Conformational Changes in Thrombin When Complexed by Serpins*

James C. Fredenburgh, Alan R. Stafford, and Jeffrey I. Weitz‡

From the Hamilton Civic Hospitals Research Centre and Department of Medicine, McMaster University, Hamilton, Ontario L8V 1C3, Canada

Thrombin possesses two positively charged surface domains, termed exosites, that orient substrates and inhibitors for reaction with the enzyme. Because the exosites also allosterically modulate thrombin’s activity, we set out to determine whether the structure or function of the exosites changes when thrombin forms complexes with antithrombin, heparin cofactor II, or α1-antitrypsin (M358R), serpins that utilize both, one, or neither of the exosites, respectively. Using a hirudin-derived peptide to probe the integrity of exosite 1, no binding was detected when thrombin was complexed with hirudin or with heparin cofactor II or α1-antitrypsin (M358R), and the peptide exhibited a 55-fold lower affinity for the thrombin-antithrombin complex than for thrombin. Bound peptide or HD-1, an exosite 1-binding DNA aptamer, was displaced from thrombin by each of the three serpins. Thrombin binding to fibrin also was abrogated when the enzyme was complexed with serpins. These data reveal that, regardless of the initial mode of interaction, the function of exosite 1 is lost when thrombin is complexed by serpins. In contrast, the integrity of exosite 2 is largely retained when thrombin is complexed by serpins, because interaction with hirudin or an exosite 2-directed DNA aptamer was only modestly altered. The disorganization of exosite 1 that occurs when thrombin is complexed by serpins is consistent with results of protease sensitivity studies and crystallographic analysis of a homologous enzyme-serpin complex.

Thrombin activates platelets, converts fibrinogen to fibrin, and amplifies its own generation by activating factors V, VIII, and XI, highlighting its central role in coagulation. To prevent excessive clotting, the activity of thrombin must be tightly regulated (1). Down-regulation of thrombin activity is mediated by two serpins, antithrombin and heparin cofactor II, which form 1:1 stoichiometric complexes with thrombin (2). Another inhibitory mechanism involves thrombomodulin, a thrombin receptor found on the surface of vascular endothelial cells. Once bound to thrombomodulin, thrombin undergoes a specificity change that converts it from a procoagulant enzyme into one that initiates an anticoagulant pathway by activating protein C (3).

Selective interactions of thrombin with its substrates, cofactors, and inhibitors reflect structural characteristics unique to thrombin. These structural features include two surface loops that limit access to the active site by protruding over the active site cleft. In addition, thrombin possesses two positively charged domains, termed exosites, located on opposite poles of the thrombin molecule (4–6). The principal role of exosite 1 is to bind substrates, cofactors, and inhibitors and orient them for optimal interaction with the active site. This exosite, which also is known as the fibrinogen recognition site (7), interacts with negatively charged domains on fibrinogen, the thrombin receptor, hirudin, thrombomodulin, and heparin cofactor II. In contrast, the other exosite, which is designated exosite 2, binds heparin, dermatan sulfate, and chondroitin sulfate, glycosaminoglycans that promote thrombin’s interactions with serine protease inhibitors and thrombomodulin.

In addition to mediating thrombin’s interactions with its substrates and inhibitors, the exosites also modulate thrombin’s activity. By binding to exosite 1, in a concerted interaction that also involves exosite 2, thrombomodulin abolishes the procoagulant activity of thrombin by hindering its reaction with fibrinogen, factors V and VIII, and the thrombin receptor (8–10). Coincidentally, thrombin’s interaction with thrombomodulin induces allosteric changes at the active site of the enzyme (11, 12) that promote its ability to activate protein C and the procarboxypeptidase B-like enzyme, thrombin-activatable fibrinolysis inhibitor (TAI) (13). Interactions at exosite 2 also modulate the structure and function of the active site of thrombin (14, 15). Furthermore, ligand binding to either exosite can influence the other exosite in a reciprocal fashion (14).

