Ligand Binding to Thrombin Exosite II Induces Dissociation of the Thrombin−Heparin Cofactor II(L444R) Complex[†]

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ABSTRACT: Heparin cofactor II (HCII) inhibits thrombin rapidly in the presence of heparin or dermatan sulfate. The product of the inhibition reaction is a kinetically stable, 1:1 complex between the two proteins. We recently observed that heparin induces dissociation of complexes containing thrombin and the reactive site mutant HCII(L444R) to yield active thrombin and cleaved inhibitor (Han, J.-H., Van Deerlin, V. M. D., and Tollefsen, D. M. (1997) *J. Biol. Chem. 272*, 8243−8249). In the current study, we have investigated the mechanism by which heparin induces dissociation of the thrombin−HCII(L444R) complex. Heparin oligosaccharides ≥6 sugars in length induce dissociation, which suggests that dissociation does not depend on binding of a heparin molecule simultaneously to both proteins in the complex. Binding of heparin to HCII(L444R) in the complex also does not appear to be required, since the heparin dose response is unaltered for complexes containing the double mutant HCII(L444R/K173Q), which has decreased affinity for heparin. By contrast, binding of heparin to thrombin appears to be necessary and sufficient to induce dissociation. First, heparin fails to induce dissociation of complexes that contain thrombin(K236E), a variant with decreased heparin affinity. Second, a monoclonal IgG that interacts with the heparin-binding site of thrombin mimicks heparin in its ability to induce dissociation of the thrombin−HCII(L444R) complex. Finally, the complex of HCII(L444R) with thrombin(desPPW), which binds normally to heparin but lacks $Pro^{60B}Pro^{60C}Trp^{60D}$ in an insertion loop ("60-loop") between the heparin-binding site and the catalytic site, does not dissociate in the presence of heparin. These results suggest that binding of heparin to thrombin induces an allosteric effect causing destabilization of the thrombin−HCII(L444R) complex and that the allosteric effect may be mediated by the 60-loop.

Heparin cofactor II (HCII)[1] is a plasma glycoprotein that belongs to the serpin superfamily (*1*). Most members of this family are inhibitors of serine proteases. The inhibitory serpins function as suicide substrates for their target proteases by providing a bait sequence at the P1−P1′ peptide bond (reactive site). When the protease attacks the P1−P1′ bond, it becomes trapped in a kinetically stable, 1:1 complex with the serpin. HCII inhibits thrombin and chymotrypsin but not a variety of other proteases (*2*, *3*). Glycosaminoglycans such as heparin and dermatan sulfate increase the rate of inhibition of thrombin by HCII >1000-fold (*4*) but have no effect on the rate of inhibition of chymotrypsin (*3*). Rapid inhibition of thrombin by HCII in the presence of a glycosaminoglycan depends on the interaction of the N-terminal acidic domain of HCII with anion-binding exosite I of thrombin (reviewed in ref *5*).

HCII inhibits chymotrypsin 3−4 times faster than thrombin in the absence of a glycosaminoglycan (*6*), which is consistent with the presence of Leu^{444} at the P1 position of the reactive site (*7*). Mutation of Leu^{444} to Arg in recombinant HCII (rHCII) increases the rate of inhibition of thrombin ∼100-fold in the absence of a glycosaminoglycan but abolishes the reaction with chymotrypsin (*6*). Recently, we and others reported that the thrombin−rHCII(L444R) complex is unstable in the presence of heparin and dissociates to yield active thrombin and the inhibitor cleaved at the reactive site (*8*, *9*). For example, we find that thrombin is inhibited rapidly and completely by a 35-fold molar excess of rHCII(L444R) in the presence of heparin; subsequently, ∼50% of the thrombin activity is regenerated after a lag phase of ∼20 min, during which the excess inhibitor is cleaved. In the presence of dermatan sulfate or in the absence of a glycosaminoglycan, however, the thrombin−rHCII(L444R) complex is stable for ≥4500 min.

In the current study, we have investigated the mechanism by which heparin induces dissociation of the thrombin−rHCII(L444R) complex. Hypothetically, dissociation could be induced by heparin binding to one or more site(s) on either the protease and/or the inhibitor portion of the complex. The heparin-binding site on thrombin has been mapped to amino acid residues in exosite II, which include Arg^{93}, Arg^{97}, Arg^{101}, Arg^{233}, Lys^{236}, and Lys^{240} (*10*−*14*).[2] Residues in HCII that are involved in binding heparin include Lys^{173}, Arg^{184}, and Lys^{185} (*15*−*17*). Experiments utilizing recombinant proteins with mutations in each of these two sites provide evidence

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[1] Abbreviations: HCII, heparin cofactor II; rHCII, recombinant HCII.

