Molecular Basis for the Susceptibility of Fibrin-bound Thrombin to Inactivation by Heparin Cofactor II in the Presence of Dermatan Sulfate but Not Heparin*

Received for publication, November 22, 2000, and in revised form, March 9, 2001
Published, JBC Papers in Press, April 9, 2001, DOI 10.1074/jbc.M010584200

Patricia C. Y. Liaw‡, Debra L. Becker, Alan R. Stafford, James C. Fredenburgh, and Jeffrey I. Weitz§

From McMaster University and the Henderson Research Centre, Hamilton, Ontario L8V 1C3, Canada

Although fibrin-bound thrombin is resistant to inactivation by heparin-antithrombin and heparin-heparin cofactor II complexes, indirect studies in plasma systems suggest that the dermatan sulfate-heparin cofactor II complex can inhibit fibrin-bound thrombin. Herein we demonstrate that fibrin monomer produces a 240-fold decrease in the heparin-catalyzed rate of thrombin inhibition by heparin cofactor II but reduces the dermatan sulfate-catalyzed rate only 3-fold. The protection of fibrin-bound thrombin from inhibition by heparin-heparin cofactor II reflects heparin-mediated bridging of thrombin to fibrin that results in the formation of a ternary heparin-thrombin-fibrin complex. This complex, formed as a result of three binary interactions (thrombin-fibrin, thrombin-heparin, and heparin-fibrin), limits accessibility of heparin-catalyzed inhibitors to thrombin and induces conformational changes at the active site of the enzyme. In contrast, dermatan sulfate binds to thrombin but does not bind to fibrin. Although a ternary dermatan sulfate-thrombin-fibrin complex forms, without dermatan sulfate-mediated binding of thrombin to fibrin, only two binary interactions exist (thrombin-fibrin and thrombin-dermatan sulfate). Consequently, thrombin remains susceptible to inactivation by heparin cofactor II. This study explains why fibrin-bound thrombin is susceptible to inactivation by heparin cofactor II in the presence of dermatan sulfate but not heparin.

Heparin, a sulfated polysaccharide, acts as an anticoagulant by accelerating the inhibition of thrombin and factor Xa by antithrombin (1). Although heparin is widely used for the treatment of acute coronary ischemic syndromes, it has limitations in patients undergoing percutaneous coronary interventions (2) or when used as an adjunct to thrombolytic therapy (3–5). These limitations have been attributed to the inability of the antithrombin-heparin complex to inactivate clotting enzymes bound to components of the thrombus, particularly thrombin bound to fibrin (6, 7).

Resistance of fibrin-bound thrombin to inactivation by the antithrombin-heparin complex reflects the incorporation of thrombin into a ternary heparin-thrombin-fibrin complex (8–10). To form this complex, heparin interacts with both exosite II on thrombin (11–13) and the D domain of fibrin (14), thereby bridging thrombin to fibrin via exosite II (8). This heightens exosite I-mediated binding of thrombin to fibrin and likely increases the overall affinity of thrombin for fibrin. The formation of the ternary complex, therefore, is a consequence of the presence of two exosites on thrombin, which independently bind fibrin and heparin. Recently, we demonstrated that protection requires ligation of both of thrombin’s exosites within the ternary heparin-thrombin-fibrin complex, a process that impairs access of inhibitor-bound heparin to exosite II on thrombin (15). Thrombin within the ternary complex is protected from inactivation by the heparin-heparin cofactor II (HCII) complex to a greater extent than the heparin-antithrombin complex (15), because access of the amino terminus of HCII to exosite I on thrombin, an obligatory part of the HCII inhibitory mechanism (16, 17), is reduced. Allosteric changes in the active site of thrombin induced upon formation of the ternary heparin-thrombin-fibrin complex may also contribute to the protection of fibrin-bound thrombin from inactivation by heparin-antithrombin and heparin-HCII complexes by limiting inhibitor reactivity with fibrin-bound thrombin (18).

Dermatan sulfate (DS), a sulfated glycosaminoglycan that has antithrombotic activity in laboratory animals (19, 20) and in humans (21–23), acts as an anticoagulant by catalyzing only HCII. Because thrombin is the exclusive plasma target of HCII, DS is considered a selective inhibitor of thrombin (16). Although fibrin-bound thrombin is protected from inactivation by the heparin-HCII complex (15), indirect studies done in plasma systems suggest that fibrin-bound thrombin is susceptible to inactivation by the DS-HCII complex (24, 25). The purpose of this study was to confirm these findings using purified reagents and to elucidate the mechanism by which the DS-HCII complex, but not the heparin-HCII complex, inactivates fibrin-bound thrombin.

