyl groups per fibrinogen and, similar to unlabeled fibrinogen, the dansyl-fibrinogen was >95% clottable. To measure Zn$^{2+}$ binding, 100 nM dansyl-fibrinogen in TBS containing 0.4% polyethylene glycol and 0.05% Tween 20 was placed in a cuvette in the fluorimeter. Fluorescence was monitored with excitation at 340 nm and emission at 520 nm in the presence of a 430-nm cutoff filter. The sample was maintained at 23 °C and titrated with aliquots of 1 mM ZnCl$_2$. Intensity values were obtained from the time drive profile and plotted as I/I$_0$ versus ZnCl$_2$ concentration. The data were analyzed by nonlinear regression of the rectangular hyperbola equation to obtain $K_d$. Experiments were performed three times, and data are reported as mean ± S.D.

**Surface Plasmon Resonance (SPR) Studies**

**Interaction of Fibrinogen with Immobilized Heparin**—Heparin was biotinylated with biotin-amidohexanoic acid hydrazide (Sigma) in 0.1 M sodium acetate, pH 5.5 (20). Streptavidin was attached to a CM5 chip using an amine coupling kit (GE Healthcare). Biotinylated heparin was adsorbed to the streptavidin flow cell to 640 resonance units (RU) in 20 mM HEPES, pH 7.4, 150 mM NaCl containing 0.005% Tween 20 (HBS-Tween 20) in a BIAcore 1000 (GE Healthcare). Aliquots of 250 nM fibrinogen in HBS-Tween 20 containing 0−15 μM ZnCl$_2$ were injected over the flow cell at 30 μl/min. Between runs, the flow cell was regenerated with 0.5% SDS and 1 mM EDTA. The sensorgrams, RU values at equilibrium ($R_{eq}$) were determined and plotted versus ZnCl$_2$ concentration. Data were analyzed by nonlinear regression of a rectangular hyperbola to determine the $K_d$ values. Studies were performed five times.

**Effect of Antibodies on the Interaction of Fibrinogen with Immobilized d-heparin**—d-heparin was biotinylated as described above and adsorbed to the flow cell of a streptavidin sensor chip (GE Healthcare) in HBS-Tween 20 in a BIAcore 1000. Binding was performed in HBS-Tween 20 containing 2 mM CaCl$_2$ or 12.5 μM ZnCl$_2$. Samples containing 125 nM fibrinogen and 500 nM anti-body were preincubated in HBS, pH 7.4, containing 12.5 μM ZnCl$_2$ and 0.005% Tween 20 for 90 min at 23 °C. Aliquots of 250 μl were injected at a flow rate of 30 μl/min for 4 min prior to injection of buffer alone. Flow cells were regenerated with 150 μl of 1.5 M NaCl, 100 μM EDTA, and 0.005% Tween 20 in HBS, pH 7.4. Using BIAcore software, peak RU values were determined for each run and corrected for signal from a blank flow cell. The percentage of fibrinogen bound to biotinylated-d-heparin in the presence of each antibody was calculated relative to that in its absence. Studies were performed two times.

**Interaction of Zn$^{2+}$ with Immobilized Fibrinogen**—Using an amine coupling kit, fibrinogen (0.1 mg/ml in 10 mM acetic buffer, pH 5.5) was immobilized on a CM5 sensor chip to 10,000 RU in a BIAcore T200 (GE Healthcare). ZnCl$_2$ (0−25.6 μM in HBS containing 2 mM CaCl$_2$ and 0.05% Tween 20) was injected into flow cells containing immobilized fibrinogen for 120 s at a rate of 30 μl/min to monitor association. Buffer lacking ZnCl$_2$ was then injected for 300 s to monitor dissociation. Between runs, flow cells were regenerated with 30 μl of 3.6% sodium citrate. Sensorgrams for each Zn$^{2+}$ concentration were background corrected using a blank flow cell. A plot of background-corrected $R_{eq}$ values versus Zn$^{2+}$ concentration was analyzed by nonlinear regression of a rectangular hyperbola to determine $K_d$ of Zn$^{2+}$ for fibrinogen. Studies were performed two times.