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that binding of heparin to exosite II of thrombin is necessary and sufficient to induce dissociation of the thrombin−rHCII-(L444R) complex. This hypothesis is supported by two additional observations: (1) heparin oligosaccharides presumed to be of insufficient length to bind both proteins simultaneously induce dissociation, and (2) a monoclonal antibody that binds specifically to Arg^{233} in exosite II of thrombin mimicks the effect of heparin. We propose that binding of a ligand to exosite II induces a conformational change in the active site of thrombin that destabilizes the thrombin−rHCII(L444R) complex, and we present evidence that this conformational change is mediated by the Pro^{60B}-$Pro^{60C}Trp^{60D}$ insertion loop ("60-loop"), which lies between exosite II and the active site (*18*).

EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin was purchased from Haematologic Technologies (Essex Junction, VT). Recombinant thrombin(K236E)[2] (*11*) was provided by Dr. J. Evan Sadler, Washington University. Recombinant thrombin(desPPW), in which Pro^{60B}, Pro^{60C}, and Trp^{60D} were deleted from the B-chain (*19*), was obtained from Dr. Charles T. Esmon, Oklahoma Medical Research Foundation. A monoclonal human IgG that binds to exosite II of thrombin (*20*)[3] was prepared by Dr. Niall S. Colwell, Washington University. Bovine lung heparin was purchased from Upjohn (Kalamazoo, MI). Porcine intestinal heparin (grade 1-A) and porcine skin dermatan sulfate were purchased from Sigma (St. Louis, MO); the dermatan sulfate was treated with nitrous acid to degrade contaminating heparin or heparan sulfate (*4*). Other materials were obtained from the following sources: isopropyl β-D-thiogalactopyranoside and deoxyribonuclease-1 from Sigma; poly(ethylene glycol) 8000 from Union Carbide (Danbury, CT); tosyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH) from Boehringer Mannheim (Indianapolis, IN); Sephadex G-10, heparin-Sepharose CL-6B, HiTrap heparin-Sepharose, and Mono Q and Mono S columns from Pharmacia Biotech (Piscataway, NJ); Bio-Gel P-10 from Bio-Rad (Hercules, CA); restriction enzymes from New England Biolabs (Beverly, MA); and medium for high-density bacterial culture from BIO 101 (Vista, CA).

Partial Depolymerization of Heparin and Fractionation of Oligosaccharides. Depolymerization of heparin was carried out by a modification of the procedure described previously (*21*). Nitrous acid was prepared by reacting 114 mg/mL $Ba(NO_2)_2 \cdot H_2O$ with an equal volume of 1 M H_2SO_4 followed by centrifugation to remove the $BaSO_4$ precipitate. Porcine heparin (500 mg in 5 mL of H_2O) was incubated with an equal volume of nitrous acid (diluted 1:10 in 1 M H_2SO_4) for 15 min at −5 °C. The pH was then adjusted to 8.5 with saturated Tris base. The oligosaccharide products were separated on a Bio-Gel P-10 column (1.0 × 200 cm) equilibrated with 1 M NaCl in 10% ethanol. Fractions were assayed with carbazole to determine the uronic acid concentration (*22*). The void and total volumes of the column were estimated with blue dextran and [^{14}C]glucose, respectively. Oligosaccharide peaks were pooled, desalted on a Sephadex G-10 column, and dried with a Speed Vac concentrator (Savant). The purity of each oligosaccharide was assessed by electrophoresis at 150 V for 30 min on a 12% polyacrylamide gel in 0.02 M sodium barbital buffer, pH 8.6. The gel was stained with 0.1% toluidine blue in 1% acetic acid.

Expression and Purification of rHCII(L444R) and rHCII-(L444R/K173Q). An expression vector containing the full-length cDNA for HCII(L444R) in pET-3d (Novagen, Madison, WI) was constructed previously (*8*). To obtain the double mutant, the 1113-bp *Nco*I/*Xho*I fragment of this vector, which spans the region encoding the glycosaminoglycan-binding site, was replaced with the *Nco*I/*Xho*I fragment isolated from a pMON−HCII construct containing the K173Q mutation (*17*). The mutation and ligation sites were verified by dideoxynucleotide sequencing (*23*). The plasmid vectors were electroporated into *E. coli* BL21(DE3)-pLysS cells for expression. The cells were grown to an optical density (600 nm) of ∼5−6 in a BioFlo III high-density fermentor (New Brunswick Scientific, Edison, NJ) at 37 °C and then induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 h. The cells were harvested and lysed at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and 2 mM EDTA. The lysate was treated with 10 μg/mL deoxyribonuclease-1 and 10 mM $MnCl_2$, and cellular debris was removed by centrifugation. The rHCII was purified to homogeneity from the lysate by chromatography on heparin−Sepharose CL-6B, Mono Q, and Mono S columns. Typically, 800 mL of lysate was applied to a 1.4 L heparin−Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.4. The column was washed and then eluted with a 0−500 mM NaCl gradient. Fractions containing HCII activity were concentrated by ultrafiltration, dialyzed against 20 mM Tris-HCl, 50 mM NaCl, pH 7.4, and applied to a Mono Q HR 10/10 column equilibrated in the dialysis buffer. The column was eluted with a 50−300 mM NaCl gradient. Fractions containing HCII activity were dialyzed against 50 mM sodium phosphate buffer, pH 7.4, and applied to a Mono S HR 10/10 column equilibrated with the dialysis buffer. The column was eluted with a 0−100 mM NaCl gradient. HCII activity in the column fractions was determined in a thrombin inhibition assay in the presence of dermatan sulfate (*6*). The concentration of purified rHCII was determined by absorbance at 280 nm using the extinction coefficient determined for plasma HCII (1.17 $mL \cdot mg^{-1} \cdot cm^{-1}$) (*24*).