* This work was supported in part by grants-in-aid from the Medical Research Council of Canada (MT-3992), the Heart and Stroke Foundation of Ontario (T-2268 and MT-3864), and the Ontario Research and Development Challenge Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Research Fellowship from the Heart and Stroke Foundation of Canada.

§ Recipient of a Career Investigator Award from the Heart and Stroke Foundation of Canada and holder of the Heart and Stroke Foundation of Ontario/J. Fraser Mustard Chair in Cardiovascular Research and the Canada Research Chair in Thrombosis at McMaster University. To whom correspondence should be addressed: Henderson Research Centre, 711 Concession St., Hamilton, Ontario L8V 1C3, Canada. Tel.: 905-574-8550; Fax: 905-575-2646; E-mail: jweitz@thrombosis.hhscr.org.

1 The abbreviations used are: HCII, heparin cofactor II; ANS, anilinonaphthalene-6-sulfonic acid; DS, dermatan sulfate; f, fluorescein-labeled; SF, soluble fibrin; FM, fibrin monomer; FTIC, fluorescein-5’-isothiocyanate; PFR, o-Phe-Pro-Arg; PPRCK, o-Phe-Pro-Arg-chloromethyl ketone; GPRP-NH2, Gly-Pro-Arg-Pro-amide; tGPR-pNA, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide acetate; ATA, N-(acetylamido)acetyl; TBS, Tris-buffered saline; PEG, polyethylene glycol.
EXPERIMENTAL PROCEDURES

Materials

Human HClII, isolated from plasma by affinity chromatography, was from Affinity Biologicals Inc. (Hamilton, Ontario, Canada). Human α- and γ-thrombin and fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Dermatan sulfate (DS) was from Mediolanum Farmaceutici (Milan, Italy). Enoxaparin, a commercial low-molecular-weight heparin, was from Rhône-Poulenc Rorer Canada (Montreal). Based on high performance liquid chromatography gel filtration analysis, the mean molecular masses of DS, heparin, and enoxaparin were 20 kDa (range 7–34 kDa), 15 kDa (range 5–30 kDa), and 4.5 kDa (range 3.5–5.5 kDa), respectively. Fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Eugene, OR). D-Pro-Arg-chloromethyl ketone (FPRCK) was from Calbiochem Novabiochem Corp. (San Diego, CA). FITC-FPRCK was from Hematologic Technologies, Inc. (Essex Junction, VT). Hexadimethrine bromide (Polybrene) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Cynogen bromide-activated Sepharose 4B was from Amersham Pharmacia Biotech (Dorval, Quebec).

Preparation of Soluble Fibrin—Human fibrinogen, treated with gelatin-agarose to remove fibronectin, was used to prepare soluble fibrin (SF), as described previously (15). Briefly, fibrin clots were centrifuged and dialyzed versus water. The fibrin was then dissolved by dialysis versus 20 mM acetic acid, aliquoted, and stored at a concentration of about 100 μM at −70 °C. Polymerization of SF was blocked by addition of 5 mM GPRP-NH₂ (26), and the material was neutralized with 1 M Tris-HCl, pH 7.5. The volume corresponding to 40% of the volume of SF, just prior to use. A molecular weight of 340,000 and ε280,280 value of 14.0 were used to calculate the soluble fibrin concentration.

Preparation of Fibrin Monomer-Sepharose—Fibrinogen was coupled to cynogen bromide-activated Sepharose 4B, treated with thrombin to convert it to fibrin monomer (FM), and washed with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) as described previously (15).

Methods

Rates of Thrombin Inhibition by HCII in the Absence or Presence of SF, Glycosaminoglycans, or Both—The influence of varying concentrations of heparin or DS on the second-order rate constants (k₂) for inhibition of thrombin by HCII were determined under pseudo-first-order conditions in the absence or presence of SF. Thrombin (10 nM) was incubated for 5 min at room temperature in TBS containing 0.6% PEG 8000 and various concentrations of heparin or DS (0–11 μM), SF (0–4 mg/ml), and 10 mM GPRP-NH₂. Reaction mixtures (10 μl) were aliquoted to 96-well round bottom microtiter plates and an equal volume of 140 nM ANS-FPR-thrombin in 50 mM HEPES, pH 7.5, 10 mM EDTA, 1 mM MgCl₂, 1 mg/ml PEG 8000, 5 mM GPRP-NH₂ was added to a semi-micro quartz cuvette. Using a PerkinElmer Life Sciences LS50B luminescence spectrometer with excitation wavelength set to 334 nm and excitation and emission slit widths set to 5 nm, the fluorescence emission spectrum from 380 to 580 nm of ANS-FPR-thrombin was monitored before and after the addition of 310 nM SF and/or 100 nM heparin or 3 μM DS. Soluble fibrin was neutralized by the addition of 40% v/v of Tris-HCl, pH 7.5, just prior to use. Addition of 1x Tris-HCI, pH 7.5, had no effect on the fluorescence spectrum of ANS-FPR-thrombin (data not shown).