Exosite 2 contributes to the inactivation of thrombin by antithrombin and heparin cofactor II by serving as a heparin-binding site, thereby promoting heparin-mediated bridging of the enzyme to the inhibitor (16, 17). Exosite 1 also plays an important role in the interaction of thrombin with heparin cofactor II. Binding of heparin or dermatan sulfate to heparin cofactor II disrupts the intramolecular interaction of the NH2-terminal domain, allowing it to bridge to exosite 1 on thrombin (17, 18). The exosites are not essential for thrombin inactivation, however, because α1-antitrypsin Pittsburgh, a naturally occurring variant with its reactive site P1 residue, Met-558, replaced with Arg (M358R), has no requirement for interaction with either exosite (19, 20).

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‡ A Career Investigator of the Heart and Stroke Foundation of Canada and recipient 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: Hamilton Civic Hospitals Research Center, 711 Concession St., Hamilton, Ontario L8V 1C3, Canada. Tel.: 905-574-8550; Fax: 905-575-2646; E-mail: jweitz@thrombosis.hhsc.ca.

1 The abbreviations used are: TAFI, thrombin activable fibrinolysis inhibitor; α1-antitrypsin M358R, α1-antitrypsin with Met-358 mutated to Arg; FITC, 5'-fluorescein isothiocyanate; FPR, D-Phe-Pro-Arg; FPRck, FPR chloromethyl ketone; HD-22, AGTCCGTGGAGGGCAGGTTGGGGTGACT oligonucleotide; HD-1, AGTCCGTGGAGGGCAGGTTGGGGTGACT oligonucleotide; hirudin 54–65; hirudin residues 54–65; tGPR-pNA, tosyl-t-Gly-Pro-Arg-p-nitroanilide; PAGE, polyacrylamide gel electrophoresis.
The sample was applied to a PD-10 column equilibrated with 20 mM NaCl, 0.6% polyethylene glycol 8000. The column was eluted under gravity, 10 min. The bead was removed, and 200 μl of a 2- to 20-fold molar excess FPRck, antithrombin, heparin cofactor II, or α1-antitrypsin M358R. After incubation for 60 min at 23°C, the residual activity of thrombin with tGPR-pNA was determined. The complex with heparin cofactor II showed 98% inhibition of chromogenic activity, whereas the others demonstrated greater than 99.5% inhibition. Concentrations of thrombin-serpin complexes were based on starting concentration of thrombin in each reaction. SDS-PAGE analysis followed by autoradiography revealed reduced mobility of 125I-thrombin in the presence of serpins, but not FPRck (Fig. 1).

**Methods**

**Titration of Fluorescein-Hirudin Complexes—** Fluorescein-hirudin, prepared as described previously (14), was added to a quartz cuvette at a concentration of 10 nM in 900 μl of TS. The sample was stirred with a mini-stirbar and maintained at 37°C in a water bath. Fluorescence of the sample (I₀) was monitored until the signal reached a plateau. The reaction was made 5 mM in EDTA and applied to a Q-Sepharose column. Fluorescein-thrombin, which eluted in the flow-through fraction, was precipitated with 80% ammonium sulfate, resuspended in TS, and dialyzed against TS overnight. By protein absorbance and chromogenic activity, the concentration was determined to be 16 μM. Fluorescein-thrombin, prepared in this manner, demonstrated clotting activity, chromogenic activity, and inhibition by antithrombin comparable to native thrombin.

**Displacement of Fluorescein-Hirudin Complexes from Thrombin in the Presence of Serpins—** The fluorescence of 10 nM fluorescein-hirudin was monitored as described above. When hirudin was added to the cuvette to a concentration of 400 nM, there was no change in fluorescence intensity. Subsequent addition of 200 nM thrombin produced a ~15% decrease in fluorescence intensity. Antithrombin, heparin cofactor II, or α1-antitrypsin M358R was added to a concentration of 400 nM, and fluorescence was monitored until the signal reached a plateau.

