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By Increasing the Affinity of Heparin for Fibrin, Zn^{2+} Promotes the Formation of a Ternary Heparin–Thrombin–Fibrin Complex That Protects Thrombin from Inhibition by Antithrombin

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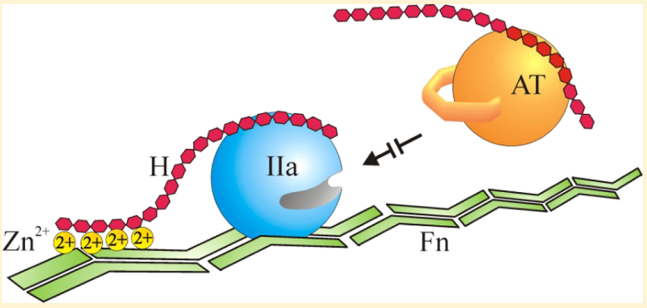
ABSTRACT: Heparin binds fibrin and, by bridging thrombin onto fibrin, promotes the formation of a ternary heparin–thrombin–fibrin complex that protects thrombin from inhibition by antithrombin. Because thrombin binds γ_A/γ'-fibrin, a variant with an extended γ-chain, with higher affinity than the bulk γ_A/γ_A-fibrin, γ_A/γ'-fibrin affords bound thrombin more protection from inhibition by antithrombin–heparin. We examined the effect of Zn^{2+} on heparin–thrombin–fibrin complex formation because Zn^{2+} modulates heparin–protein interactions. Zn^{2+} increased the affinity of heparin for γ_A/γ_A- and γ_A/γ'-fibrin by 4.3- and 3.7-fold, respectively, but had no effect on the affinity of thrombin for either form of fibrin. In contrast, in the presence of heparin, Zn^{2+} increased the affinity of thrombin for γ_A/γ_A-fibrin 4-fold (from a K_d value of 0.8 to 0.2 μM) and slowed the rate of thrombin dissociation from γ_A/γ_A-fibrin clots. These findings suggest that Zn^{2+} enhances the formation of ternary heparin–thrombin–fibrin complexes with γ_A/γ_A-fibrin but does not influence the already high affinity interaction of thrombin with γ_A/γ'-fibrin. Consistent with this concept, in the presence of Zn^{2+}, γ_A/γ_A-fibrin protected thrombin from inhibition by antithrombin–heparin to a similar extent as γ_A/γ'-fibrin. Therefore, by enhancing the binding of heparin to fibrin, physiological concentrations of Zn^{2+} render fibrin-bound thrombin more protected from inhibition by antithrombin. Because fibrin-bound thrombin can trigger thrombus expansion, these findings help to explain why recurrent thrombosis can occur despite heparin treatment.



Thrombin plays a pivotal role in hemostasis. As a procoagulant, thrombin converts fibrinogen to fibrin, activates factor (F) XIII, which cross-links and stabilizes the fibrin monomers, catalyzes the hydrolysis of protease-activated receptors to induce platelet activation,[1,2] and amplifies its own generation through feedback activation of FV, FVIII, and FXI.[3] When bound to thrombomodulin, its receptor on the endothelial cell surface, thrombin initiates anticoagulant and antifibrinolytic pathways by activating protein C and thrombin-activatable fibrinolysis inhibitor, respectively. Therefore, thrombin not only promotes coagulation but also regulates its own generation.

The specificity of thrombin activity is mediated by two exosites; positively charged domains that flank the active site of the enzyme. Exosite 1 mediates thrombin's interaction with substrates, such as fibrinogen. However, about 10% of circulating fibrinogen possesses a variant γ-chain designated as the γ'-chain that binds thrombin with higher affinity.[4−6] Because it is a dimer, fibrinogen containing the predominant γ_A-chains is designated γ_A/γ_A-fibrinogen, whereas the variant fibrinogen is a heterodimer designated γ_A/γ'-fibrinogen because it possesses one γ_A-chain and one γ'-chain.[4,6] The higher affinity interaction of thrombin with γ_A/γ'-fibrinogen results from bivalent binding, where exosite 1 binds to the NH_2-terminus of the α- or β-chain, and exosite 2 engages the COOH-terminus of the γ'-chain.[5−9] After thrombin-mediated conversion of fibrinogen to fibrin, some thrombin remains bound to the fibrin clot. Thrombin binding to γ_A/γ_A-fibrin is mediated via a single class of binding sites with a K_d of about 3−4 μM, whereas binding to γ_A/γ'-fibrin displays both high and lower affinity, with K_d values of 11 nM and 1.1 μM, respectively.[5,6,8]

One consequence of residual binding to fibrin is that thrombin retains activity even in the face of abundant serpin inhibitors, namely, antithrombin and heparin cofactor II.[10−13] Because it is protected from inhibition, fibrin-bound thrombin can trigger thrombus expansion by locally activating platelets, amplifying its own generation and converting more fibrinogen to fibrin. Moreover, we have shown that the bivalent interaction of thrombin with γ_A/γ'-fibrin affords the enzyme greater protection from serpin inhibition than its univalent interaction with γ_A/γ_A-fibrin.[14]

Heparin augments the binding of thrombin to fibrin because it simultaneously binds to fibrin and to exosite 2, thereby bridging more thrombin onto the fibrin surface.[10,11] Formation

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of this ternary heparin−thrombin−fibrin complex not only positions more thrombin onto the fibrin surface but also heightens the apparent affinity of thrombin for fibrin and renders the thrombin more resistant to inhibition by heparin-catalyzed antithrombin or heparin cofactor II.[10,11,15] Thus, formation of the ternary heparin−thrombin−fibrin complex may account for heparin resistance where thrombi remain thrombogenic both during and after heparin treatment.[16]

Ternary heparin−thrombin−fibrin complex formation involves three binary interactions: those between heparin and fibrin, heparin and exosite 2 on thrombin, and thrombin and fibrin.[10,11] Previous studies have demonstrated that Zn^{2+} enhances the binding of heparin to high molecular weight kininogen[17] and other plasma proteins.[18−22] Although free Zn^{2+} exists in serum at a concentration of 0.2−0.5 μM,[23,24] the total concentration of Zn^{2+} ranges from 12 to 22 μM and local levels can rise in response to its release from platelets, which accumulate Zn^{2+} in their α-granules in concentrations 30−60 times higher than that in serum.[25] Because Zn^{2+} modulates known heparin−protein interactions, we hypothesized that Zn^{2+} would (a) promote heparin binding to fibrin, (b) heighten the apparent affinity of thrombin for fibrin in the presence of heparin, (c) render thrombin within the ternary heparin−thrombin−fibrin complex even more resistant to inactivation by heparin-catalyzed antithrombin, and (d) attenuate the inhibitory effect of heparin on thrombin generation in human plasma. The current studies were undertaken to explore these hypotheses so as to determine whether Zn^{2+}, which is released locally upon platelet activation, augments the protective effect of ternary complex formation, thereby further stabilizing fibrin-bound thrombin.