Time Course of Inhibition of Thrombin by rHCII. Purified rHCII was incubated with thrombin with or without bovine heparin at room temperature in 10 mM Tris-HCl, 150 mM NaCl, 1 mg/mL poly(ethylene glycol), pH 7.5. Thrombin was added last to initiate the reaction (t_0). At specified times ranging from 0.1 to 2000 min, 100 μL of the reaction mixture was placed in a disposable polystyrene cuvette, 500 μL of 100 μM Chromozym TH in the buffer above was added, and the absorbance at 405 nm was recorded continuously for 100 s. Residual thrombin activity was estimated from the rate of substrate hydrolysis (ΔA405/min). For short time points, 100 μL incubations were carried out directly in

[2] Thrombin residues are identified according to their topologic equivalence with chymotrypsinogen as suggested by Bode et al. (*18*). For residues discussed in this paper, the chymotrypsinogen numbering corresponds to positions numbered from the N-terminal end of the B-chain (in parentheses) as follows: $Pro^{60B(48)}$, $Pro^{60C(49)}$, $Trp^{60D(50)}$, $Arg^{93(89)}$, $Arg^{97(93)}$, $Arg^{101(98)}$, $Arg^{221A(233)}$, $Arg^{233(245)}$, $Lys^{236(248)}$, and $Lys^{240(252)}$.

[3] Colwell, N. S., Blinder, M. A., and Tollefsen, D. M. Manuscript in preparation.

cuvettes. Under the conditions of these experiments, heparin alone did not have a significant effect on the rate of hydrolysis of Chromozym TH.

Determination of the Second-order Rate Constant for Inhibition of Thrombin by rHCII(L444R/K173Q). The second-order rate constant for thrombin inhibition by rHCII-(L444R/K173Q) in the absence of a glycosaminoglycan was determined by the discontinuous method under pseudo-first-order conditions. Purified rHCII (100 nM) was incubated with thrombin (10 nM) in 10 mM Tris-HCl, 150 mM NaCl, 1 mg/mL poly(ethylene glycol), pH 7.5. At various times after the addition of thrombin, 100 μL of the reaction mixture was assayed for residual thrombin activity as described above. The pseudo-first-order rate constant (k_{obs}) was obtained from the slope of a plot of ln(E) versus time (t) according to the equation $\ln(E)_t = \ln(E)_0 - k_{obs}t$, in which $(E)_t$ and $(E)_0$ are thrombin activity at time t and time zero, respectively. The second-order rate constant (k) was determined by $k = k_{obs}/[I]_0$, where $[I]_0$ is the initial rHCII concentration.

RESULTS

Heparin Fragments Induce Dissociation of the Thrombin−rHCII(L444R) Complex. To identify the smallest heparin oligosaccharide that induces dissociation of the thrombin−rHCII(L444R) complex, we used nitrous acid to randomly cleave the heparin polymer at α1−4 glycosidic linkages between N-sulfated D-glucosamine and uronic acid residues. This procedure yields a series of oligosaccharides containing an even number of residues, the smallest of which is a disaccharide. The products were fractionated on a Bio-Gel P-10 column (Figure 1A), and peaks containing 4−10 monosaccharide units were judged to be >90% pure based on polyacrylamide gel electrophoresis (Figure 1B). We then incubated thrombin with rHCII(L444R) in the presence of 200 μg/mL of intact heparin or each of the purified oligosaccharides and monitored thrombin activity for ~1200 min. In the absence of heparin or in the presence of the tetrasaccharide (Figure 1C), thrombin is inhibited rapidly and remains inactivated for the duration of the experiment. By contrast, reversible inhibition of thrombin by rHCII(L444R) occurs in the presence of the hexa-, octa-, and decasaccharide fragments, although the rate and extent of dissociation of the complex are somewhat reduced in comparison with intact heparin. These results suggest that a single heparin molecule does not need to bind simultaneously to both thrombin and rHCII(L444R) to induce dissociation of the complex.