**Binding of Fluorescein- HD to Fluorescein-Thrombin Complexes—** Fluorescein-thrombin or fluorescein-thrombin-serpin complexes were titrated with HD-22 for determination of Kᵣ. A 2-ml sample containing 100 nM fluorescein-thrombin or fluorescein-thrombin-serpin complexes was titrated with HD-22 for determination of Kᵣ. A 2-ml sample containing 100 nM fluorescein-thrombin or fluorescein-thrombin-serpin complexes was titrated with HD-22 for determination of Kᵣ.
thrombin was incubated with 200 nM α1-antitrypsin M358R, 1 μM antithrombin, or 1 μM heparin cofactor II for more than 15 min, a time sufficient to achieve complete inhibition of thrombin activity. Fluorescence was monitored while fluorescein-thrombin or fluorescein-thrombin-serpin complexes were then titrated with HD-22 and $K_d$ values were determined as described above.

**Binding of Pentasaccharide to Antithrombin-Anti-thrombin Complex**—Binding of synthetic pentasaccharide to antithrombin was assessed by monitoring the intrinsic protein fluorescence of antithrombin in the absence and presence of pentasaccharide. Antithrombin at 100 nM, in the absence or presence of 300 nM thrombin, was equilibrated in 500 μl of TS in a cuvette for 1 h. The sample was monitored with excitation and emission wavelengths of 280 and 340 nm, respectively, with slitswidths of 5 nm and a 290-nm cutoff filter in the emission beam. Aliquots of pentasaccharide were added, and the fluorescence was monitored. $I/I_0$ values were determined and $K_d$ values calculated as described above.

**Heparin-Sepharose Chromatography**—Heparin-Sepharose affinity chromatography was used for qualitative assessment of the affinities of thrombin- and thrombin-serpin complexes for heparin. Samples containing 1–2 μg of 125I-thrombin or 125I-thrombin-serpin complexes were loaded onto a 0.6 × 10 cm column containing 4 ml of heparin-Sepharose equilibrated with 10 mM Tris-HCl, pH 7.4, at a flow rate of 1 ml/min using a Beckman System Gold high performance liquid chromatography system. After washing, a 50-min linear gradient to 1 or 2 M NaCl was initiated. Fractions (2 ml) were collected and counted for radioactivity. In supplemental experiments, unlabeled thrombin or thrombin-serpin complexes (50–400 μg) were subjected to chromatography, and the elution was monitored at 280 nm with an online UV detector. Fractions of 1 ml were collected, and peak protein-containing fractions were lyophilized, reconstituted in water, and subjected to SDS-PAGE analysis. Gels were stained with Fast Stain (Zoion Research, Shrewsbury, MA), and the bands were quantified by densitometry with an ImageMaster VDS (Amersham Pharmacia Biotech).

**Immunoprecipitation of Thrombin-Serpin Complexes**—Autoantibody D, an IgG that inhibits clotting, binds to exosite 1 on thrombin (29). The antibody was affinity-purified from a crude IgG fraction on a thrombinagarose column, prepared with biotin-FPRc and streptavidin-agarose (14). The IgG fraction was applied to the column and the flow-through material reapplied three times. After the column was washed with TS, bound IgG was eluted with Gentle Elution Buffer (Pierce). The eluate was dialyzed versus TS and concentrated by ultrafiltration with a Centricon 30 (Amicon Corp., Beverly, MA). For binding studies, 0.5 μM 125I-thrombin or preformed 125I-thrombin-serpin complexes was incubated with 2.4 μg autoantibody D for 1 h. An equal volume of protein G-agarose was added to the tubes, and the samples were mixed for 15 min on an end-over-end rotator. The samples were centrifuged, and the supernatants were removed and counted for radioactivity. The agarose was washed two times with TS, mixed with an equal volume of SDS gel sample buffer, boiled, and aliquots were subjected to SDS-PAGE. Autoradiographs of the dried gels were used to identify radioactive bands, which were excised and counted in a γ-counter. The amount of protein immunoprecipitated by autoantibody D/protein G-agarose was calculated as a fraction of the radioactivity of aliquots of the reactions taken prior to autoantibody D addition.