Determination of the Affinities of f-heparin, f-DS, and f-FPR-thrombin for Fibrin Clots—fluorescent derivatives of heparin and DS were prepared as follows (29). 10 mg of heparin or DS were incubated with 15 mg of FITC in 2.5 ml of 1.0 M Na₂CO₃ pH 9.0 for 2 h at 23 °C. After centrifugation at 13,000 × g for 5 min, 1 ml of the supernatant was applied to duplicate 10-μl gel filtration columns (Millipore Corp., Bedford, MA), equilibrated with H₂O, and eluted with H₂O under gravity. 0.5-ml fractions were collected, frozen, and lyophilized. Recovered material was pooled, weighed, and dissolved in TBS to a concentration of 10 mg/ml. FITC-labeled active site-blocked thrombin (f-FPR-thrombin) was prepared as previously described (28). Briefly, thrombin was incubated with fluorescein-n-Phe-Pro-Arg-chloromethyl ketone (f-FPRCK) until no residual thrombin chromogenic activity was detected. After dialysis to remove unincorporated f-FPRCK, the concentration of fluorescently labeled thrombin was determined by measuring absorbance at 390 nm of the fluorescence spectrum of ANS-FPR-thrombin (data not shown).

I₂ = 1 + [K₂ + L]P × ΔL 2

where ΔL is the maximum fluorescence change, P is the initial concentration of f-heparin, f-DS, or f-FPR-thrombin, L is the total fibrinogen concentration, and a stoichiometry of 1 is assumed (28).
changes in fluorescence were monitored (∆I). $K_d$ values were calculated by plotting $II_o$ versus glycosaminoglycan concentration and the data were fit by nonlinear regression to the equation given above.

**Determination of the Affinities of HCII for Heparin, DS, and SF**—The association between HCII and either heparin, DS, or fibrin was monitored by the ligand-dependent fluorescence intensity change of anilinonaphthalene-6-sulfonic acid labeled HCII (ANS-HCII). ANS-HCII, prepared as previously described (30), was added to a concentration of 100 nM to a quartz cuvette. The initial fluorescence intensity ($I_o$) of ANS-HCII was determined at excitation and emission wavelengths set to 280 and 437 nm, respectively, and excitation and emission slit widths set to 10 nm, and an emission filter of 290 nm. Known quantities of either heparin, DS, or SF were then added to the cuvette and, after mixing, changes in fluorescence was monitored (∆I). $K_d$ values were calculated by plotting $II_o$ versus ligand concentration, and the data were fit by nonlinear regression to the equation given above.

**Displacement of Thrombin from FM-Sepharose by HCII—**500-µl suspensions of FM-Sepharose (9 µM FM) containing 50 nM $^{125}$I-FPR-thrombin without glycosaminoglycan or with 2.5 µM DS in TBS0.6% PEG/0.01% Tween 20 were mixed for 2 min. The amount of unbound $^{125}$I-FPR-thrombin in the suspension after each addition of an aliquot of 80 µM HCII was determined as described above.

**RESULTS**

**Comparison of the Effect of SF on Heparin- and DS-catalyzed Rates of Thrombin Inhibition by HCII**—To verify the protective effect of SF on thrombin inhibition by HCII, we determined the rates of thrombin inhibition in the absence or presence of SF and heparin. As shown in Fig. 1A, SF caused a dose-dependent decrease in the heparin-catalyzed rates of thrombin inhibition by HCII, which by two-way analysis of variance performed using Minitab (software version 11, State College, PA), was highly significant ($p < 0.001$). At 1 µM heparin and 4 µM SF, a maximal 240-fold decrease in the rate was observed, a value consistent with that reported previously (15). In contrast, at a concentration of 4 µM SF, a significant ($p < 0.001$) but modest 3-fold decrease in the DS-catalyzed rates of thrombin inhibition by HCII was observed (Fig. 1B).