Binding of Heparin to rHCII(L444R) Is Not Required To Dissociate the Complex. We used the double mutant rHCII-(L444R/K173Q) to investigate whether heparin binding to the inhibitor portion of the complex induces dissociation. Previously, Whinna et al. (*17*) found that rHCII(K173Q) elutes at a lower ionic strength from heparin−Sepharose and requires a 30-fold higher concentration of heparin to inhibit thrombin in comparison with native rHCII. As shown in Figure 2A, rHCII(L444R/K173Q) elutes from heparin−Sepharose at a lower ionic strength than rHCII(L444R); the elution profiles are similar to those reported for rHCII-(K173Q) and native rHCII, respectively (*17*). In addition, the second-order rate constant for inhibition of thrombin by rHCII(L444R/K173Q) in the absence of a glycosaminoglycan

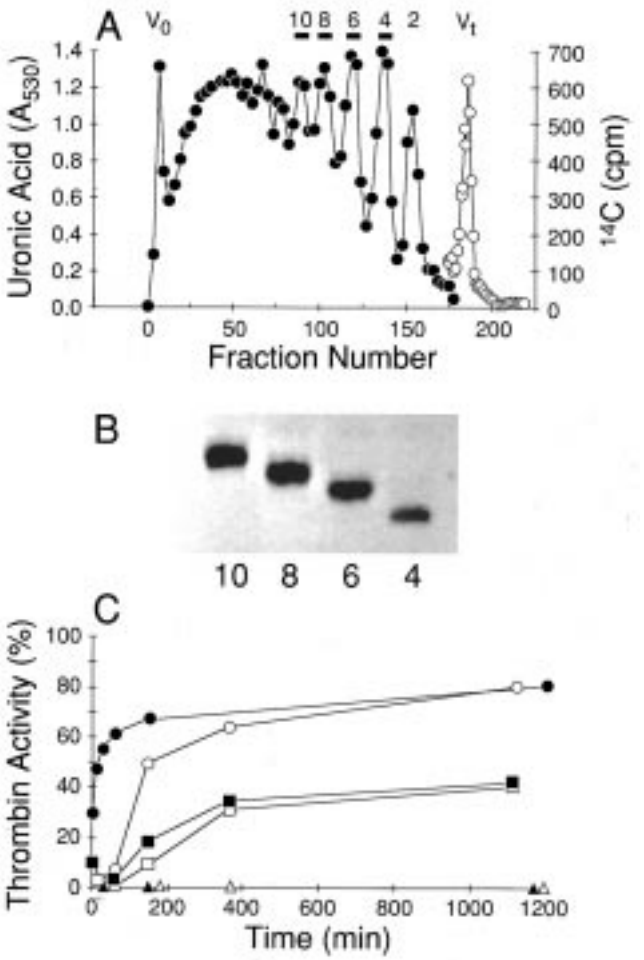

FIGURE 1: Inhibition of thrombin by rHCII(L444R) in the presence of heparin oligosaccharides. Panel A: Partial depolymerization of heparin was carried out as described in "Experimental Procedures." The heparin oligosaccharides were fractionated by gel filtration chromatography on a Bio-Gel P-10 column. Peaks containing fragments 4−10 monosaccharide units in length were pooled as indicated (bars). ●, carbazole assay for uronic acid; ○, [^{14}C]glucose standard; V_0, void volume; V_t, total volume. Panel B: Approximately 6 μg of the tetra- (4), hexa- (6), octa- (8), and decasaccharide (10) fractions from panel A were analyzed by polyacrylamide gel electrophoresis as described in "Experimental Procedures." Panel C: Thrombin (10 nM) and rHCII(L444R) (200 nM) were incubated without heparin (△) or with 200 μg/mL of the heparin tetra- (▲), hexa- (■), octa- (□), or decasaccharide (○), or full-length heparin (●). Thrombin was added last to initiate the reaction. At various times (0.1−1200 min), the remaining thrombin activity was determined by hydrolysis of Chromozym TH. Activity is expressed as a percentage of that present in an incubation containing thrombin alone. In the absence of rHCII(L444R), thrombin activity was stable (±5%) during the course of the experiment (data not shown).

is 2.5×10^6 M^{-1} min^{-1} (data not shown), which is similar to that of rHCII(L444R) (2.1×10^6 M^{-1} min^{-1}) and is ~100 times greater than that of native rHCII. The double mutant, therefore, has the expected properties, including a decreased heparin affinity and an increased basal rate of thrombin inhibition. To characterize the double mutant further, various concentrations of either rHCII(L444R) or rHCII(L444R/K173Q) were incubated for 1 min with 10 nM thrombin in the presence of heparin, and the residual thrombin activity was determined. As shown in Figure 2B, the stoichiometry of inhibition of thrombin by both rHCII variants is ~28:1, which is similar to our previous result (*8*). Longer incubations in the presence of heparin showed that the thrombin−