**Binding of Thrombin-Serpin Complexes to Fibrin**—Complexes of 125I-thrombin with serpins or FPRc were incubated in microcentrifuge tubes at a concentration of 50 nM in the presence of 0–10 μM fibrinogen and 10 mM CaCl2. Atroxin was added to each sample to a final concentration of 5% (v/v), and samples were incubated for 60 min at 23 °C. Resultant fibrin was compacted by centrifugation at 13,000 × g for 10 min, and duplicate 30-μl aliquots of the supernatant were counted for radioactivity. Plots of concentration of 125I-thrombin-complexes bound versus the fibrin concentration were subjected to rectangular hyperbola analysis to calculate $K_d$ values (30).

**RESULTS**

**Integrity of Exosite 1 on Thrombin Complexed by Serpins**—To determine whether the integrity of exosite 1 on thrombin is compromised when the enzyme is complexed by serpins, we first compared the affinity of hirudin54–65 a peptide that binds exclusively to exosite 1 on thrombin, for active thrombin with that for thrombin complexed by the various serpins. Titration of a fluorescein derivative of hirudin54–65 with thrombin produced a saturable decrease in fluorescence intensity (Fig. 2). Fluorescein-hirudin54–65 bound thrombin with a $K_d$ of 41 nM, a value consistent with that reported previously (15, 21). Although saturable binding also was observed when fluorescein-hirudin54–65 was titrated with thrombin-antithrombin complexes, the affinity of the interaction was 55-fold weaker than that with thrombin ($K_d$ value of 2270 nM). Bock et al. (21) also demonstrated reduced affinity of fluorescein-hirudin54–65 to the thrombin-antithrombin complex could not be demonstrated, suggesting that the $K_d$ value was greater than 5 μM.

In contrast to the results obtained with thrombin-antithrombin complexes, when thrombin-heparin cofactor II or thrombin-α1-antitrypsin M358R complexes were titrated with fluorescein-hirudin54–65, no binding was detected (Fig. 2). These findings suggest that the structure of thrombin may differ depending on the serpin with which it is complexed. To further explore this concept, the binding of fluorescein-hirudin54–65 to thrombin was monitored in real time as the sample was titrated with each of the three serpins. As illustrated in Fig. 3, addition of thrombin to fluorescein-hirudin54–65 produced a ~12% decrease in fluorescence intensity, and this value was unchanged upon the addition of 400 nM heparin. When 100 nM antithrombin was added, the fluorescence intensity rapidly returned to a value approaching that obtained with fluorescein-hirudin54–65 alone, suggesting that antithrombin reduced the amount of fluorescein-hirudin54–65 bound to thrombin. When parallel experiments were performed with heparin cofactor II and α1-antitrypsin M358R, similar results were obtained. Based on fluorescence intensity values recorded after serpin addition, heparin cofactor II or α1-antitrypsin M358R displaced 99% of fluorescein-hirudin54–65 from thrombin, whereas only 92% displacement was observed with antithrombin. Reduced displacement with antithrombin relative to the other two serpins is consistent with the observation that fluorescein-hirudin54–65 retains affinity for the thrombin-antithrombin complex, albeit much less than that for thrombin (Fig. 2).