EXPERIMENTAL PROCEDURES

Reagents. Human α-thrombin and plasminogen-free fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Activated FXIII was from Haematologic Technologies Inc. (Essex Junction, VT). Unfractionated heparin and polybrene were from Sigma-Aldrich Canada Ltd. (Oakville, ON). Venom from *Bothrops atrox moojani* (Batroxobin) was from Pentapharm (Centerchem Inc., Norwalk, CT). Human antithrombin was from Affinity Biologicals Inc. (Ancaster, ON). A tyrosine-phosphorylated peptide analogue of the COOH-terminus sequence of the γ'-chain of fibrinogen, designated as γ'-peptide (VRPEHPAETEYDSLYPEDDL), and a rabbit polyclonal antibody directed against this peptide were prepared by Bachem Bioscience, Inc. (King of Prussia, PA). The thrombin-directed chromogenic and fluorogenic substrates CS-01-38 (H-D-Phe-Pip-Arg-pNA-2HCl), tos-Gly-Pro-Arg 7-amido-4-methylcoumarin (tGPR-AMC), and Z-Gly-Gly-Arg-AMC were from Aniara Corp. (Mason, OH), Sigma, and Bachem, respectively. D-Phe-Pro-Arg-chloromethyl-ketone (FPR) and D-Tyr-Pro-Arg-chloromethyl-ketone (YPR) were from EMD Chemicals Inc. (La Jolla, CA). γ_A/γ_A- and γ_A/γ'-fibrinogen were isolated by DEAE-Sepharose chromatography and characterized as previously described.[14]

Preparation of Radiolabeled Thrombin and Heparin. Thrombin was reacted with ^{125}I-labeled YPR, prepared using Iodo-beads (Thermo Fisher Scientific Inc., Ottawa, ON) as previously described.[14] The specific activity of ^{125}I-YPR-thrombin was 7.2×10^5 cpm/μg protein. Active-site blocked thrombin was prepared by incubating thrombin with a 3-fold molar excess of FPR in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) for 1 h at 24 °C. When no chromogenic activity against 200 μM CS-01-38 was detected, the reaction mixture was dialyzed against TBS to remove excess FPR. Complete removal of FPR was verified by the absence of thrombin inhibition in the presence of postdialysis mixture containing 10 nM FPR-thrombin.

To prepare ^{125}I-heparin, lyophilized heparin (45 mg) was dissolved in 2 mL of 200 mM borate, pH 9.0, and mixed with 3 mL of 1 mg/mL water-soluble Bolton-Hunter reagent (Thermo Scientific). After 3 h incubation, the mixture was dialyzed against water and lyophilized. Two IODO-BEADS were first washed with phosphate buffered saline (PBS). After incubation for 5 min at 23 °C with 200 μL of PBS and 1 mCi Na^{125}I (McMaster University Nuclear Reactor), 4.2 mg of modified heparin in 100 μL of PBS was added. The reaction mixture was mixed every 5 min for 30 min and then removed from the beads. After application to a PD-10 column (GE Healthcare) equilibrated with TBS containing 0.005% Tween 20, 1 mL fractions were collected and the radioactivity of aliquots was determined. Based on the results of an Azure A assay,[26] the final concentration of ^{125}I-heparin was 575 μM and the specific radioactivity was 42 400 cpm/μg.

Affinity of ^{125}I-Heparin for γ_A/γ_A- or γ_A/γ'-Fibrin. The dose response for the effect of Zn^{2+} on ^{125}I-heparin binding to fibrin was determined in a pelleted-clot assay. Tubes containing 400 nM ^{125}I-heparin, 650 nM γ_A/γ_A- or γ_A/γ'-fibrinogen, and 2 mM $CaCl_2$ in TBS with 0.005% Tween 20 were prepared in the presence of $ZnCl_2$ in concentrations ranging from 0 to 25 μM. After addition of 50 nM thrombin and 60 min incubation at 24 °C, clots were pelleted by centrifugation at 14000*g* for 4 min, and aliquots of supernatant were removed and counted for radioactivity. The concentration of bound ^{125}I-heparin was then calculated by subtraction and comparison with a standard curve containing known concentrations of ^{125}I-heparin. The EC_{50} for Zn^{2+} promotion of heparin binding to fibrin was determined by nonlinear regression of a rectangular hyperbola using Table Curve (Jandel Scientific, San Rafael, CA).

To further evaluate the effect of Zn^{2+} on the affinity of ^{125}I-heparin for γ_A/γ_A- or γ_A/γ'-fibrin, ^{125}I-heparin (100 nM) was added to a series of tubes containing γ_A/γ_A- or γ_A/γ'-fibrinogen in concentrations ranging from 0 to 3.5 μM in the absence or presence of 12.5 μM $ZnCl_2$. Clotting was initiated by the addition of 5 nM thrombin and 2 mM $CaCl_2$. The concentrations of fibrin-bound ^{125}I-heparin were calculated and plotted against the fibrinogen concentration. The data were then analyzed by nonlinear regression of the binding isotherm equation (eq 1)[14]

$$B = B_0 + \frac{\alpha}{2}\left(1 + \frac{K_d + L_0}{P_0} - \sqrt{\left(1 + \frac{K_d + L_0}{P_0}\right)^2 - 4 \times \frac{L_0}{P_0}}\right) \quad (1)$$

where B is the fraction bound, B_0 is the initial fraction bound without titrant, L_0 is the concentration of ^{125}I-heparin added, P_0 is the concentration of fibrinogen, and α is the maximum concentration of ^{125}I-heparin bound.

Binding of Thrombin to γ_A/γ_A- or γ_A/γ'-Fibrin. The effect of Zn^{2+} on thrombin binding to fibrin was determined in the absence and presence of heparin. Experiments were performed in TBS containing 0.01% Tween 20 and 2 mM $CaCl_2$ (TBS-Tw-Ca). Samples containing 650 nM fibrinogen, 2 mM $CaCl_2$, 0 or 400 nM heparin, and increasing concentrations of $ZnCl_2$ were clotted with 50 nM thrombin. After incubation for 1 h at 24 °C, fibrin was pelleted by centrifugation at 14000*g*

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for 10 min, and three 10 μL aliquots of supernatant were removed. The chromogenic activity of thrombin in the aliquots was assessed in a 96-well plate containing 200 μL of 200 μM CS-01-38 and 10 mg/mL polybrene. Substrate hydrolysis was monitored at 405 nm in a plate reader (Molecular Devices, Sunnyvale, CA), and the unbound thrombin concentration was determined by comparison with a standard curve. The fraction of bound thrombin was then calculated by subtraction and plotted versus Zn^{2+} concentration. Subsequently, the effect of Zn^{2+} on the affinity of thrombin for fibrin was determined in the absence or presence of heparin. Increasing concentrations of γ_A/γ_A- or γ_A/γ'-fibrinogen were added to a series of microcentrifuge tubes containing 250 nM heparin in the absence or presence of 12.5 μM $ZnCl_2$. Fibrinogen was clotted by the addition of 100 nM thrombin containing 400 nM FPR-thrombin in a final reaction volume of 200 μL. The fraction of fibrin-bound thrombin was determined as described above and plotted versus the fibrinogen concentration, and the apparent K_d values were determined by nonlinear regression analysis of eq 1.