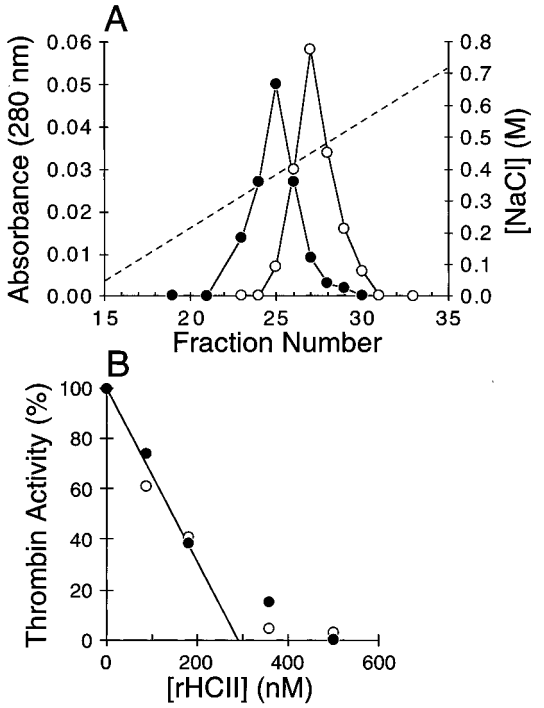

FIGURE 2: Heparin binding and stoichiometry of inhibition of rHCII(L444R/K173Q) and rHCII(L444R). Panel A: Samples of purified rHCII(L444R/K173Q) (●) or rHCII(L444R) (○) were applied to a 5 mL HiTrap heparin−Sepharose column in 50 mM Tris-HCl, 50 mM NaCl, pH 7.5, and eluted with a linear NaCl gradient (- -). Protein was detected by absorbance at 280 nm. Panel B: Various concentrations of rHCII(L444R/K173Q) (●) or rHCII-(L444R) (○) were incubated with thrombin (10 nM) for 1 min in the presence of 50 μg/mL heparin, and the remaining thrombin activity was then determined by hydrolysis of Chromozym TH.

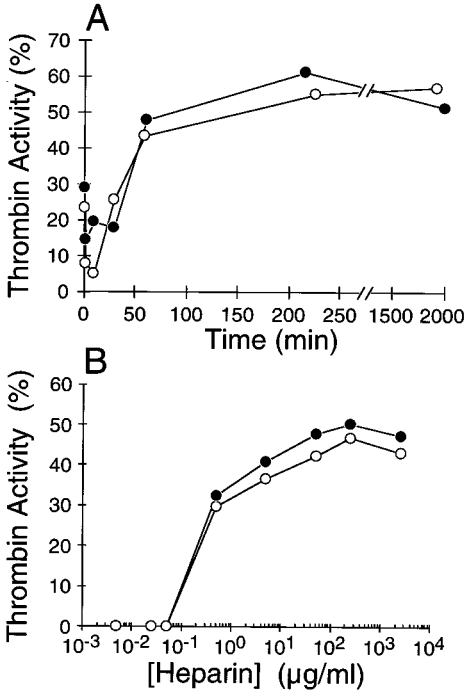

FIGURE 3: Time course and heparin dependence of inhibition of thrombin by rHCII(L444R/K173Q). Panel A: rHCII(L444R/K173Q) (●) or rHCII(L444R) (○) (360 nM) was incubated with thrombin (10 nM) in the presence of 50 μg/mL heparin. Thrombin was added last to initiate the reaction. At various times (0.1−2000 min), the remaining thrombin activity was determined by hydrolysis of Chromozym TH. Activity is expressed as a percentage of that present in an incubation containing thrombin alone. Panel B: rHCII-(L444R/K173Q) (●) or rHCII(L444R) (○) (390 nM) was incubated with thrombin (10 nM) for 1000 min in the presence of various concentrations of heparin. The remaining thrombin activity was then determined by hydrolysis of Chromozym TH.

rHCII(L444R) and thrombin−rHCII(L444R/K173Q) complexes dissociate with similar time courses and yield similar plateau levels of thrombin activity (Figure 3A). More importantly, the concentrations of heparin required to dissociate the two complexes are identical (Figure 3B) despite the lower affinity of rHCII(L444R/K173Q) for heparin. This result suggests that binding of heparin to the inhibitor portion of the complex is not required to induce dissociation.