To determine whether formation of a covalent complex between thrombin and a non-serpin, active-site-directed inhibitor is sufficient to alter the integrity of exosite 1, FPRc (500 nM) was added to a cuvette containing fluorescein-hirudin54–65 and thrombin (not shown). In contrast to the results with the serpins, chloromethyl ketone addition had no effect on fluores-
Thrombin Exosite Function in Thrombin-Serpin Complexes

The fluorescence of 10 nM fluorescein-hirudin54-65 was monitored continuously in time-drive profile. Addition of heparin to 400 nM had no effect on fluorescence, whereas addition of thrombin (200 nM) produced an ~18% decrease in intensity. At about 200 s, serpin was added to a final concentration of 400 nM. The time-drive profiles are shown for antithrombin ( ), heparin cofactor II ( ), and α1-antitrypsin M358R ( ) with symbols shown only every 100 s for clarity. The inset shows the percent recovery of the fluorescence signal after serpin addition as a percentage of the initial fluorescein-hirudin fluorescence measured prior to thrombin addition. Standard errors are less than 1.1% based on two to three determinations (not shown).

Displacement experiments also were performed using an exosite 1-directed DNA aptamer, HD-1, in place of the hirudin54-65 peptide (22). The fluorescent derivative of HD-1 binds thrombin with a $K_d$ value of 235 nM, producing an ~11% increase in fluorescence intensity (Fig. 3, inset). Under the conditions of the displacement experiment, addition of 100 nM thrombin to 20 nM fluorescein-HD-1 resulted in a ~3.5% increase in fluorescence intensity (Fig. 4). When 500 nM antithrombin, heparin cofactor II, or α1-antitrypsin M358R was added in the presence of 4 nM heparin, the fluorescence intensity rapidly returned to a value similar to that of unbound fluorescein-HD-1. These results are in agreement with those obtained with hirudin54-65, suggesting that exosite 1 function is compromised when thrombin is complexed by serpins. The observation that antithrombin addition results in complete displacement of fluorescein-HD-1, but not of fluorescein-hirudin54-65, may reflect differing binding interactions between the two ligands and thrombin.

To determine whether the interaction of exosite 1 with physiological ligands also was altered when thrombin was complexed with serpins, we compared the binding of thrombin-serpin complexes to fibrin with that of thrombin (Fig. 5). Samples containing 50 nM 125I-Thrombin or 125I-thrombin-serpin complexes and 0–10 μM fibrinogen were clotted with Atoxin. After 60-min incubation, clots were prepared by centrifugation and unbound 125I-thrombin or 125I-thrombin-serpin complexes in the supernatants were quantified by counting aliquots for radioactivity. Under these conditions, thrombin bound fibrin with a $K_d$ value of 1.6 μM, a value comparable to that reported previously (31, 32). In contrast, no binding of thrombin-serpin complexes was observed. Thus, the loss of function of exosite 1 observed with synthetic ligands extends to physiological ligands such as fibrin.

As a second macromolecular probe of exosite 1, binding of a thrombin-specific antibody to thrombin and thrombin serpin complexes was examined. Autoantibody D is a human-derived antibody that binds to thrombin to exosite 1, as demonstrated by the lack of binding to thrombin variants with mutations in this region (29). Using protein G-agarose to extract thrombin-antibody complexes, autoantibody D bound 66% of 125I-thrombin (not shown). The antibody recovered 11, 10, and 13% of the radioactivity of thrombin-antithrombin, thrombin-heparin cofactor II, or thrombin-α1-antitrypsin M358R complexes, respectively. Thus, reactivity with the exosite 1-directed antibody was greatly reduced when thrombin was complexed with serpins.
Thrombin Exosite Function in Thrombin-Serpin Complexes