**Quantification of Binary Interactions That Comprise Ternary Thrombin-Fibrin-Glycosaminoglycan Complexes**—The assembly of the ternary heparin-thrombin-fibrin complex is postulated to occur through a series of binary interactions between thrombin-fibrin, thrombin-heparin, and fibrin-heparin (8, 10, 18). In this study, we determined the dissociation constants for these interactions, as well as those involving DS. The affinities of DS and heparin for thrombin were determined by monitoring changes in intrinsic protein fluorescence of thrombin when titrated with DS or heparin (not shown). DS and heparin bind saturably to thrombin with $K_d$ values of 2600 and 116 nM, respectively (Table I). The $K_d$ value for the thrombin-heparin interaction is in agreement with that determined by titration of ANS-labeled thrombin with increasing concentrations of heparin ($K_d = 59$ nM) (10).

Current evidence suggests that both heparin and DS bind to exosite II on thrombin (11–13, 31). This was confirmed in two ways. First, competitive binding studies were performed where thrombin-bound f-thrombin was displaced by DS (Fig. 2). In this experiment, addition of thrombin to f-thrombin resulted in an approximately 3.5% decrease in fluorescence intensity. Subsequent titration of the sample with DS resulted in a dose-dependent increase of fluorescence intensity to the value observed for f-thrombin prior to binding to thrombin ($II_o$ of 1). Likewise, in the reciprocal experiment, the fluorescence decrease that occurred upon addition of thrombin to f-DS was negated in a dose-dependent fashion by titration with heparin (not shown). In a second approach, we used thrombin variants with impaired exosites to identify the DS binding site (not shown). DS binds to γ-thrombin, a proteolytic derivative of α-thrombin lacking exosite I (11), with a 2.6-fold lower affinity than α-thrombin ($K_d$ values of 6.8 µM and 2.6 µM, respectively).

In contrast, DS binds RA-thrombin, a thrombin variant with three point mutations in exosite II that lower its affinity for heparin 20-fold (32), with a 7-fold lower affinity ($K_d = 17$ µM). These studies confirm that, like heparin, DS also binds to exosite II on thrombin, albeit with lower affinity (Table I).

The affinities of f-FPR-thrombin, f-heparin, and f-DS for fibrin were monitored by clotting varying concentrations of fibrinogen with a catalytic amount of thrombin in the presence of a fluorescently labeled ligand, and quantifying unbound ligand in the clot supernatant (not shown). As listed in Table I, f-FPR-thrombin and f-heparin bind to fibrin with $K_d$ values of...
1500 and 187 nm, respectively, values consistent with previous reports (8, 10). In contrast, f-DS does not bind to fibrin.

Both heparin and DS bind to ANS-HCII, but the affinity of heparin for HCII is 5-fold higher (13 and 71 μM, respectively). Thus, DS binds to both HCII and thrombin with lower affinity than heparin. No binding of ANS-HCII to fibrin was detected (Table I).

**Effect of Heparin and DS on the Binding of **\( ^{125} \text{I}-\text{FPR-thrombin} \) to Fibrin**—It has been shown previously that heparin enhances the binding of thrombin to fibrin, an effect that occurs regardless of whether heparin has high or low affinity for antithrombin, but occurring only with heparin chains of 11,200 Da or more (8). In this study, we compared the ability of DS to promote thrombin binding to FM-Sepharose with that of heparin and low-molecular-weight heparin. As shown in Fig. 3, DS has little effect on \( ^{125} \text{I}-\text{FPR-thrombin} \) binding to FM-Sepharose, even at concentrations up to 10 μM. In contrast, at concentrations up to 250 nM, heparin enhances \( ^{125} \text{I}-\text{FPR-thrombin} \) binding to FM-Sepharose in a dose-dependent manner. At heparin concentrations above 250 nM, \( ^{125} \text{I}-\text{FPR-thrombin} \) binding to fibrin monomer decreases, likely reflecting the accumulation of distinct heparin/h fibrin and heparin/thrombin populations (8). When thrombin and FM-Sepharose were titrated with low-molecular-weight heparin (enoxaparin), there was only a small increase in the amount of thrombin bound, comparable to that observed with DS. Similar results were obtained with fibrin clots in place of FM-Sepharose (not shown).