Thrombin(K236E)−rHCII(L444R) Is Stable in the Presence of Heparin. If binding of heparin to the protease portion of the thrombin−rHCII(L444R) complex is necessary to cause dissociation, then mutation of the heparin-binding site (exosite II) of thrombin should make the complex resistant to dissociation. Figure 4 shows an experiment in which rHCII(L444R) was incubated with either native thrombin or thrombin(K236E),[2] an exosite II mutant with decreased affinity for heparin but normal catalytic activity (*11*). In the presence of heparin, ∼45% of the native thrombin−rHCII(L444R) complexes dissociate, whereas the thrombin-(K236E)-rHCII(L444R) complexes do not dissociate during a prolonged incubation. Control incubations performed in the absence of rHCII(L444R) and heparin demonstrated that both native thrombin and thrombin(K236E) are stable for the duration of the experiment (Figure 4). This result supports the hypothesis that binding of heparin to thrombin destablizes the thrombin−rHCII(L444R) complex.

A Monoclonal Antibody against Thrombin Exosite II Dissociates the Thrombin−rHCII(L444R) Complex. We have isolated, from a patient with multiple myeloma, a

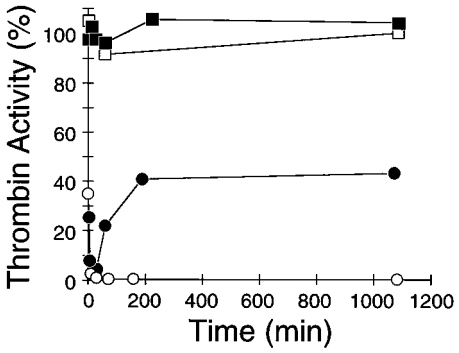

FIGURE 4: Stability of the thrombin(K236E)−rHCII(L444R) complex. rHCII(L444R) (350 nM) was incubated with 10 nM native thrombin (●) or thrombin(K236E) (○) in the presence of 50 μg/mL heparin. Thrombin was added last to initiate the reaction, and the activity was determined at various times (0.1−1100 min) with Chromozym TH. Control incubations without the inhibitor and heparin are also shown for native thrombin (■) and thrombin-(K236E) (□).

monoclonal IgG that binds to thrombin (*20*). The epitope for this IgG has been mapped with a panel of thrombin surface mutants (*14*)[3] and includes Arg^{233} in exosite II. In the experiment shown in Figure 5, we incubated thrombin with rHCII(L444R) for 5 min to allow complex formation and then added heparin, the monoclonal IgG, or control human IgG. The monoclonal IgG induces dissociation of the thrombin−rHCII(L444R) complex, although the rate of dissociation is somewhat reduced in comparison with hep-

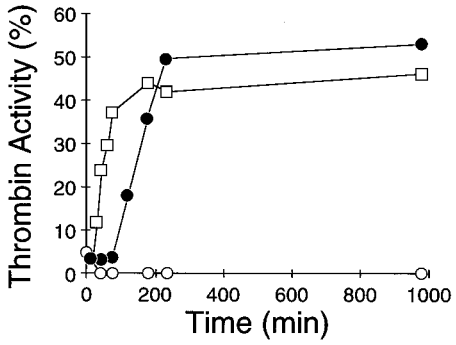

FIGURE 5: Antibody-induced dissociation of thrombin−rHCII-(L444R). rHCII(L444R) (350 nM) was incubated with thrombin (10 nM) for 5 min followed by the addition of heparin (50 μg/mL) (□), monoclonal IgG against exosite II of thrombin (90 μg/mL) (●), or control IgG (90 μg/mL) (○). Thrombin activity was determined at various times by hydrolysis of Chromozym TH. The activity is expressed as a percentage of that determined in the absence of rHCII(L444R).

arin. By contrast, the control IgG does not cause dissociation. This result indicates that binding of a ligand other than heparin to exosite II of thrombin can destabilize the thrombin−rHCII(L444R) complex.

Heparin Does Not Dissociate the Thrombin(desPPW)−rHCII(L444R) Complex. Thrombin(desPPW) is a mutant that lacks the Pro^{60B}, Pro^{60C}, and Trp^{60D} residues in the 60-loop of thrombin. Deletion of these residues alters the kinetics of synthetic substrate hydrolysis and interaction with macromolecular inhibitors (*19*). Because these residues lie between exosite II and the active site of thrombin, it is possible that a ligand bound to exosite II could transmit a conformational change to the active site through the 60-loop. Therefore, we were interested in determining whether heparin induces dissociation of the thrombin(desPPW)−rHCII-(L444R) complex. As shown in Figure 6A, thrombin-(desPPW) binds to heparin−Sepharose and elutes at the same ionic strength as native thrombin, which indicates that the affinity of thrombin(desPPW) for heparin is normal. Thrombin(desPPW) is inhibited rapidly by rHCII(L444R) with a second-order rate constant of $\sim 1 \times 10^7$ M^{-1} min^{-1}, and the thrombin(desPPW)−rHCII(L444R) complex is stable during prolonged incubation either in the presence or absence of heparin (Figure 6B). Under similar conditions, ∼50% of the native thrombin−rHCII(L444R) complexes dissociate. These results suggest that dissociation of the complex may depend on a conformational change in thrombin induced by binding of heparin to exosite II and mediated by the 60-loop.