**Heparin Binding to Fibrin Clots**

d-heparin was labeled with $^{125}$I after modification with 4-hydroxybenzhydrazide. Thus, d-heparin (10 mg) was dissolved in 400 μl of acetic buffer, pH 5.5, and mixed with 100 μl of dimethyl sulfoxide containing 2 mg 4-hydroxybenzhydrazide (Sigma). The solution was mixed overnight at 23 °C and then passed over a Sephadex G10 column equilibrated with water. Fractions containing heparin were pooled, lyophilized, and resuspended in phosphate-buffered saline. For labeling, 6 mg of modified d-heparin was reacted with 3 iodobeads (Pierce) and 2 mCi of Na$^{125}$I (McMaster University Nuclear Reactor) as described (13). The concentration of $^{125}$I-d-heparin was determined by Azure A assay.

Binding of $^{125}$I-d-heparin to fibrin clots was performed by measuring the radioactivity in clot supernatants. Samples containing 1 μM fibrinogen and 100 nM $^{125}$I-d-heparin in TBS were clotted with 5 nM thrombin in the presence of 12.5 μM ZnCl$_2$ or 2 mM CaCl$_2$ and containing 20 μM of either the αC-20 directed IgG or non-immune sheep IgG. After incubation for 1 h, clots were compacted by centrifugation. Aliquots of the supernatant were counted for radioactivity to determine the concentration of free $^{125}$I-d-heparin. The fraction of $^{125}$I-d-heparin bound was normalized and plotted against the IgG concentration. The data were analyzed by nonlinear regression of a rectangular hyperbola to determine the IC$_{50}$ for inhibition of $^{125}$I-d-heparin binding by the αC-20-directed IgG.

**Factor Xa Inhibition**

Second order rate constants for factor Xa inhibition by anti-thrombin were determined under pseudo first order conditions (22). Aliquots from incubations containing 15 nM factor Xa, 100 nM antithrombin, 1 μg/ml heparin, and 12.5 μM ZnCl$_2$ were removed at fixed time intervals and assayed for residual chro-
mogenic activity with the factor Xa-directed substrate BioPhen CS-11(65). Second order rate constants were determined in the absence or presence of varying concentrations of fibrinogen or fragment X.

**Statistical Analysis**

Data are expressed as means ± S.D. Unless otherwise stated, significance of differences was examined using paired *t* tests. In all cases, *p* values < 0.05 were considered statistically significant.

**RESULTS**

**Heparin-Sepharose Chromatography**—To examine the potential effect of Zn$^{2+}$ on the heparin-fibrinogen interaction, fibrinogen was subjected to chromatography on heparin-Sepharose. In the presence of 2 mM CaCl$_2$, the bulk of fibrinogen was eluted at 200 mM NaCl, and a minor fraction was eluted at 400 mM NaCl (Fig. 1A). A similar profile was obtained in the presence of EDTA (data not shown). Chromatography was then repeated in the presence of 12.5 μM ZnCl$_2$. Under these conditions, fibrinogen eluted at a higher ionic strength, with two peaks at 300 and 400 mM NaCl, respectively, and a trailing edge that eluted with NaCl concentrations >500 mM. These findings suggest that fibrinogen binds heparin with higher affinity in the presence of Zn$^{2+}$.