Integrity of Exosite 2 on Thrombin Complexed with Serpins—Having demonstrated that the function of exosite 1 was impaired when thrombin was complexed by serpins, it was of interest to examine the integrity of exosite 2 in the thrombin-inhibitor complexes. Initial experiments utilized an exosite 2-directed DNA aptamer, HD-22 (23). Titration of fluorescein-thrombin with HD-22 produced a saturable decrease in fluorescence intensity as HD-22 bound thrombin with a $K_d$ value of 227 nM (Fig. 6). When complexes of fluorescein-thrombin with antithrombin, heparin cofactor II, or α1-antitrypsin M358R were titrated with HD-22, there was a saturable decrease in fluorescence intensity. Although the decreases in fluorescence intensity with the complexes were less than that observed with thrombin, the $K_d$ values of HD-22 for fluorescein-thrombin complexed with antithrombin, heparin cofactor II, and α1-antitrypsin M358R were 370, 220, and 272 nM, respectively, values less than 2-fold higher than that of HD-22 for fluorescein-thrombin. These data suggest that, in contrast to exosite 1, the integrity of exosite 2 is minimally affected when thrombin is complexed by serpins. Addition of FFRek to a sample of HD-22 and fluorescein-thrombin caused no change in fluorescence intensity, indicating that low molecular weight active site aducts also have no effect on the integrity of exosite 2 (not shown).

To confirm these concepts, we used heparin, a more physiological ligand, to probe the integrity of exosite 2 on thrombin complexed by serpins. Elution from heparin-Sepharose was not detected by this method. To positively identify the species eluting from the column, 50–400 μg of unlabeled thrombin or thrombin-serpin complexes was applied to the column and elution was monitored spectrophotometrically at 280 nm (not shown). Peak fractions were concentrated and electrophoresed on SDS-PAGE gels. Under these conditions, antithrombin eluted at ~1200 mM NaCl, consistent with its higher affinity for heparin relative to thrombin (33). The thrombin-antithrombin reaction mixture eluted in two peaks, one at a position coincident with thrombin and a second eluting with a slightly lower concentration of NaCl. SDS-PAGE analysis revealed that these two peaks were thrombin and thrombin-antithrombin complex, respectively. The elution profile confirms that the thrombin-antithrombin complex retains affinity for heparin comparable to that of thrombin. Heparin cofactor II demonstrated lower affinity for heparin-Sepharose than thrombin, likely reflecting intramolecular interactions between its positively charged heparin binding domain and its anionic NH$_2$-terminal domain (34). The thrombin-heparin cofactor II reaction mixture eluted in two peaks, one coincident with unlabeled heparin cofactor II and the other, identified as thrombin-heparin cofactor II complex, at a NaCl concentration close to that of thrombin. α1-Antitrypsin M358R demonstrated only weak heparin affinity, consistent with its lack of a heparin binding site (19). The thrombin-α1-antitrypsin M358R reaction mixture eluted from heparin-Sepharose in three peaks, two equivalent to free thrombin and α1-antitrypsin M358R, and the other at an intermediate NaCl concentration. PAGE analysis identified the latter peak as thrombin-α1-antitrypsin M358R complex. Taken together, these results indicate that thrombin-serpin complexes retain their affinity for heparin, suggesting that the integrity of exosite 2 is not compromised when thrombin is complexed by serpins.

To demonstrate that the thrombin-antithrombin complex was not adsorbing to the heparin-Sepharose complex via its antithrombin moiety, binding of pentasaccharide to antithrombin and thrombin-antithrombin complex was assessed (not shown). Pentasaccharide binding to antithrombin, monitored by changes in intrinsic fluorescence of antithrombin, yielded a $K_d$ value of 25 nM and a 30% increase in fluorescence intensity. In contrast, addition of pentasaccharide to thrombin-antithrombin complex had no effect on intrinsic fluorescence, sug-
gestive of no interaction. Because antithrombin within the thrombin-antithrombin complex has reduced affinity for pentasaccharide, the retention of the thrombin-antithrombin complex on heparin-Sepharose is mediated by the thrombin moiety.