DISCUSSION

The inhibitory mechanism of a serpin is generally thought to involve rapid formation of a tetrahedral or acyl intermediate (E−I) between the catalytic serine hydroxyl group of the protease (E) and the carbonyl group of the P1 amino acid residue of the inhibitor (I) (*25*, *26*). This intermediate has two alternative fates (reviewed in ref 1): (1) conversion to a kinetically stable complex (E−I*) brought about by partial insertion of the reactive site loop into β-sheet A of the serpin and (2) dissociation to yield the free protease (E) and a modified inhibitor (I_M) that has been cleaved proteolytically at the P1−P1′ peptide bond. Partitioning of E−I between

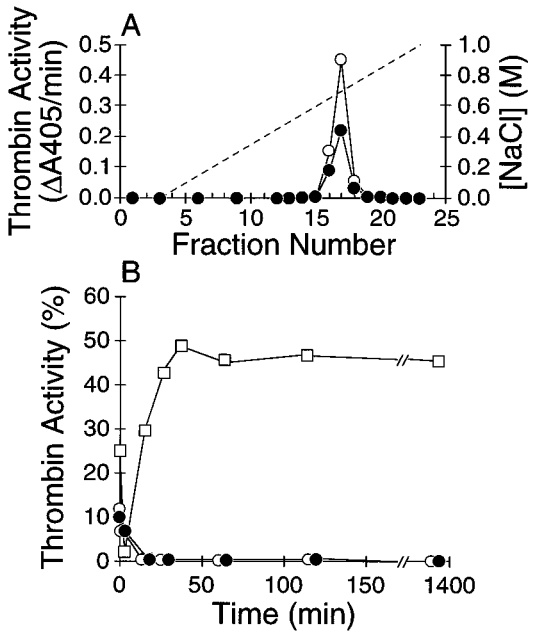

FIGURE 6: Heparin binding of thrombin(desPPW) and stability of the thrombin(desPPW)−rHCII(L444R) complex. Panel A: Samples of purified native thrombin (○) and thrombin(desPPW) (●) were applied to a 1 mL HiTrap heparin−Sepharose column and eluted with a linear NaCl gradient (- -). The activity of each fraction was assayed with Chromozym TH. Panel B: rHCII(L444R) (350 nM) was incubated with thrombin(desPPW) (10 nM) in the presence (●) or absence (○) of 50 μg/mL heparin or with native thrombin (10 nM) in the presence of 50 μg/mL heparin (□). Thrombin was added last to initiate the reaction, and the activity was determined at various times (0.3−1300 min) with Chromozym TH. The activity is expressed as a percentage of that determined in the absence of rHCII(L444R).

these two pathways determines the stoichiometry of inhibition. The kinetically stable E−I* complex generally remains associated for many hours and can be considered the final product of the inhibition pathway. However, in some cases E−I* slowly dissociates to yield E + I_M (*27*, *28*) or undergoes further modification to yield a nondissociable or "locked" form of the complex (E−I**) (*29*).

Mutation of Leu^{444} to Arg at the P1 position of rHCII affects several steps in the mechanism outlined above. Consequently, the rate of inhibition of thrombin by rHCII-(L444R) is ∼100-fold greater than that of native rHCII in the absence of a glycosaminoglycan (*6*). Furthermore, the stoichiometry of inhibition of thrombin is several-fold higher for rHCII(L444R) than for native rHCII in the presence of heparin (*8*, *9*), indicative of increased partitioning of E−I into the substrate pathway. Perhaps the most striking effect of this mutation is that it enables heparin to induce dissociation of the thrombin−rHCII(L444R) complex, releasing the active protease and the cleaved inhibitor (*8*, *9*). Dissociation occurs after partitioning of E−I to E−I* and is best explained by accelerated conversion of E−I* to E + I_M (discussed in ref 8). Thus, heparin appears to increase the rate at which the cleavage of rHCII(L444R) by thrombin proceeds to completion after being temporarily arrested at an intermediate stage of the proteolytic reaction.

In this study, we investigated the mechanism by which heparin induces dissociation of the thrombin−rHCII(L444R) complex. Initially, we asked whether a heparin molecule must bind simultaneously to sites on both thrombin and

rHCII(L444R) to induce dissociation. It is known that heparin oligosaccharides containing ≥18 monosaccharide units are necessary to form a bridge between thrombin and antithrombin (*30*). By analogy, fragments of heparin shorter than this are unlikely to bind to HCII and thrombin simultaneously (*9*). We found that heparin oligosaccharides containing as few as six monosaccharide units induce dissociation of the complex (Figure 1C), which argues against a bridging mechanism for dissociation. Our results differ from those of Ciaccia et al. (*9*), who reported that heparin oligosaccharides <16 units in length do not induce dissociation of thrombin−rHCII(L444R). This discrepancy is most likely explained by differences in the concentrations of oligosaccharide used in the experiments. Thrombin binds to heparin hexasaccharides with a $K_d \approx 6-10$ μM (*31*). The concentration of oligosaccharide employed by Ciaccia et al. (1.4 μM) was well below the K_d, whereas we used ∼100 μM oligosaccharide. Both sets of results, therefore, are consistent with the hypothesis that binding of short oligosaccharides to thrombin induces dissociation of the complex.