**Incorporation of Thrombin into Ternary Glycosaminoglycan-Thrombin-Fibrin Complexes**—Our binding studies indicate that thrombin can interact with both fibrin and DS, via exosites I and II, respectively. These findings suggest that, like heparin, DS can form a three-component complex with thrombin and fibrin, even though DS does not bind fibrin. To explore this possibility, a mixture of 500 nM \( ^{125} \text{I}-\text{DS} \) and 5 μM FM-Sepharose was titrated with increasing concentrations of thrombin or γ-thrombin (Fig. 4). After centrifugation, residual \( ^{125} \text{I}-\text{DS} \) in the supernatant was quantified and used to determine the fraction of \( ^{125} \text{I}-\text{DS} \) bound to FM-Sepharose. In the absence of thrombin, minimal amounts of \( ^{125} \text{I}-\text{DS} \) bound to FM-Sepharose. With thrombin addition, \( ^{125} \text{I}-\text{DS} \) binds to FM-Sepharose in a concentration-dependent fashion. In contrast, when γ-thrombin, a proteolytic derivative of thrombin that lacks exosite 1, is substituted for thrombin, there is no increase in the amount of \( ^{125} \text{I}-\text{DS} \) that binds to FM-Sepharose. These data indicate that a DS-thrombin-fibrin complex can form despite the lack of direct DS-fibrin interactions.

**Influence of Ternary Thrombin-Fibrin-Glycosaminoglycan Complex Formation on the Conformation of the Active Site of Thrombin**—Incorporation of thrombin into a ternary heparin-thrombin-fibrin complex alters the rate of thrombin-mediated hydrolysis of chromogenic substrates (9, 10, 15), suggesting that ternary complex formation induces conformational changes in the active site of thrombin that may limit its reactivity with macromolecular inhibitors (18). To determine whether thrombin in a DS-thrombin-fibrin complex also experiences conformational changes in its active site, the fluorescence of ANS-FPR-thrombin was monitored upon addition of
to the results obtained with heparin, the addition of 3
binary thrombin
active site of thrombin beyond those produced by formation of
thrombin
L
combination of SF and heparin (•), or the
presence of SF (○), DS (□), or the combination of SF and DS (△). For clarity, only every 15th point of each spectrum is plotted.

DS and/or SF, as was described previously for heparin or SF (10). As illustrated in Fig. 5A, addition of 310 nM SF or 100 nM heparin to 140 nM ANS-FPR-thrombin produces a 19 and 48% increase in fluorescence, respectively, compared with the fluorescence of ANS-thrombin alone. When 100 nM heparin is added to the ANS-FPR-thrombin/SF mixture, however, there is an 86% increase in fluorescence. The observation that the addition of both SF and heparin produces a greater change in the emission spectrum of ANS-FPR-thrombin than the two components alone is in agreement with a previous report by Hogg et al. (10) and suggests that formation of the ternary heparin-thrombin-fibrin complex induces conformational changes in the active site of thrombin beyond those produced by formation of binary thrombin-fibrin or thrombin-heparin complexes. In contrast to the results obtained with heparin, the addition of 3 μM DS, a concentration above the Kd value for the DS-thrombin interaction (Table I), to either ANS-FPR-thrombin alone or to the ANS-FPR-thrombin/SF mixture has minimal effect on the emission spectra (Fig. 5B). These findings suggest that, unlike heparin, DS does not induce conformational changes in the active site environment of thrombin in the absence or presence of fibrin.

Effect of Heparin and Low-molecular-weight Heparin on the Rate of Inhibition of Fibrin-bound Thrombin by the DS/HCII Complex—Although fibrin-bound thrombin is susceptible to inactivation by the DS/HCII complex (Fig. 1), we predicted that heparin, by virtue of its higher affinity for both fibrin and thrombin than DS, would impair DS activity by bridging thrombin to fibrin, thereby enhancing the fibrin-thrombin interaction. As a control, we used low-molecular-weight heparin, because only heparin chains of >11.2 kDa are of sufficient length to bridge thrombin to fibrin (8). In the presence of 4 μM SF, heparin concentrations of 0.1 μM or higher cause a decrease in the 3.3 μM DS-catalyzed rate of thrombin inhibition by HCII (Fig. 6). In contrast, a low-molecular-weight heparin, enoxaparin, has no effect on the rate of inhibition of fibrin-bound thrombin by the DS/HCII complex. Neither heparin nor low-molecular-weight heparin influenced the DS-catalyzed rate of thrombin inhibition in the absence of SF (data not shown).