**DISCUSSION**

This study demonstrates that the functional integrity of exosite 1 on thrombin is essentially lost when the enzyme is complexed by serpins. In contrast, exosite 2 on thrombin complexed by serpins retains its affinity for its ligands. Results were obtained using small, synthetic exosite-binding ligands as well as macromolecular targets of thrombin binding. The thrombin-antithrombin complex exhibited 55-fold lower affinity for fluorescein-hirudin–65 than thrombin in a direct binding experiment (Fig. 1). This result corroborates a previous report (21). The discrepancy in values may reflect differences in the methods used to label the hirudin–65 peptide. Isothiocyanate was used in the present study, whereas a succinimidyl ester-labeling procedure was employed in the previous study. We have extended the results of the earlier study by examining other serpins. In contrast to the result with antithrombin, fluorescein-hirudin–65 does not bind to thrombin-heparin cofactor II or thrombin-α1-antitrypsin M358R complexes. Similarly, peptidyl and oligonucleotidyl ligands bound to exosite 1 were displaced when thrombin was inhibited by serpins. Hirudin–65 was quantitatively displaced when thrombin was complexed by heparin cofactor II or α1-antitrypsin M358R, but not antithrombin. Thrombin-serpin complexes also have greatly reduced affinity for fibrin, an interaction mediated by exosite 1, and for an antibody directed against exosite 1. These observations indicate that the function of exosite 1 is compromised when thrombin forms complexes with serpins.

The integrity of exosite 2 is maintained when thrombin is complexed by serpins. Thus, an exosite 2-directed oligonucleotide binds thrombin-serpin complexes with affinities similar to that for thrombin. Studies using heparin, a physiological ligand for exosite 2, verified that this exosite retained measurable functional activity when thrombin was complexed by serpins. The use of a non-heparin binding serpin, α1-antitrypsin M358R, confirmed that binding of thrombin-serpin complexes to heparin was not mediated by the heparin-binding domains of antithrombin or heparin cofactor II. In addition, we demonstrated that, once complexed with thrombin, antithrombin no longer binds pentasaccharide, a finding in agreement with a previous report (35). Therefore, when complexed by serpins, the integrity of exosite 2 on thrombin is retained, whereas that of exosite 1 is lost.

Three serpins with different modes of interaction with thrombin were used to examine whether impairment of exosite function was influenced by different use of the exosites during the inhibition reaction. The interactions of antithrombin and α1-antitrypsin M358R with thrombin are exosite 1-independent, as evidenced by inhibition studies with γ-thrombin or exosite 1 mutants (36, 37).2 Despite this, when thrombin formed a complex with either serpin, exosite 1 function was lost. Heparin cofactor II differs from antithrombin and α1-antitrypsin M358R, because its NH2-terminal domain interacts with exosite 1 on thrombin during the inhibition reaction. However, based on the results with the other two serpins, the NH2-terminal domain of heparin cofactor II is unlikely to remain bound to the impaired exosite. Indirect verification of this concept is provided by our observation that the thrombin-heparin cofactor II complex displaces thrombin bound to fibrin monomer-Sepharose (38). This suggests that the NH2-terminal domain of heparin cofactor II within the thrombin-serpin complex is capable of binding to other thrombin molecules. The fact that hirudin–65 binds, albeit weakly, to the thrombin-antithrombin complex, but not to thrombin complexed by heparin cofactor II or α1-antitrypsin M358R, may indicate subtle differences among the complexes. However, it also is possible that the fluorophore did not register interactions with the latter two inhibitors.

Loss of function of exosite 1 on thrombin in complex with serpins occurs in the presence or absence of heparin. When fluorescein-hirudin–65 or fluorescein-HD-1 was displaced from thrombin upon addition of serpins, heparin was added to catalyze the inhibition reactions. However, the preformed thrombin-serpin complexes, which were used to titrate fluorescein-hirudin–65, were prepared in the absence of heparin. Therefore, the similarity of results obtained with exosite-dependent and -independent inhibitors in the absence and presence of heparin demonstrates that impairment of exosite 1 function is a generalized response to thrombin inhibition by serpins.