Protection of Thrombin Bound to γ_A/γ_A- or γ_A/γ'-Fibrin from Inhibition by Antithrombin−Heparin. Fibrin-bound thrombin inhibition by antithrombin was measured in a continuous assay in TBS containing 6 mg/mL PEG 8000.[14] Mixtures containing 0−2 μM antithrombin, 2 μM γ_A/γ_A- or γ_A/γ'-fibrinogen, 50 μM tGPR-AMC, 60 nM heparin, and 2 mM $CaCl_2$ were added to a multiwell plate in the absence or presence of 12.5 μM $ZnCl_2$. Fibrinogen was clotted by addition of 1 nM thrombin and 10 units/mL batroxobin, which was added to ensure rapid and uniform clotting of the fibrinogen even in the presence of antithrombin.[14] Reactions were monitored at 10 s intervals for 15 min at 24 °C in a Gemini M3 fluorescence plate reader (Molecular Devices) with excitation and emission wavelengths of 360 and 460 nm, respectively, and a 455 nm cutoff filter. Under these conditions, the fibrinogen clotted within 45 s. Because of accelerated thrombin inhibition in the absence of fibrinogen, the concentrations of antithrombin and tGPR-AMC were decreased to 100 nM and 35 μM, respectively. Background-corrected fluorescence values (F) were analyzed with respect to time (t) by nonlinear regression analysis of eq 2 to obtain the apparent, pseudo-first-order rate constant of inhibition (k_1).[27]

$$F = V_f \times t + (V_0 - V_f)(1 - e^{-k_1 \times t})/k_1 \quad (2)$$

where V_0 and V_f are the initial and final reaction rates, respectively.[28] The k_1 value was multiplied by (1 + [tGPR-AMC]/K_m) to correct for competition between the fluorogenic substrate and antithrombin for thrombin binding using a K_m of 6.4 μM, which was determined in a separate experiment. The apparent second-order rate constant (k_2 app) was calculated by dividing k_1 by the antithrombin concentration.

Dissociation of Thrombin from γ_A/γ_A- or γ_A/γ'-Fibrin Clots. Dissociation of ^{125}I-YPR-thrombin from fibrin clots was monitored to examine the effects of Zn^{2+} on this process.[14] Stock solutions containing 3 μM γ_A/γ_A- or γ_A/γ'-fibrinogen, 18.5 nM ^{125}I-YPR-thrombin, and 30 nM FXIIIa in TBS-Tw-Ca were prepared in the absence or presence of 12.5 μM $ZnCl_2$ and/or 250 nM heparin. Aliquots of 125 μL were added to microcentrifuge tubes, and clots were formed around truncated plastic inoculation loops (Bac-Loop; Thermo-Fisher Scientific, Waltham, MA) by incubation with 5 μL of 100 nM thrombin for 30 min at 24 °C. After incubation, clots were removed from the tubes, washed with TBS-Tw-Ca for 5 min, and suspended in 12 × 55 mm polypropylene tubes (Sarstedt Inc., Montreal, QC) containing 2 mL of TBS-Tw-Ca. γ_A/γ_A-fibrin clots were incubated at 24 °C with gentle agitation for 8 h, whereas γ_A/γ'-fibrin clots were incubated under the same conditions for up to 90 h. Heparin and/or $ZnCl_2$ were added to the bathing buffer to match the conditions under which the fibrin clots were formed. A control set of clots was incubated in bathing buffer containing 2 M NaCl to monitor nonspecific binding. Under all conditions, 0.5 mL aliquots of buffer removed at intervals were counted for radioactivity and then returned to the tubes. The concentration of clot-associated ^{125}I-YPR-thrombin at each time point was calculated as a percentage of the initial amount bound. The time course was then fit to a two-phase exponential decay curve with a zero end-point to determine the rates of dissociation.[14] The two phases reflect the rapid fluid-phase diffusion of unbound thrombin in the bulk solvent followed by the slower dissociation of thrombin that is reversibly bound to fibrin.

Thrombin Generation in Human Plasma in the Absence or Presence of Zn^{2+}. Platelet-poor plasma obtained from blood collected from 10−12 donors was pooled, dialyzed versus TBS, and stored in aliquots at −80 °C. Plasma was thawed at 37 °C, and 1 M $ZnCl_2$ was added to 0−80 μM. In polystyrene black plates (Costar), 5 μL aliquots of heparin were added to yield final concentrations of 0.5, 1, or 2 μg/mL, which are the equivalent of 0.1, 0.2, and 0.4 U/mL of heparin, respectively—concentrations that span the range of heparin used for prevention or treatment of thrombosis in humans.[29] To each well, 40 μL of plasma was added, and the plate was warmed at 37 °C for 10 min prior to the addition of 5 μL of 1/50 diluted thromboplastin reagent (RecombiPlasTin, Instrumentation Laboratory) that contained 0.3 μg/mL tissue factor.[30] The reaction was initiated by addition of 50 μL of a prewarmed solution containing 1 mM Z-Gly-Gly-Arg-AMC and 50 mM $CaCl_2$. Plates were read in a fluorescent plate reader at 60 s intervals for 90 min at 37 °C using excitation and emission wavelengths of 360 and 460 nm, respectively, and a 455 nm cutoff filter. Data were analyzed using TechnoThrombin TGA software (TechnoClone GmbH, Vienna, Austria) to obtain peak thrombin concentration and endogenous thrombin potential, determined by area under the curve.

Statistical Analyses. Results are reported as mean ± SEM. Significance of differences was determined using paired Student's t tests. Two-way ANOVA was used to analyze the effect of heparin on thrombin generation in the absence or presence of varying concentrations of Zn^{2+}. In all cases, p-values less than 0.05 were considered statistically significant.