The same experiment was then repeated using fragment X in place of fibrinogen (Fig. 1B). Fragment X was examined because this high molecular weight, plasmin-derived degradation product of fibrinogen lacks the COOH-terminal degradation product of fibrinogen lacks the COOH-terminal half of the Aα chains. In the presence of Ca$^{2+}$ (or EDTA), fragment X eluted with a profile similar to that of intact fibrinogen. However, in the presence of Zn$^{2+}$, only a small fraction of fragment X bound heparin with higher affinity than fibrinogen. Based on SDS-PAGE analysis (data not shown), this minor population may represent a small residual amount of intact fibrinogen in the fragment X preparation. The difference in the results obtained with fragment X and fibrinogen suggests that the region of fibrinogen responsible for Zn$^{2+}$-mediated binding to heparin resides in the αC domain. Furthermore, these results reveal that other interactions of heparin with fibrinogen also are promoted by Zn$^{2+}$.

**Affinity of Fibrinogen or Fragment X for Fluorescently Labeled Heparin**—To determine more quantitative data, the affinity of d-heparin for fibrinogen or fragment X was determined in the presence of Ca$^{2+}$ or Zn$^{2+}$. Binding was determined by monitoring the fluorescence intensity of d-heparin as it was titrated with aliquote of fibrinogen (Fig. 1B). Fluorescence intensity (I) values were recorded and converted to a fraction of the initial fluorescence (I$_0$) and plotted versus the titrant concentration. The data were analyzed by nonlinear regression analyses to determine $K_d$ (lines). Representative data from three experiments are plotted.

**FIGURE 1.** Binding of fibrinogen or fragment X to heparin-Sepharose in the presence of Ca$^{2+}$ or Zn$^{2+}$. A 1-ml heparin-Sepharose column was equilibrated in 10 mM Tris-HCl, pH 7.4. Chromatography of 1 mg of fibrinogen (A) or fragment X (B) was performed in the presence of 2 mM CaCl$_2$ (solid lines) or 12.5 μM ZnCl$_2$ (dashed lines) at a flow rate of 1 ml/min. After a 5-min wash, a linear gradient of 0–1 M NaCl was run for 40 min, and absorbance at 280 nm ($A_{280}$) was monitored. Representative data from three experiments are plotted.

**FIGURE 2.** Binding of fibrinogen or fragment X to f-d-heparin. The fluorescence of 150 nM f-d-heparin in TBS was monitored with excitation and emission wavelengths of 492 and 535 nm, respectively. Samples containing 2 mM EDTA (triangles), 2 mM CaCl$_2$ (squares), or 12.5 μM ZnCl$_2$ (circles) were titrated with aliquots of fibrinogen (A) or fragment X (B). Fluorescence intensity (I) values were recorded and converted to a fraction of the initial fluorescence (I$_0$) and plotted versus the titrant concentration. The data were analyzed by nonlinear regression analyses to determine $K_d$ (lines). Representative data from three experiments are plotted.
100 nM determined kinetically (23). In the presence of EDTA, the affinity was 2-fold lower, and a smaller change in fluorescence was observed. However, in the presence of 12.5 μM ZnCl₂, there was a greater decrease in fluorescence at lower concentrations of fibrinogen, such that the affinity of d-heparin for fibrinogen in the presence of Zn²⁺ was 16-fold higher than that determined in its absence (Kₐ values of 60 ± 32 and 975 ± 110 nM, respectively; p < 0.002).

The experiment was then repeated using fragment X in place of fibrinogen (Fig. 2B). No binding to f-d-heparin was observed in the presence of Ca²⁺ or EDTA. The lack of binding in the presence of Ca²⁺ contrasts with the results obtained using heparin-Sepharose chromatography. This difference may reflect the high density of higher molecular weight heparin molecules attached to the Sepharose beads. When f-d-heparin was titrated with fragment X in the presence of Zn²⁺, binding was observed and a Kₐ value of 539 ± 185 nM was obtained. Therefore, removal of the αC domain results in a 9-fold reduction in the affinity of fibrinogen for f-d-heparin (from 60 ± 32 to 539 ± 185 nM; p = 0.03).