Disruption of protease structure is not simply a function of the formation of a covalent adduct at the active site, because exosite 1 function is not impaired when thrombin is complexed with a chloromethyl ketone derivative. Thus, in contrast to serpins, inhibition by FPRck does not displace fluorescein-hirudin–65 from thrombin. Likewise, FPR-thrombin retains its affinity for fibrin, whereas thrombin-serpin complexes do not bind to fibrin. These data are consistent with the suggestion that the interaction of proteases with macromolecular inhibitors produces greater conformational changes in the enzyme than interaction with inhibitors of lower molecular mass (39).

The failure of thrombin-serpin complexes to bind hirudin–65 or fibrin is consistent with previous observations that the thrombin-antithrombin complex does not bind to thrombomodulin. Consequently, thrombomodulin recovers its cofactor activity for protein C activation when thrombin is inhibited by antithrombin (40). Although this observation is complicated by the fact that thrombomodulin also binds exosite 2 on thrombin via its chondroitin sulfate moiety, only the exosite 1-mediated interaction is sufficient to endow thrombin with the capacity to activate protein C. Previous studies have demonstrated that FPRck modification of thrombin does not affect its interaction with thrombomodulin (41), further supporting the contention that loss of exosite 1 function only occurs when thrombin interacts with macromolecular ligands (39).

Numerous studies have focused on the structural changes that occur in serpins when they complex their target proteases (42, 43). Covalent complex formation produces considerable movement of the enzyme relative to the serpin, as the portion of the reactive site loop to which the enzyme is covalently attached inserts itself into the body of the serpin. Insertion moves the enzyme from one pole of the serpin to the other. Crystallographic analysis of the covalent trypsin-α1-antitrypsin complex indicates that the enzyme moiety also undergoes structural alterations that extend beyond the active site to involve a significant portion of the body of the enzyme (43). Thus, these data suggest that the loss of exosite function that occurs when thrombin is complexed by serpins may be a universal phenomenon common to all serpin-protease interactions.

Based on the crystal structure of a representative protease-serpin complex, the loss of exosite 1 function that occurs when thrombin is complexed by serpins is more likely to reflect structural changes than steric inhibition (43). This is supported

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2 D. L. Becker and J. I. Weitz, unpublished.
by our observation that even small ligands, such as hirudin and HD-1, exhibit reduced or absent binding to the thrombin-antithrombin complex. Analysis of the trypsin-antithrombin complex reveals disorganization of about a third of the enzyme moiety, including the regions that correspond to exosite 1 on thrombin. Thus, the loss of function of exosite 1 may be a generalized phenomenon reflecting denaturation of the protease once it is complexed by a serpin. Selective disorganization of this region renders the enzyme more susceptible to proteolysis. Numerous studies have demonstrated increased sensitivity of the protease portion of the protease-serpin complex to proteolysis (39, 44). Protease denaturation and subsequent proteolysis may serve a role in facilitating clearance of protease-serpin complexes.

The targeted inactivation of exosite 1 leaves open the question as to why the function of exosite 2 is spared when thrombin is complexed by serpins. Consistent with the functional studies presented here, the trypsin-antithrombin crystal structure reveals less disorganization in the exosite 2 region of the molecule (43). Retention of the heparin-binding domain in the thrombin moiety of the thrombin-antithrombin complex may promote clearance via low density lipoprotein receptor-related protein (45). However, the lack of heparin binding sites in other serine proteases argues against this being a universal clearance mechanism for enzyme-serpin complexes.

As a general phenomenon, the role of enzyme denaturation upon interactions with serpins is unclear. In the case of thrombin, however, this process ensures that, once thrombin is inhibited, it no longer remains bound to thrombomodulin, thereby preventing thrombin-antithrombin complexes from acting as competitive inhibitors of thrombin-mediated protein C or TAFI activation. Much like the release of heparin from antithrombin within the thrombin-antithrombin complex, recovery of thrombomodulin upon inhibition of thrombin permits cofactor recycling.

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