RESULTS

Effect of Zn^{2+} on the Binding of ^{125}I-Heparin to γ_A/γ_A- or γ_A/γ'-Fibrin. Binding of ^{125}I-heparin to fibrin clots was assessed in the absence or presence of $ZnCl_2$ at concentrations up to 25 μM. Fibrinogen was clotted in the presence of ^{125}I-heparin, and clots were compacted by centrifugation. Unbound ^{125}I-heparin in the clot supernatants was used to calculate the fraction bound. There was a dose-dependent and saturable increase in the concentration of ^{125}I-heparin bound to fibrin with increasing $ZnCl_2$ concentrations (Figure 1). Half-maximal binding occurred at 1.3 μM $ZnCl_2$, which is well within the physiological Zn^{2+} concentration of 12−22 μM, whereas maximal binding was observed at 12.5 μM. Having demonstrated that $ZnCl_2$ promotes heparin binding to fibrin, the affinity of ^{125}I-heparin for γ_A/γ_A- or γ_A/γ'-fibrin was then

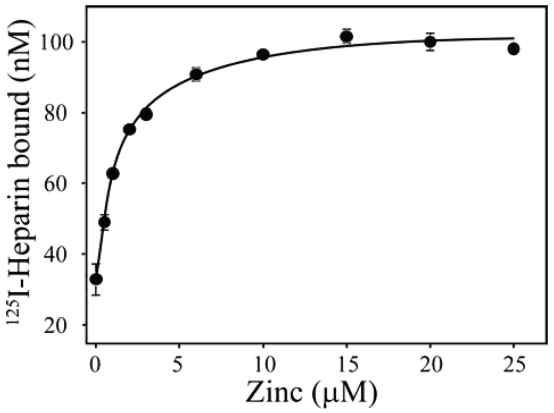

Figure 1. Effect of increasing concentrations of Zn^{2+} on the binding of ^{125}I-heparin to fibrin. In a series of microcentrifuge tubes, samples containing 400 nM ^{125}I-heparin, 650 nM fibrinogen, 2 mM $CaCl_2$, and 0–25 μM $ZnCl_2$ were clotted with 50 nM thrombin. After 60 min incubation, fibrin was sedimented by centrifugation and aliquots of the supernatant were used to determine the concentration of unbound ^{125}I-heparin. The amount bound was then calculated and plotted against the concentration of $ZnCl_2$. Data were analyzed by nonlinear regression of a rectangular hyperbola (line) to determine the concentration of half-maximal effect. The symbols represent the mean ± SEM of three determinations.

measured in the absence or presence of 12.5 μM $ZnCl_2$. A series of tubes containing ^{125}I-heparin (100 nM) and γ_A/γ_A- or γ_A/γ'-fibrinogen in concentrations ranging from 0 to 3.5 μM were prepared, and the fibrinogen was then clotted with 5 nM thrombin. The percentage of ^{125}I-heparin bound to fibrin was plotted against the fibrinogen concentration for determination of the K_d value by nonlinear regression (Figure 2). ^{125}I-heparin bound γ_A/γ_A-fibrin with a K_d value of 1.4 ± 0.2 μM in the absence of Zn^{2+}, which falls between the values of 0.28 and 5.7 μM that were reported previously.[11,15] In the presence of Zn^{2+},

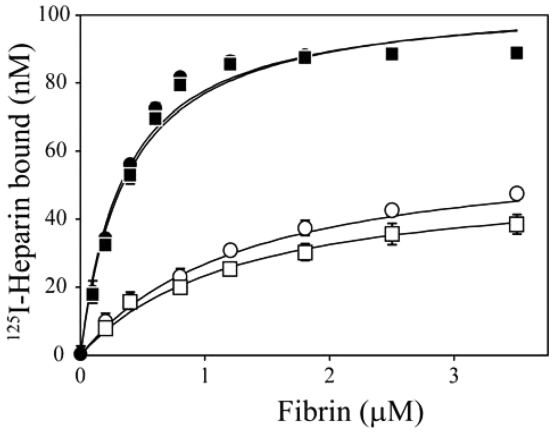

Figure 2. Effect of Zn^{2+} on the binding of ^{125}I-heparin to fibrin. In microcentrifuge tubes, 100 nM of ^{125}I-heparin and 2 mM $CaCl_2$ were mixed with increasing concentrations of γ_A/γ_A-fibrinogen (squares) or γ_A/γ'-fibrinogen (circles), and the samples were clotted with 5 nM thrombin in the absence (open) or presence (closed) of 12.5 μM $ZnCl_2$. After centrifugation, the radioactivity of the unbound ^{125}I-heparin in the supernatant was quantified to calculate the fraction bound. The plot of concentration of ^{125}I-heparin bound versus fibrinogen concentration was subjected to nonlinear regression analysis to determine K_d (lines). The symbols represent the mean ± SEM of three independent determinations.

the K_d was 0.3 ± 0.02 μM—a statistically significant 4.3-fold increase in affinity ($p < 0.05$). Similar results were obtained with γ_A/γ'-fibrin, where the K_d values were 1.3 ± 0.2 and 0.4 ± 0.02 μM in the absence and presence of Zn^{2+}, respectively. Thus, Zn^{2+} promotes the binding of ^{125}I-heparin to both γ_A/γ_A- and γ_A/γ'-fibrin, producing a 4-fold increase in affinity.

Effect of Zn^{2+} on the Affinity of Thrombin for γ_A/γ_A- or γ_A/γ'-Fibrin in the Absence or Presence of Heparin. Having shown that Zn^{2+} enhances the interaction between heparin and γA/γA- or γA/γ′-fibrin, we next examined the effect of Zn^{2+} on the thrombin–fibrin interaction in the absence or presence of heparin. A series of tubes containing 650 nM fibrinogen, 2 mM $CaCl_2$, and increasing concentrations of $ZnCl_2$ were prepared in the absence or presence of 400 nM heparin. After incubation with 50 nM thrombin, the resultant fibrin clots were sedimented by centrifugation, and aliquots of supernatant were removed to determine the concentration of unbound thrombin by chromogenic assay. Without heparin, $ZnCl_2$ had little effect on thrombin binding to fibrin (Figure 3).

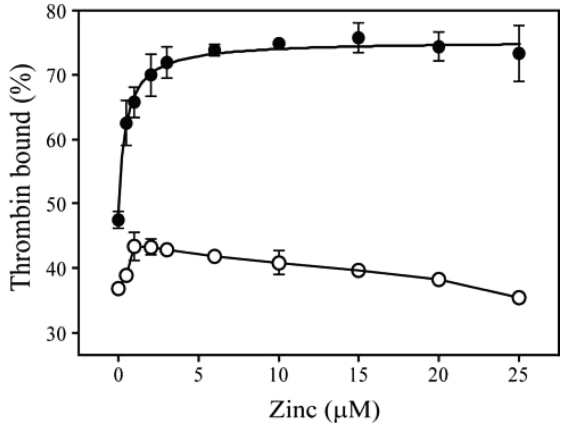

Figure 3. Effect of Zn^{2+} on thrombin binding to fibrin in the absence or presence of heparin. To a series of tubes containing 650 nM fibrinogen and 2 mM $CaCl_2$, 50 nM thrombin was added in the absence (open circles) or presence (closed circles) of 400 nM heparin and increasing concentrations of $ZnCl_2$. The resultant clots were pelleted by centrifugation, and the concentration of free thrombin in the clot supernatants was determined by chromogenic assay. The percentage of thrombin bound was determined and plotted versus $ZnCl_2$ concentration. The symbols represent the mean ± SEM of three determinations.

In contrast, in the presence of heparin, the fraction of thrombin bound to fibrin increased as a function of $ZnCl_2$ concentration. The half-maximal increase was achieved with 0.45 μM $ZnCl_2$, a concentration comparable with that which promoted heparin binding to fibrin.