Displacement of Ha from FM-Sepharose by HCII—Because catalysis of thrombin inhibition by HCII is not impaired by fibrin in the presence of DS, exosite I on thrombin must be accessible to the DS/HCII complex even though thrombin binds to fibrin via this site. This observation predicts that the DS/HCII complex is capable of displacing thrombin from fibrin by binding the amino-terminal tail of HCII to exosite I of thrombin. This was tested by monitoring the amount of 125I-FPR-thrombin displaced from FM-Sepharose by increasing concentrations of HCII in the absence or presence of DS (Fig. 7). HCII alone, at concentrations up to three times physiological concentration, had limited capacity to displace thrombin from FM-Sepharose. However, in the presence of 2.5 μM DS, dose-dependent displacement of thrombin by HCII was observed. Maximal displacement was achieved with physiological concentrations of HCII, with half-maximal effect at about 250 nM HCII. Thus, the amino terminus of HCII is able to compete effectively with fibrin for binding to thrombin exosite I.
**DISCUSSION**

In this study, we demonstrate that fibrin-bound thrombin is readily inhibited by the DS-HCII complex, but not by the heparin-HCII complex, even though heparin and DS demonstrate comparable catalysis of HCII in the absence of fibrin (Fig. 1). The resistance of fibrin-bound thrombin to inhibition by the heparin-HCII complex is a consequence of formation of a ternary heparin-thrombin-fibrin complex (15). This ternary complex forms as a result of mutual binary interactions between each of the three components, resulting in increased affinity of each of the individual interactions (10, 18). Formation of this ternary complex protects thrombin from inhibition by heparin-HCII by reducing access of the amino terminus of HCII to exosite I on thrombin, impairing the ability of HCII-bound heparin to bridge HCII to exosite II on thrombin, and inducing allosteric changes in the active site of thrombin that may limit its reactivity with HCII (10, 15).

Like heparin, DS binds to exosite II on thrombin, even when thrombin is fibrin-bound (Fig. 2). However, DS and heparin are distinguished by the fact that only heparin binds fibrin (Table 1). With high affinity for thrombin and fibrin, heparin augments exosite I-mediated binding of thrombin to fibrin, impairing the ability of HCII to bind and inhibit thrombin (Fig. 3). Because DS does not heighten the thrombin-fibrin interaction, the amino terminus of DS-activated HCII retains access to exosite I on thrombin. The failure of DS to induce a conformational change in the active site of thrombin (Fig. 5R) may also permit reactivity of HCII with fibrin-bound thrombin. These results confirm the hypothesis that all three binary interactions (thrombin-fibrin, thrombin-glycosaminoglycan, glycosaminoglycan-fibrin) within the ternary complex are integral to convey protection of fibrin-bound thrombin from inhibition by heparin-catalyzed inhibitors (15).

Heparin compromises the DS-catalyzed rate of thrombin inhibition by HCII in the presence of fibrin (Fig. 6). This reduction in rate is consistent with the formation of a ternary thrombin-fibrin-heparin complex. Heparin can bridge thrombin to fibrin in the presence of DS, because the affinity of heparin for thrombin is higher than that of DS (Table 1). In contrast, low-molecular-weight heparin (enoxaparin) does not reduce the DS-catalyzed rate of thrombin inhibition by HCII, because only heparin chains greater than 11.2 kDa are of sufficient length to bind simultaneously to thrombin and fibrin (8).

The DS-HCII complex displaces thrombin from FM-Sepharose with an $IC_{50}$ value of 0.17 $\mu$m (Fig. 7). This value reflects the ability of the amino terminus of DS-activated HCII to bind to thrombin exosite I. A previous study determined that a 22-residue peptide, corresponding to amino acids 54–75 of HCII, inhibited the clotting activity of thrombin with an $IC_{50}$ of 28 $\mu$m (33). The lower $IC_{50}$ value with intact HCII suggests that there are additional interactions with thrombin provided by the remainder of the amino terminus or the body of the HCII molecule. A homologous peptide derived from hirudin residues 54–66 inhibited thrombin clotting activity with an $IC_{50}$ of 0.8 $\mu$m (33). This compares with the $K_i$ value of 0.6 $\mu$m derived for the initial encounter of intact hirudin with thrombin (34). Thus, in addition to its ability to inhibit fibrin-bound thrombin, the DS-HCII complex displaces thrombin from fibrin allowing both HCII and antithrombin to inhibit thrombin. A similar displacement role for the hirudin peptide has been proposed (35).