Zn²⁺ Dependence of Fibrinogen Binding to Immobilized Biotinylated Heparin—The effect of Zn²⁺ on fibrinogen binding to immobilized biotinylated heparin was determined using SPR. Increasing concentrations of Zn²⁺ resulted in a saturable increase in Rₑq values that yielded half-maximal binding at 4.5 ± 1.1 μM Zn²⁺, which is within the physiological Zn²⁺ concentration range of ~10 μM (Fig. 3). Identical results were obtained with immobilized biotinylated-d-heparin, demonstrating that heparin deamination does not influence its interaction with fibrinogen (data not shown).

Affinity of Zn²⁺ for Fibrinogen—The affinity of Zn²⁺ for fibrinogen was quantified using SPR. Increasing concentrations of ZnCl₂ were injected into flow cells containing immobilized fibrinogen. Sensorgrams revealed a rapid and concentration-dependent increase in RU values with a subsequent rapid decrease upon injection of buffer lacking ZnCl₂ (Fig. 4). The increase in RU values is attributed to conformational changes in fibrinogen that occur upon Zn²⁺ binding that alter the refractive index of fibrinogen (24, 25). Based on analysis of the plot of Rₑq values versus Zn²⁺ concentration, Zn²⁺ binds fibrinogen with a Kₐ value of 9.4 ± 2.2 μM, a value similar to the Kₐ of 18 μM reported previously (12).

A second binding assay was performed to confirm the results obtained using SPR. Fluorescence was monitored as dansyl-fibrinogen as determined by fluorescence. In a cuvette, 100 nm dansyl-fibrinogen was titrated with aliquots of 1 μM ZnCl₂. Fluorescence intensity was monitored by excitation at 340 nm and emission at 520 nm. The plot of I/ΔI versus ZnCl₂ concentration was analyzed by nonlinear regression to obtain Kₐ (line). Data represent mean ± S.D. of three determinations.
Further confirmation of Zn\(^{2+}\) binding to fibrinogen was obtained in a competition experiment with the Zn\(^{2+}\)-binding fluorophore, FluoZin-1. Addition of Zn\(^{2+}\) to FluoZin-1 results in an increase in fluorescence, which is reversed upon titration with a Zn\(^{2+}\)-binding ligand (26). Titration of the Zn\(^{2+}\)/FluoZin-1 mixture with fibrinogen resulted in a saturable decrease in fluorescence, indicative of near quantitative binding of Zn\(^{2+}\) to the peptide (data not shown). Accounting for the affinity of Zn\(^{2+}\) for FluoZin-1, a K<sub>d</sub> value of 0.67 ± 0.11 μM was obtained. In contrast, Zn\(^{2+}\) bound fragment X with 8.5-fold lower affinity, exhibiting a K<sub>d</sub> value of 5.7 ± 0.5 μM.

Effect of αC Domain-directed Antibodies on the Heparin-fibrinogen Interaction—To validate the existence of a Zn\(^{2+}\)-dependent heparin binding site in the αC domain of fibrinogen and to begin to localize this site, we used SPR to examine the effect of various αC domain-directed monoclonal antibodies on the heparin-fibrinogen interaction. The antibodies were directed against the following epitopes: Aα 563–578 (F102), Aα 259–276 (F103), Aα 566–580 (134B-29), and Aα 529–549 (TF-359). Binding of fibrinogen to biotinylated-d-heparin was monitored in the presence of Zn\(^{2+}\) and in the absence or presence of the antibodies; in all cases, binding was normalized relative to that observed in the absence of antibody (data not shown). Compared with control, F102 and F103 reduced fibrinogen binding by 40 and 30%, respectively, whereas 134B-29 had no effect. TF-359 reduced fibrinogen binding to heparin by 90%.