To quantify the effect of Zn^{2+} on thrombin binding, increasing concentrations of γ_A/γ_A- or γ_A/γ'-fibrinogen were clotted with 100 nM active thrombin in the presence of 400 nM FPR-thrombin in the absence or presence of heparin. Concentrations of unbound thrombin were determined as described above and plotted against fibrinogen concentrations for calculation of the K_d value for thrombin binding to fibrin (Table 1). In the absence of heparin, the K_d of thrombin for γ_A/γ_A-fibrin was 2.6 ± 0.1 μM, and Zn^{2+} had no statistically significant effect on this value. Although, as expected, thrombin bound γ_A/γ'-fibrin with higher affinity (K_d of 0.1 ± 0.04 μM), Zn^{2+} had no statistically significant effect on this value. Studies

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Table 1. Effect of Zn^{2+} and/or Heparin on the Affinity of Thrombin for γ_A/γ_A- or γ_A/γ'-Fibrin

addition	K_d (μM)	
	γ_A/γ_A-fibrin	γ_A/γ'-fibrin
control	2.6 ± 0.1	0.1 ± 0.04
Zn^{2+}	2.9 ± 0.5	0.2 ± 0.02
heparin	0.8 ± 0.1[a]	0.09 ± 0.01
heparin and Zn^{2+}	0.2 ± 0.01[a,b]	0.1 ± 0.03

[a] $p < 0.001$ compared with the γ_A/γ_A-fibrin control. [b] $p < 0.001$ compared with γ_A/γ_A-fibrin plus heparin.

were then repeated in the presence of heparin to examine the effect of Zn^{2+} on the affinity of thrombin for fibrin under these conditions. In the presence of heparin, there was a statistically significant ($p < 0.001$) 3.3-fold increase in the affinity of thrombin for γ_A/γ_A-fibrin to a K_d value of 0.8 ± 0.1 μM, a finding consistent with previous observations.[14] Zn^{2+} produced a further statistically significant ($p < 0.001$) 4-fold increase in the affinity of thrombin for γ_A/γ_A-fibrin in the presence of heparin to a K_d value of 0.2 ± 0.01 μM. When experiments were repeated with γ_A/γ'-fibrin, neither heparin nor the combination of heparin plus Zn^{2+} significantly affected the affinity of thrombin for γ_A/γ'-fibrin. These findings are consistent with the concept that, because Zn^{2+} potentiates the heparin–fibrin interaction, the formation of ternary heparin–thrombin–fibrin complex with γ_A/γ_A-fibrin is enhanced. In contrast, γ_A/γ'-fibrin binds thrombin with high affinity even in the absence of heparin. Therefore, with γ_A/γ'-fibrin, neither heparin nor the combination of heparin plus Zn^{2+} significantly affects the affinity of thrombin for γ_A/γ'-fibrin.

Effect of Zn^{2+} on the Dissociation of Thrombin from γ_A/γ_A- or γ_A/γ'-Fibrin Clots. On the basis of the observation that Zn^{2+} promotes the formation of ternary heparin–γ_A/γ_A-fibrin–thrombin complexes, we hypothesized that the dissociation of thrombin from intact γ_A/γ_A-fibrin clots would be slower in the presence of the combination of Zn^{2+} and heparin than with Zn^{2+} or heparin alone. To test this hypothesis, fibrin clots containing ^{125}I-YPR-thrombin were immersed in buffer, and dissociation was monitored over time. In the control without heparin, only 20 ± 1% of ^{125}I-YPR-thrombin was retained in the γ_A/γ_A-fibrin clots at 8 h (Figure 4A). On its own, Zn^{2+} had no effect on the rate of ^{125}I-YPR-thrombin dissociation (data not shown). Although heparin slowed the initial rate of dissociation of ^{125}I-YPR-thrombin from γ_A/γ_A-fibrin clots, the extent of dissociation at 8 h in the presence of heparin was comparable with that in its absence. When heparin and Zn^{2+} were combined, the rate of ^{125}I-YPR-thrombin dissociation was considerably slower such that 42 ± 4% of the total ^{125}I-YPR-thrombin remained clot-associated at 8 h.

The rate of dissociation of ^{125}I-YPR-thrombin from γ_A/γ'-fibrin clots was slower than that from γ_A/γ_A-fibrin clots (Figure 4B) such that even by 90 h, about 70% of the ^{125}I-YPR-thrombin remained clot-associated. Heparin produced a modest increase in the rate of dissociation because only 63 ± 3% of the total ^{125}I-YPR-thrombin remained clot-associated at 90 h. Heparin plus Zn^{2+} increased the rate of dissociation still further such that 55 ± 2% of ^{125}I-YPR-thrombin remained clot-associated at 90 h. To assess the influence of the interaction of thrombin with the γ'-chain on its rate of dissociation from γ_A/γ'-fibrin clots, dissociation was measured in the presence of an antibody directed against the γ'-peptide (Figure 4B). In the presence of this antibody, only 19 ± 3% and 22 ± 4% of the

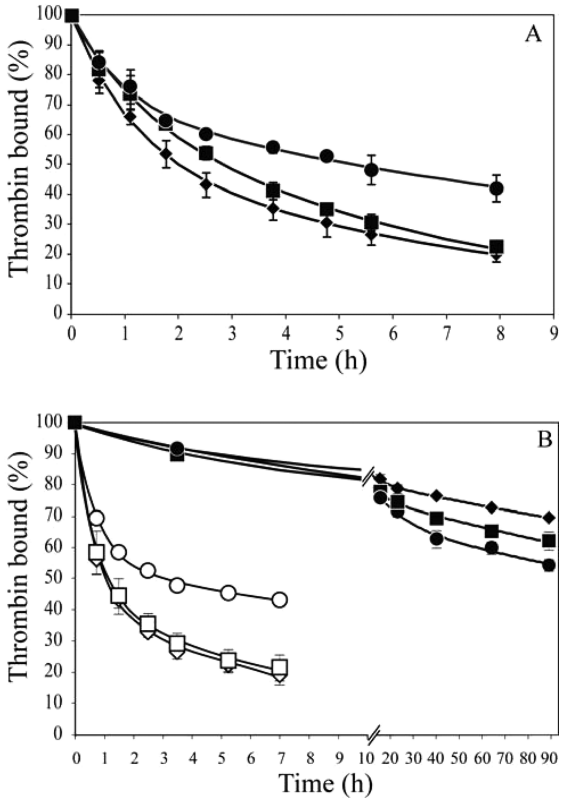

Figure 4. Dissociation of ^{125}I-YPR-thrombin from γ_A/γ_A- or γ_A/γ'-fibrin clots. Fibrin clots formed around plastic loops were prepared with 125 μL of 3 μM γ_A/γ_A-fibrinogen (panel A) or γ_A/γ'-fibrinogen (panel B), 100 nM thrombin, and 18.5 nM ^{125}I-YPR-thrombin in the absence (diamonds) or presence of 250 nM heparin (squares) or 250 nM heparin plus 12.5 μM $ZnCl_2$ (circles). Clots were washed and suspended in 2 mL of TBS-Tw-Ca containing $ZnCl_2$ and/or heparin, as indicated. In panel B, duplicate clots were incubated without (closed symbols) or with (open symbols) an antibody directed against the γ'-peptide. The radioactivity in the incubation buffer was measured at intervals to determine the amount of ^{125}I-YPR-thrombin retained in the clot, which was calculated as a percent of the total and then plotted against time. Lines represent nonlinear regression analysis of the data using a two-component exponential decay model. Data represent the mean ± SEM of six experiments.