Binding of heparin to rHCII(L444R) does not appear to be involved in dissociation of the thrombin−rHCII(L444R) complex. As shown in Figures 2B and 3, the stoichiometry of inhibition, time course of dissociation, and heparin dose response are unaltered for complexes containing the double mutant rHCII(L444R/K173Q), even though rHCII(L444R/K173Q) has a decreased affinity for heparin−Sepharose (Figure 2A) and rHCII(K173Q) requires a 30-fold higher concentration of heparin to stimulate thrombin inhibition in comparison with native rHCII (*17*). By contrast, binding of heparin to thrombin appears to be necessary and sufficient to induce dissociation of the thrombin−rHCII(L444R) complex. In agreement with Ciaccia et al. (*9*), we observe that complexes which contain a thrombin variant with a mutation in the heparin-binding site are stable in the presence of heparin (Figure 4). Furthermore, a monoclonal IgG that interacts with Arg^{233} of thrombin mimicks heparin in its ability to induce dissociation of the thrombin−rHCII(L444R) complex (Figure 5). Arg^{233} occurs in exosite II and has been shown by mutagenesis to be involved in binding of both heparin and dermatan sulfate to thrombin (*32*). It is important to note that dermatan sulfate does not induce dissociation of thrombin−rHCII(L444R) (*8*), suggesting that interaction of heparin or the monoclonal IgG with additional residues in the vicinity of Arg^{233} is required to induce dissociation.

Recent data support the idea that thrombin is an allosteric enzyme. For example, binding of a Na^+ ion in the vicinity of Arg^{221A} converts thrombin from a form that preferentially cleaves protein C ("slow" form) to a form that preferentially cleaves fibrinogen ("fast" form) (*33*, *34*). Alterations in the catalytic activity of thrombin can be detected after binding of thrombomodulin or other ligands to exosite I (*35*−*37*). Binding of prothrombin fragment 2 to exosite II of thrombin (*38*) also produces a conformational change that can be detected with active site-directed fluorescence probes (*39*). Interaction of substrates and inhibitors with the active site is controlled, in part, by two prominent insertion loops (i.e., the 60-loop and the 148-loop) that border the active site cleft of thrombin (*18*, *19*, *40*). The 60-loop restricts access of macromolecules to the active site, and deletion of part of this loop in thrombin(desPPW) increases the affinity for bovine pancreatic trypsin inhibitor ∼3000-fold (*19*). By contrast, the affinity (K_m) of thrombin(desPPW) for the fibrinogen Aα chain is unaffected, although the k_{cat} for cleavage of this substrate is decreased ∼50-fold (*19*). The 60-loop, therefore, appears to be important for the proper orientation of the scissile bond relative to the catalytic triad.

Deletion of part of the 60-loop in thrombin(desPPW) or substitution of alanine for Trp^{60D} decreases the rate of inhibition of thrombin by antithrombin ∼200-fold (*41*, *42*). By contrast, the rate of inhibition of thrombin(desPPW) by protease nexin-1 is essentially normal, which indicates that the interactions of the 60-loop with antithrombin are somewhat specific (*41*). Inhibition of thrombin by antithrombin is characterized by low-affinity binding in the initial encounter complex followed by rapid formation of the stable complex. Studies with thrombin(W60A) indicate that the presence of Trp^{60D} in the 60-loop weakens the affinity of the encounter complex but accelerates stable complex formation (*42*). Although the 60-loop has been considered to be a relatively rigid structure, recent crystallographic studies indicate that this loop can move a considerable distance (∼2−8 Å) when the active site is occupied by a substrate or an inhibitor (*43*, *44*). Because of its mobility and its position between the active site and exosite II on the surface of thrombin, the 60-loop could potentially mediate allosteric effects of ligands that bind to exosite II. This idea is consistent with our observation that heparin does not induce dissociation of the thrombin(desPPW)−rHCII(L444R) complex even though thrombin(desPPW) binds normally to heparin (Figure 6A).

In summary, the results of our study are compatible with a model in which binding of heparin or the monoclonal IgG to exosite II of thrombin induces an allosteric effect that destabilizes the thrombin−rHCII(L444R) complex. Although the molecular basis for the destabilization remains to be determined, the data suggest that the allosteric effect may be mediated by the 60-loop of thrombin.

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