Binding of Heparin and Zn\(^{2+}\) to αC-20 or αC-20AA—The results with αC domain-directed antibodies suggest that the major Zn\(^{2+}\)-dependent heparin binding site resides in the region of Aα 529–549, near the COOH terminus. The sequence was examined for clusters of basic or His residues, which could serve as heparin or Zn\(^{2+}\) binding sites, respectively. The most likely candidate was the consecutive His residues at positions 544 and 545. Consequently, a peptide analog of the Aα 529–548 sequence (αC-20) was synthesized and used to raise a polyclonal antibody in sheep. As a control, a second peptide with the two His residues substituted with Ala (αC-20AA) was prepared.

To examine heparin binding, the peptides were labeled at their NH\(_2\) termini with 5-IAF, and binding to heparin was examined in the absence or presence of Zn\(^{2+}\) or Ca\(^{2+}\). In the presence of Ca\(^{2+}\), no change in fluorescence intensity was observed (data not shown). However, in the presence of 12.5 μM Zn\(^{2+}\), a saturable decrease in f-αC-20 fluorescence intensity was observed and yielded a K<sub>d</sub> of 691 ± 268 nM (Fig. 5A). In contrast, no binding of heparin to αC-20AA was observed. Similar results were obtained in the reciprocal experiment with unlabeled αC-20 and f-d-heparin (data not shown). These results confirm the presence of a Zn\(^{2+}\)-dependent heparin binding site in the αC domain.

Binding of Zn\(^{2+}\) to αC-20 also was compared with that to αC-20AA using FluoZin-1. Titration of the Zn\(^{2+}\)/FluoZin-1 mixture with αC-20 resulted in a saturable decrease in fluorescence (Fig. 5B), yielding a K<sub>d</sub> value for the interaction of Zn\(^{2+}\) with αC-20 of 12.7 ± 3.0 μM. In contrast, when αC-20AA was used in place of αC-20, no binding was detected. These results indicate that the αC-20 peptide contains a Zn\(^{2+}\)-binding site that is dependent on the His residues at positions 544 and 545.

Effect of αC-20-directed Antibody on the Interaction of Heparin with Fibrin—To examine whether the αC-20-directed antibody could antagonize the binding of heparin to fibrin, fibrinogen was clotted in the presence of \(^{125}\)I-d-heparin and increasing concentrations of αC-20-directed or non-immune sheep IgG in the absence or presence of Zn\(^{2+}\) or Ca\(^{2+}\). After a 60-min incubation, clots were subjected to centrifugation, and the fraction of bound \(^{125}\)I-d-heparin was determined. In the presence of Ca\(^{2+}\), the antibody blocked the binding of \(^{125}\)I-d-heparin to fibrin, yielding an IC_{50} value of 0.22 ± 0.06 μM (Fig. 6). This confirms that the antibody and heparin bind to the same site on fibrin. Non-immune sheep IgG had no effect on \(^{125}\)I-d-heparin binding to fibrin. However, in the presence of Zn\(^{2+}\), the ability of the antibody to block d-heparin binding to fibrin was compromised, yielding a 9-fold higher IC_{50} value of 1.9 ± 0.19 μM (p < 0.002). This finding suggests that the more
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Effect of Fibrinogen or Fragment X on the Heparin-catalyzed Rate of Factor Xa Inhibition by Antithrombin—As another measure of the consequence of Zn\(^{2+}\) promotion of heparin binding to fibrinogen, we used a functional assay to compare the effects of fibrinogen and fragment X on the catalytic activity of heparin. Previous studies have shown that heparin binding to fibrin reduces the rate of inhibition of thrombin by antithrombin (27). It also was observed that inhibition of factor Xa by antithrombin was reduced by fibrin, albeit to a lesser extent because, unlike thrombin, factor Xa does not bind fibrin (23). In the presence of Zn\(^{2+}\), fibrinogen produced a concentration-dependent reduction in the heparin-catalyzed rate of factor Xa inhibition by antithrombin, with a 5-fold reduction in rate at a fibrinogen concentration of 2 \(\mu\)M (\(p < 0.05\) by two-way analysis of variance; Fig. 7). In contrast, the heparin-catalyzed rate of factor Xa inhibition by antithrombin decreased \(<2\)-fold in the presence of increasing concentrations of fibrinogen when Ca\(^{2+}\) was added in place of Zn\(^{2+}\) (data not shown). These results demonstrate that the increased binding of heparin to fibrinogen in the presence of Zn\(^{2+}\) reduces the catalytic activity of heparin. When fragment X was substituted for fibrinogen, no reduction in the rate of inhibition was observed in the presence of Zn\(^{2+}\) (Fig. 7) These data further confirm the role of the \(\alpha\)C domain in the Zn\(^{2+}\)-dependent binding of heparin.