total ^{125}I-YPR-thrombin remained associated with γ_A/γ'-fibrin clots at 7 h in the absence or presence of heparin, respectively. With the combination of heparin and Zn^{2+}, 43 ± 1% of the ^{125}I-YPR-thrombin remained clot-associated at 7 h. Thus, when thrombin's interaction with the γ'-chain is blocked with the antibody, the rate of ^{125}I-YPR-thrombin dissociation from γ_A/γ'-fibrin clots is similar to that from γ_A/γ_A-fibrin clots, confirming that the γ'-chain is responsible for the heightened interaction of thrombin with γ_A/γ'-fibrin.

Dissociation of thrombin from fibrin clots depends on two independent processes: rapid diffusion of unbound thrombin that is not associated with the fibrin meshwork and slower dissociation of thrombin that is reversibly bound to fibrin. Values for the rapid diffusion phase half-lives ranged from 0.9 ± 0.2 h for γ_A/γ_A-fibrin to 1.3 ± 1.0 h for γ_A/γ'-fibrin (not shown)—results consistent with those observed previously.[14] In the slow phase, ^{125}I-YPR-thrombin adsorbed to γ_A/γ_A-fibrin dissociated with a half-life of 5.3 ± 0.8 h (Table 2). Although heparin alone had no significant effect on the dissociation half-life, heparin plus Zn^{2+} produced a statistically significant ($p <$

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Table 2. Slow-Phase Dissociation Half-Lives of Thrombin from γ_A/γ_A- or γ_A/γ'-Fibrin Clots in the Absence or Presence of Heparin, Zn^{2+}, or the γ'-Peptide-Directed Antibody

	$t_{1/2}$ (h)		
	control	heparin	heparin + Zn^{2+}
γ_A/γ_A-fibrin	5.3 ± 0.8	4.5 ± 0.9	10.4 ± 2.9[b]
γ_A/γ'-fibrin	368.0 ± 5.1[a]	266.0 ± 97.2[a]	250.7 ± 15.0[a]
γ_A/γ'-fibrin + γ'-antibody	5.8 ± 1.9	6.5 ± 2.3	20.1 ± 3.3[a,c]

[a] $p < 0.05$ compared with the respective γ_A/γ_A-fibrin value. [b] $p < 0.05$ compared with γ_A/γ_A-fibrin alone. [c] $p < 0.05$ compared with γ_A/γ'-fibrin in the presence of the γ'-peptide directed antibody.

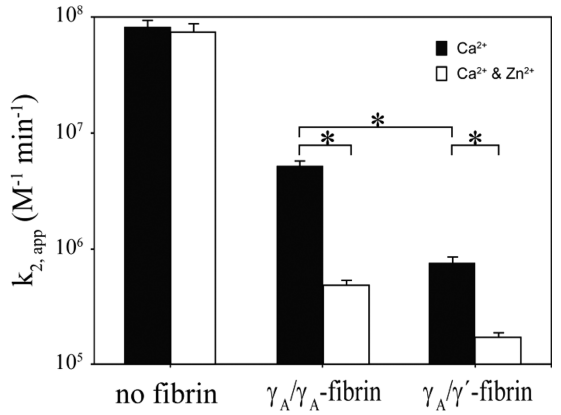

Figure 5. Protection of thrombin bound to γ_A/γ_A- or γ_A/γ'-fibrin clots from antithrombin inhibition. In a multiwell plate, 2 μM γ_A/γ_A- or γ_A/γ'-fibrinogen was clotted with 1 nM thrombin and 10 U/mL batroxobin in the presence of 2 mM $CaCl_2$, 0–2000 nM antithrombin, 60 nM heparin, and 50 μM tGPR-AMC. Under these conditions, clots formed within 45 s. Fluorescence was monitored continuously in a plate-reader, and after plotting corrected values against time, the data were analyzed by nonlinear regression to obtain the apparent second-order rate constant of inhibition ($k_{2,app}$). Samples without fibrinogen were used as controls. Studies were performed in the absence or presence of 12.5 μM $ZnCl_2$. The bars reflect the means ± SEM of six experiments. The asterisks denote significant ($p < 0.001$) differences for the indicated comparisons.

0.01) increase in the half-life to 10 ± 2.9 h. With γ_A/γ'-fibrin, the calculated dissociation half-life of thrombin was 368 ± 5.1 h, a value significantly ($p < 0.001$) longer than that from γ_A/γ_A-fibrin. In both the absence and presence of Zn^{2+}, heparin significantly ($p < 0.05$) reduced the half-life of thrombin dissociation from γ_A/γ'-fibrin clots to 266 ± 97 and 251 ± 15 h, respectively. That heparin accelerates the rate of ^{125}I-YPR-thrombin dissociation from γ_A/γ'-fibrin is consistent with the fact that heparin competes with the γ'-chain for thrombin exosite 2 binding.[14] When the interaction of thrombin with the γ'-chain was blocked with the antibody directed against the γ'-peptide, the dissociation half-life of ^{125}I-YPR-thrombin from γ_A/γ'-fibrin was 5.8 ± 1.9 h, a value similar to the dissociation of half-life from γ_A/γ_A-fibrin. There was a nonsignificant increase in the half-life to 6.5 ± 2.3 h in the presence of heparin and a significant ($p < 0.05$) increase to 20 ± 3.3 h in the presence of heparin plus Zn^{2+}. These half-lives are comparable with those obtained with γ_A/γ_A-fibrin.