Our observation that fibrin-bound thrombin is susceptible to inactivation by the DS-HCII complex suggests that DS should be effective for the prevention and treatment of thrombosis. In a randomized trial comparing subcutaneous DS with low dose subcutaneous heparin for thromboprophylaxis in patients undergoing general surgical procedures, both agents had comparable efficacy (22). More recently, DS was shown to be more effective than heparin at preventing postoperative venous thrombosis in patients undergoing general surgical procedures (36). Studies in rabbits suggest that DS suppresses thrombus accretion to a greater extent than heparin when the two agents are given in doses that have the same inhibitory activity against thrombin in vitro (25).

DS produces only minimal inhibition of platelet and fibrin deposition in a high shear, arterial-type thrombus model in baboons (37). The limited efficacy of DS in this setting may reflect the fact that DS only inhibits thrombin and has no effect on factor Xa-mediated thrombin generation, an important contributor to thrombus growth (38). Consequently, if selective thrombin inhibitors are used as monotherapy for treatment of arterial thrombosis, high concentrations may be necessary to prevent thrombus propagation. This concept is supported by our recent studies demonstrating that both DS and hirudin, agents that inhibit fibrin-bound thrombin, as well as free thrombin, are limited in their ability to block clotting in a thrombogenic extracorporeal circuit (30). In contrast, Vasoflux, a drug that not only inhibits fibrin-bound thrombin but also blocks factor Xa generation, prevents clotting in the circuit (30). These findings raise the possibility that adding heparin to DS may improve its efficacy, because heparin will block factor Xa-mediated thrombin generation. Our observation that unfractionated heparin compromises the rate of thrombin inhibition by the DS-HCII complex, whereas low-molecular-weight heparin does not (Fig. 6), suggests that low-molecular-weight heparin may be a better adjunct to DS than unfractionated heparin. The observation that DS and low-molecular-weight heparin display additive anticoagulant activity in vitro provides further support for this approach (39).

In summary, our results indicate that fibrin-bound thrombin is readily inhibited by the DS-HCII complex because, unlike heparin, DS does not bridge thrombin to fibrin. Although DS and heparin can form thrombin-fibrin-glycosaminoglycan complexes, the additional heparin-fibrin interaction strengthens the ternary complex, thereby limiting accessibility of thrombin exosites and inducing conformational changes in the active site of thrombin. These findings reveal that resistance of thrombin to inhibition by serpins results from a glycosaminoglycan-fibrin interaction, as well as interactions between thrombin-fibrin and thrombin-glycosaminoglycan. These mechanistic insights also demonstrate the advantages of glycosaminoglycans whose activities are not compromised by fibrin.