DISCUSSION

Several groups have identified domains on fibrinogen that contribute to heparin binding. The NH\(_2\)-terminal region of the B\(\beta\)-chain represents one consensus site. Interaction is enhanced upon removal of fibrinopeptide B, endowing fibrin with a higher affinity for heparin than fibrinogen (28, 29). In contrast to earlier reports (30), these groups failed to observe significant binding of heparin to the D or \(\alpha\)C domains. The heparin binding sites on the NH\(_2\) termini of the \(\beta\)-chains of fibrin are proximal to putative thrombin binding sites, consistent with the ability of heparin to promote formation of a ternary heparin-thrombin-fibrin ternary complex (6). The current work provides evidence for a Zn\(^{2+}\)-dependent heparin binding site in the \(\alpha\)C domain. Although Zn\(^{2+}\) was not included in previous studies that examined the heparin-fibrinogen interaction, Zn\(^{2+}\) exists in plasma at a concentration of \(\sim 10 \mu\)M (8). In addition, Zn\(^{2+}\) is stored in platelet \(\alpha\)-granules, providing a mechanism whereby local concentrations may be elevated at sites of platelet activation (31). Our results provide further insight into the mechanism by which heparin interacts with fibrinogen.

The Zn\(^{2+}\)-dependence of the interaction of heparin with fibrinogen is consistent with the reported capacity of fibrinogen to bind Zn\(^{2+}\) (12, 32). However, the Zn\(^{2+}\) binding sites on fibrinogen have not been identified. In this study, we localized the Zn\(^{2+}\)-dependent heparin binding site to a 20-residue segment near the COOH terminus of the \(\alpha\)-chain. The location of this site was predicted by inspection of the sequence and confirmed with the use of antibodies and peptide analogs. A monoclonal antibody directed against \(\alpha\)529–549 reduced binding to heparin by 90%, whereas antibodies directed against \(\alpha\)563–578 (F102) or \(\alpha\)259–276 (F103) reduced binding by \(<40\%\). A peptide analog of the \(\alpha\)529–548 sequence bound f-d-heparin in a Zn\(^{2+}\)-dependent fashion. Heparin-binding sites in proteins are typically populated with basic residues (33), and the \(\alpha\)-chain COOH terminus has five basic residues in the final 10 amino acids (\(\alpha\)601–610). However, apart from Lys-538, the adjacent His residues at positions 544 and 545 are the only basic residues in the \(\alpha\)529–548 segment. Binding of heparin to His residues is also consistent with the Zn\(^{2+}\) dependence of the interaction. His residues represent the principal coordination site for metal ions in numerous metal-binding proteins (34, 35). Confirming
that this segment binds Zn$^{2+}$, a fluorescein-labeled analog of the peptide demonstrated a Zn$^{2+}$-dependent change in fluorescence intensity. Furthermore, substitution of the two His residues in αC-20 with Ala abrogated Zn$^{2+}$ binding. Interestingly, interaction of Zn$^{2+}$ with amyloid-β peptide also is mediated by adjacent His residues (36). Thus, these results identify the portion of the αC domain responsible for the Zn$^{2+}$-dependent interaction of heparin with fibrin.