Effect of Zn^{2+} on the Extent to Which Thrombin Bound to γ_A/γ_A- or γ_A/γ'-Fibrin Is Protected from Inhibition by Antithrombin–Heparin. In previous studies we showed that thrombin bound to fibrin is protected from inhibition by antithrombin in the presence of heparin because access of antithrombin-bound heparin to exosite 2 on thrombin is impaired when thrombin is bound to fibrin.[10] Thrombin bound to γ_A/γ'-fibrin is more protected than that bound to γ_A/γ_A-fibrin because thrombin binds γ_A/γ'-fibrin with higher affinity as a result of the interaction of exosite 2 with the γ'-chain.[14] Because Zn^{2+} promotes heparin-mediated thrombin binding to γ_A/γ_A-fibrin, but not γ_A/γ'-fibrin, we evaluated whether Zn^{2+} increases the extent to which fibrin-bound thrombin is protected from inhibition by antithrombin–heparin. In these experiments, γ_A/γ_A- or γ_A/γ'-fibrinogen was clotted with a combination of thrombin and batroxobin; the latter was added to ensure rapid and uniform clot formation even in the presence of antithrombin. A continuous assay was used to monitor the residual chromogenic activity of clot-associated thrombin, and the apparent second-order rate constant of inhibition ($k_{2,app}$) was determined under pseudo-first-order conditions. The heparin-catalyzed second-order rate constant for thrombin inhibition by antithrombin was 8.3 ± 3.0 × 10^7 M^{-1} min^{-1} (Figure 5)—a value comparable with that obtained by discontinuous assay in a previous study.[31] In the absence of fibrin, Zn^{2+} had no statistically significant effect on the k_2 value. However, in the presence of γ_A/γ_A-fibrin clots, the heparin-catalyzed rates of thrombin inhibition were significantly ($p < 0.001$) reduced by 16- and 172-fold in the absence or presence of Zn^{2+}, respectively [to (5.2 ± 0.6) × 10^6 and to (4.8 ± 0.6) × 10^5 M^{-1} min^{-1}, respectively]. The statistically significant ($p < 0.001$) 11-fold increase in protection in the presence of Zn^{2+} is consistent with the concept that Zn^{2+} promotes the formation of heparin–thrombin–fibrin complexes that protect thrombin from inhibition by antithrombin–heparin. With γ_A/γ'-fibrin clots, the rate of thrombin inhibition by antithrombin was significantly ($p < 0.001$) reduced by 111-fold to (7.4 ± 1.2) × 10^5 M^{-1} min^{-1} in the presence of heparin and by 482-fold to (1.7 ± 0.2) × 10^5 M^{-1} min^{-1} ($p < 0.001$) in the presence of both Zn^{2+} and heparin, demonstrating that Zn^{2+} enhances the protection that γ_A/γ'-fibrin clots afford thrombin by 4-fold. These results demonstrate that when Zn^{2+} is present, the protection observed in the presence of γ_A/γ_A-fibrin is comparable with the elevated protection afforded by γ_A/γ'-fibrin.

Effect of Zn^{2+} on Heparin-Induced Inhibition of Thrombin Generation. Having shown that the protection of thrombin bound to fibrin clots is enhanced in the presence of Zn^{2+} in a purified system, we used thrombin generation assays to determine whether Zn^{2+} has a similar effect in human plasma. We reasoned that if Zn^{2+} augments the heparin–fibrin interaction in a plasma system, it would attenuate the inhibitory effect of heparin on thrombin generation. Studies were performed in citrated plasma that was first dialyzed to remove citrate and was then reconstituted with 2 mM $CaCl_2$ and 0–80 μM $ZnCl_2$. In the absence of heparin, Zn^{2+} had no effect on endogenous thrombin potential or peak thrombin concentration (Figure 6). As expected, heparin produced a dose-dependent reduction in the indices of thrombin generation. Zn^{2+} attenuated the inhibitory effect of heparin in a concentration-dependent fashion, and the dose response with Zn^{2+} was magnified as the heparin concentration increased as evidenced by a highly significant value for the interaction between heparin and Zn^{2+} determined in the two-way ANOVA ($p < 0.0001$). These results suggest that by promoting the heparin–fibrin interaction, Zn^{2+} compromises the anticoagulant

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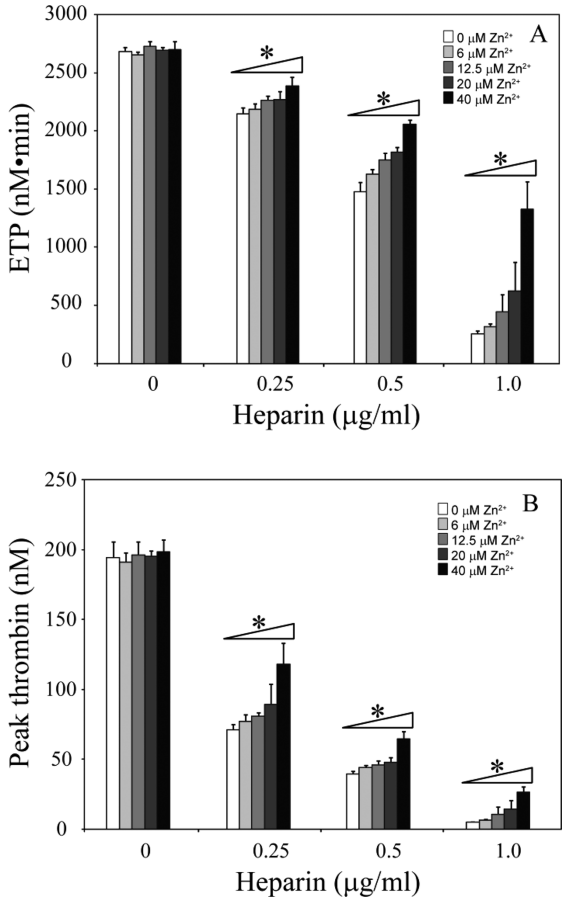

Figure 6. Effect of Zn^{2+} on thrombin generation in the absence or presence of heparin. Plasma samples containing 0−2 μg/mL heparin and 0−40 μM $ZnCl_2$ were placed in wells of a 96-well plate and warmed to 37 °C. After addition of thromboplastin and an equal volume of 1 mM Z-Gly-Gly-Arg-AMC and 2 mM $CaCl_2$, fluorescence was monitored at 1 min intervals at excitation and emission wavelengths of 360 and 460 nm, respectively. Endogenous thrombin potential (ETP), as determined by area under the curve (panel A), and peak thrombin concentration (panel B) were calculated using the instrument software. The bars represent the mean ± SEM of six determinations. Zn^{2+} had no effect on the indices of thrombin generation in the absence of heparin but, as indicated by the asterisks, in the presence of heparin, had a successively greater dose-dependent effect ($p < 0.0001$) as the heparin concentration increased.

activity of heparin. In support of this concept, Zn^{2+} had minimal effect on the anticoagulant activity of heparin when thrombin generation assays were performed in fibrinogen-depleted plasma (not shown).