**REFERENCES**

1. Rosenberg, R. D. (1987) in *Haemostasis and Thrombosis: Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 1377–1382. J. B. Lippincott, Philadelphia
2. Popma, J. J., Ohman, E. M., Weitz, J., Laneaf, A. M., Harrington, R. A., and Berger, P. (2001) *Chest* 119, 3215–3265
3. The International Study Group (1996) *Lancet* 336, 71–75
4. ISIS-3 Collaborative Group (1992) *Lancet* 339, 750–770
5. Collins, R., MacMahon, S., Flahme, M., Bajent, C., Renivg, L., Mortensen, S., Appleby, P., Godwin, J., Yusuf, S., and Peto, R. (1996) *Br. Med. J.* 313, 652–659
6. Hogg, P. J., and Jackson, C. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 3619–3623
7. Weitz, J. I., Hudoba, M., Massel, D., Maraganoire, J., and Hirsh, J. (1990) *J. Clin. Invest.* 86, 385–391
8. Hogg, P. J., and Jackson, C. M. (1990) *J. Biol. Chem.* 265, 241–247
9. Hogg, P. J., and Jackson, C. M. (1990) *J. Biol. Chem.* 265, 248–255
10. Hogg, P. J., Jackson, C. M., Labanowski, J. K., and Bock, P. E. (1996) *J. Biol. Chem.* 271, 26083–26095
11. Church, F. C., Pratt, C. W., Noyes, C. M., Kalayanaritit, T., Sherrill, G. B., Tobin, R. B., and Meade, J. B. (1989) *J. Biol. Chem.* 264, 18419–18425
12. Gan, Z. R., Li, Y., Chen, Z., Lewis, S. D., and Shader, J. A. (1994) *J. Biol. Chem.* 269, 13031–13035
13. Sheehan, J. P., and Sadler, J. E. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 5518–5522
14. Oldrin, T., Shainoff, J. R., Lawrence, S. O., and Simpson-Haidaris, P. J. (1996) *Blood* 88, 2050–2061
15. Becker, D. L., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) *J. Biol. Chem.* 274, 6226–6233
16. Tollefsen, D. M. (1995) *Thromb. Haemost.* 74, 1209–1214
17. Liaw, P. C., Austin, R. C., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) *J. Biol. Chem.* 274, 27397–27404
18. Hegg, P. J., and Bock, P. E. (1997) *Thromb. Haemost.* 77, 424–433
19. Merton, R. E., and Thomas, D. P. (1987) *Thromb. Haemost.* 58, 839–842
20. Van Ryn-McKenna, J., Ofosu, F. A., Gray, E., Hirsh, J., and Buchanan, M. R. (1989) *Ann. N. Y. Acad. Sci.* 556, 394–312
21. Lane, D. A., Ryan, K., Ireland, H., Curtis, J. R., Nurmohamed, M. T., Krediet, R. T., Roggekamp, M. C., Stevens, P., and ten Cate, J. W. (1992) *Lancet* 339, 334–335
22. Prandoni, P., Meduri, F., Cuppini, S., Teniato, A., Zangrandi, F., Polistena, P., Gianese, F., and Maffei Faccioli A. (1992) *Br. J. Surg.* 79, 505–509
23. Nagasawa, K., and Uchiyama, H. (1978) *Biochim. Biophys. Acta* 544, 430–440
24. Ye, J., Rezaie, A. R., and Esmon, C. T. (1994) *J. Biol. Chem.* 269, 17965–17970
25. Okwusidi, J. I., Anvari, N., Kulczycky, M., Blajchman, M. A., Buchanan, M. R., and Ofosu, F. A. (1991) *J. Lab. Clin. Med.* 117, 359–364
26. Kawasaki, K., Hirase, K., Miyano, M., Tsuji, T., and Iwamoto, M. (1992) *Chem. Pharm. Bull. (Tokyo)* 40, 3253–3260
27. Olson, S. T., Björk, I., and Shore, J. D. (1993) *Methods Enzymol.* 222, 525–560
28. Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1997) *J. Biol. Chem.* 272, 25495–25499
29. Nagasawa, K., and Uchiyama, H. (1978) *Biochim. Biophys. Acta* 544, 430–440
30. Weitz, J. I., Young, E., Johnston, M., Stafford, A. R., Fredenburgh, J. C., and Hirsh, J. (1999) *Circulation* 99, 682–689
31. Sheehan, J. P., Tollefsen, D. M., and Sadler, J. E. (1994) *J. Biol. Chem.* 269, 32747–32751
32. Ye, J., Rezaie, A. R., and Esmon, C. T. (1994) *J. Biol. Chem.* 269, 17965–17970
33. Hortin, G. L., Tollefsen, D. M., and Benuto, B. M. (1989) *J. Biol. Chem.* 264, 13979–13982
34. Jackman, M. P., Parry, M. A. A., Hofsteenge, J., and Stone, S. R. (1992) *J. Biol. Chem.* 267, 15375–15383
35. Naski, M. C., Fenton, J. W. I., Maraganore, J. M., Olson, S. T., and Shafer, J. A. (1990) *J. Biol. Chem.* 265, 13484–13489
36. Di Carlo, V., Agnelli, G., Prandoni, P., Coccheri, S., Gensini, G. F., Gianese, F., and Mannucci, P. M. (1999) *Thromb. Haemost.* 82, 30–34
37. Cadroy, Y., Hanson, S. R., and Harker, L. A. (1993) *Arterioscler. Thromb.* 13, 1213–1217
38. Eisenberg, P. R., Siegel, J. E., Abendschein, D. R., and Miletich, J. P. (1993) *J. Clin. Invest.* 91, 1877–1883
39. Cosmi, B., Agnelli, G., Young, E., Hirsh, J., and Weitz, J. (1993) *Thromb. Haemost.* 70, 443–447
Molecular Basis for the Susceptibility of Fibrin-bound Thrombin to Inactivation by Heparin Cofactor II in the Presence of Dermatan Sulfate but Not Heparin
Patricia C. Y. Liaw, Debra L. Becker, Alan R. Stafford, James C. Fredenburgh and Jeffrey I. Weitz

J. Biol. Chem. 2001, 276:20959-20965.
doi: 10.1074/jbc.M010584200 originally published online April 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010584200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 19 of which can be accessed free at http://www.jbc.org/content/276/24/20959.full.html#ref-list-1