The ability of Zn$^{2+}$ to promote heparin binding to fibrinogen is not unique. This response is observed with other Zn$^{2+}$ binding proteins, such as HRG and HK. These two homologous proteins have unique His-rich domains, which bind Zn$^{2+}$. At neutral pH, binding of heparin to HRG or HK is mediated by His residues and is dependent on Zn$^{2+}$ (4, 5). Although these proteins have high His content, Zn$^{2+}$ also binds to peptide sequences containing only two or three His residues (37, 38). Zn$^{2+}$ also promotes heparin binding to other proteins such as heparin cofactor II and endostatin (39, 40).

There are two mechanisms by which Zn$^{2+}$ may promote heparin-protein interaction. One is by inducing a conformational change in the protein to expose a latent heparin binding site (37), whereas the other involves Zn$^{2+}$ serving as a coordinator to bridge heparin to the protein (41). Although it is known that Zn$^{2+}$ binds heparin (42), Zn$^{2+}$ is unable to promote the interaction of thrombin or antithrombin with heparin (39, 43). This suggests that Zn$^{2+}$-dependent binding to heparin is protein-specific (44). Consistent with this, SPR and fluorescence analyses suggest that Zn$^{2+}$ induces conformational changes in fibrinogen. These observations reveal that Zn$^{2+}$ is a common mediator of heparin-protein interaction.

One of the consequences of the interaction of heparin with proteins other than antithrombin is a reduction in its anticoagulant activity. This has been observed with HRG (44, 45), HK (5), and platelet factor 4 (46). Interaction with these proteins decreases the anticoagulant activity of heparin by reducing the concentration of heparin available to bind to antithrombin (3). Fibrinogen is a recognized member of this group of heparin-binding proteins (28, 29). Further compromise of heparin activity occurs when fibrinogen is clotted because heparin promotes thrombin binding to fibrin and induces the formation of a ternary complex that limits inhibition by antithrombin (6, 27). The importance of the heparin-fibrin interaction is highlighted by the fact that higher doses of heparin are needed to achieve a therapeutic anticoagulant response in patients with venous thromboembolism than in those with acute coronary syndrome; a phenomenon that has been attributed to the larger thrombus burden in patients with venous thrombosis (47). Recently, we demonstrated that Zn$^{2+}$ promotes ternary complex formation, thereby augmenting the protection of fibrin-bound thrombin from inhibition by antithrombin (10). The current work shows that fibrinogen also compromises heparin-catalyzed inhibition of factor Xa in the presence of Zn$^{2+}$. Because factor Xa does not bind fibrin, this impairment must result from the inability of fibrin-bound heparin to catalyze antithrombin. This likely is the result of fibrinogen competitively inhibiting heparin binding to antithrombin.

The current work reveals a novel interaction of heparin with fibrinogen via the αC domain. Recent work has suggested that the E domain, which encompasses the NH$_2$ termini of the β-chains, is the predominant site of fibrinogen interaction with heparin (28, 29). Because Zn$^{2+}$ was not included, these studies overlooked the αC interaction. Our findings highlight the potential regulatory role that Zn$^{2+}$ may have in biological interactions in blood when this metal ion is released from activated platelets (4, 31). The Zn$^{2+}$-dependent interaction of heparin with fibrinogen adds to the numerous roles that Zn$^{2+}$ is proposed to play in hemostasis (9, 48), including recent reports of interaction of Zn$^{2+}$ with protein S and factor VIIa (49, 50). Zn$^{2+}$-mediated promotion of the interaction of heparin with fibrinogen may contribute to the unpredictable anticoagulant response observed with heparin (2). In addition, by promoting formation of ternary heparin-thrombin-fibrin complexes (10), Zn$^{2+}$ may also render fibrin thrombi more thrombogenic. These results illustrate the possibility that many reactions affected by Zn$^{2+}$ may have been overlooked in plasma because of the use of citrate as an anticoagulant.

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