DISCUSSION

Previous studies have shown that fibrin forms a ternary complex with heparin and thrombin and that this complex protects thrombin from inhibition by antithrombin.[10,11,32] It has also been shown that γ_A/γ'-fibrin forms a protective complex with thrombin, even in the absence of heparin.[14] Protection from inhibition occurs because engagement of exosite 2 with the carboxyl-terminus of γ_A/γ'-fibrin or with fibrin-bound heparin renders it inaccessible to antithrombin-bound heparin.[10,14,33] The current study extends these observations by demonstrating that Zn^{2+} promotes thrombin binding to γ_A/γ_A-fibrin in the presence of heparin, reduces the dissociation of thrombin from the ternary complex, and augments the extent to which fibrin-bound thrombin is protected from inhibition by antithrombin−heparin. In contrast, neither Zn^{2+} nor heparin enhances the high affinity binding of thrombin to γ_A/γ'-fibrin. Indeed, high concentrations of heparin may displace thrombin from γ_A/γ'-fibrin, possibly due to competition with exosite 2. However, at catalytic concentrations, heparin's ability to access exosite 2 is compromised because the exosite is already engaged with the γ'-chain. In support of this concept, we have previously shown that access of exosite 2-directed ligands to thrombin is impaired when the enzyme is bound to γ_A/γ'-fibrin.[8] The different effects of Zn^{2+} and heparin on these two complexes can be explained by the major structural difference between γ_A/γ_A- and γ_A/γ'-fibrin. Unlike γ_A/γ_A-fibrin, γ_A/γ'-fibrin possesses a γ'-chain that binds thrombin via exosite 2. When the interaction between the γ'-chain and thrombin exosite 2 is blocked with an antibody, γ_A/γ'-fibrin no longer retains thrombin with high affinity, and the dissociation half-life is reduced to a value similar to that with γ_A/γ_A-fibrin. In the presence of this antibody and heparin, Zn^{2+} enhances thrombin retention by γ_A/γ'-fibrin to an extent similar to that with γ_A/γ_A-fibrin. These observations confirm that γ'-chain binding to thrombin exosite 2 accounts for the different effects of Zn^{2+} on thrombin binding to these two forms of fibrin in the presence of heparin.

The effect of Zn^{2+} on ternary complex formation may involve any of the three binary heparin−fibrin, heparin−thrombin, or thrombin−fibrin interactions. Two lines of evidence indicate that Zn^{2+} does not affect the thrombin−γ_A/γ_A-fibrin interaction. First, Zn^{2+} has little effect on the K_d of thrombin for γ_A/γ_A-fibrin. Second, in the absence of heparin, Zn^{2+} has no effect on the rate of dissociation of ^{125}I-YPR-thrombin from γ_A/γ_A-fibrin clots. Although it has previously been reported that Zn^{2+} modulates thrombin adsorption to fibrin, the effect only occurred with Zn^{2+} concentrations above 50 μM.[34] In contrast, in our studies, we used a more physiological concentration of Zn^{2+} of 12.5 μM. It is unlikely that Zn^{2+} affects the thrombin−heparin binary interaction because Zn^{2+} does not influence the affinity of heparin for fluorescein−FPR-thrombin (data not shown). The remaining bivalent interaction between heparin and fibrin was shown to be augmented by Zn^{2+}. The affinity of heparin for fibrin is 5-fold higher in the presence of 12.5 μM Zn^{2+} than in its absence. Therefore, the binary heparin−fibrin interaction in the ternary complex is the interaction most likely responsible for the enhancing effect of Zn^{2+} on the formation of the ternary complex.

Both heparin and fibrin bind Zn^{2+}; therefore, the enhancing effect of Zn^{2+} on heparin binding to fibrin may be mediated by Zn^{2+} binding to heparin, fibrin, or both. Although heparin is highly negatively charged, previous studies have shown that the interaction between heparin and Zn^{2+} may reflect more than simply an electrostatic association.[35,36] Zn^{2+} binds heparin more readily than other negatively charged glycosaminoglycans,[37,38] and there is evidence that each disaccharide unit of heparin binds one Zn^{2+} ion.[39] However, Zn^{2+} had no effect on the interaction between heparin and thrombin, nor did it affect the rate of thrombin inhibition by antithrombin−heparin. Therefore, the enhanced binding of heparin to fibrin in the presence of Zn^{2+} is more likely to reflect Zn^{2+} binding to fibrin rather than to heparin. The Zn^{2+}-binding site on fibrin is distinct from that of calcium,[40,41] but its location is unknown. In support of the concept that Zn^{2+} binds to fibrin, Zn^{2+} has been shown to accelerate fibrin polymerization and to generate thicker fibrin fibers with larger pores and more branch points.[42−46] Efforts

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are underway to better characterize the role of Zn^{2+} on heparin binding to fibrin.

Heparin is widely used as an anticoagulant for prevention and treatment of venous and arterial thrombosis. Despite therapeutic levels of heparin, however, patients remain at risk for recurrent thrombotic events suggesting incomplete inactivation of thrombin by heparin.[47−53] In support of this concept, active thrombin has been recovered from thrombi harvested from patients who died of venous or arterial thrombosis, many of whom were treated with heparin.[54] Thrombin is protected from inhibition by antithrombin−heparin when it binds to fibrin, fibrin degradation products, or extracellular matrix.[11−13,55] Fibrin-bound thrombin can propagate thrombus growth by continuous activation of FV, FVIII, and FXI.[56] Bound thrombin also activates platelets,[57] which release Zn^{2+} from their α-granules,[58] thereby increasing the concentration of Zn^{2+} in the vicinity of the thrombus—a phenomenon that may compromise thrombin inhibition by enhancing the formation of heparin−thrombin−fibrin complexes. The potential for this response was demonstrated by the Zn^{2+}-mediated diminution of heparin anticoagulant activity in plasma in the thrombin generation assay. These results provide further evidence that the heparin−fibrinogen interaction compromises heparin activity.[59]

Thus, this study gives further support for the concept that Zn^{2+} is an important modulator of hemostasis. Recent reports have revealed Zn^{2+} binding to protein S, activated protein C, and FVIIa.[60−63] Moreover, Zn^{2+} promotes the binding of histidine-rich glycoprotein to FXIIa which attenuates contact-mediated activation of coagulation.[64] The potential for modulation of Zn^{2+} levels by localized platelet activation provides a mechanism by which Zn^{2+}-dependent interactions can be regulated. These observations highlight the need for investigation into the potential roles of Zn^{2+} and other metal ions in hemostatic reactions, which may have been overlooked because of the almost universal use of citrate as an anticoagulant.

In conclusion, our study shows that Zn^{2+} enhances the formation of heparin−thrombin−γ_A/γ_A-fibrin complexes, likely reflecting the modulating effect of Zn^{2+} on heparin binding to fibrin. In contrast, because γ_A/γ'-fibrin provides an additional thrombin binding site on its γ'-chain, heparin and Zn^{2+} do not augment thrombin binding to γ_A/γ'-fibrin. Because platelets release Zn^{2+} upon activation,[25] the extent of protection of fibrin-bound thrombin from inhibition by antithrombin−heparin is likely greater than previously reported. As an important trigger of thrombus growth, the resistance of fibrin-bound thrombin to inhibition helps to explain, at least in part, the limitations of heparin in patients with thrombosis.

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Notes

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ABBREVIATIONS

ETP, endogenous thrombin potential; F, coagulation factor; TBS, Tris buffered saline; Tw, Tween 20; FPR, D-Phe-Pro-Arg-chloromethyl-ketone; YPR, D-Tyr-Pro-Arg-chloromethyl ketone.

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