A heparin cofactor II (HCII) mutant with an Arg substituted for Leu at the P1 position (L444R-rHCII) was previously found to have altered proteinase specificity (Derechin, V. M., Blinder, M. A., and Tollefsen, D. M. (1990) J. Biol. Chem. 265, 5623–5628). The present study characterizes the effect of glycosaminoglycans on the substrate versus inhibitor activity of L444R-rHCII. Heparin increased the stoichiometry of inhibition of L444R-rHCII with α-thrombin (compared with minus glycosaminoglycan) but decreased it with R93A,R97A,R101A-thrombin, a mutant thrombin that does not bind glycosaminoglycans. Dermatan sulfate decreased the stoichiometry of inhibition of L444R-rHCII with both proteinases. SDS-polyacrylamide gel electrophoresis showed no proteolysis of L444R-rHCII when incubated with R93A,R97A,R101A-thrombin in the absence of the presence of glycosaminoglycan or with α-thrombin and dermatan sulfate. In contrast, greater than 75% of the L444R-rHCII converted to a lower molecular weight form when incubated with α-thrombin/heparin. A time course of α-thrombin inhibition by L444R-rHCII/heparin showed a rapid but transient inhibition with approximately 80% of the α-thrombin activity being regained after 6 h of incubation. In contrast, all other combinations of inhibitor, proteinase, and glycosaminoglycan resulted in complete and sustained inhibition of the proteinase. Heparin fragments of 8–20 polysaccharides in length rapidly accelerated L444R-rHCII inhibition of both α-thrombin and R93A,R97A,R101A-thrombin. After extended incubations, R93A,R97A,R101A-thrombin was completely inhibited by L444R-rHCII with all the heparin fragments, but approximately 30–50% of α-thrombin activity remained with fragments long enough to bridge HCII-thrombin. These results collectively indicate that ternary complex formation, mediated by heparin, increases L444R-rHCII inactivation by α-thrombin.

Serine proteinase inhibitors (serpins) are a highly conserved family of proteins whose primary role is to regulate the activity of a wide variety of serine proteinases involved in processes such as blood coagulation, fibrinolysis, inflammation, and cancer metastasis (1–3). Serpins inhibit their target proteinase by functioning as suicide substrates. The serpin contains a peptide bond, termed P1-P1′ by the nomenclature of Schechter and Berger (4), within an exposed region, called the reactive site loop, that is recognized as a substrate by the proteinase (2). The initial binding of the serpin and proteinase results in the formation of a Michaelis complex, which can then partition between a kinetically stable intermediate, resulting in proteinase inhibition, and a cleaved inactive inhibitor (3, 5, 6). Although the sequence of the amino acids in the reactive site loop and on the P1-P1′ residues in particular determines in part the proteinase specificity of the serpin, they also determine the partitioning of the serpin into an inhibitor versus substrate pathway with a given proteinase.

Heparin cofactor II (HCII), protein C inhibitor, protease nexin-1, plasminogen activator inhibitor-1, and antithrombin III (AT) belong to a subgroup of serpins whose inhibitory activity is accelerated upon binding to glycosaminoglycans, such as heparin, heparan sulfate, and dermatan sulfate (Refs. 1–3 and 7–9 and references therein). AT has a P1-P1′ Arg-Ser reactive site, and this serpin is important for regulating hemostasis because it inhibits thrombin and most other coagulation proteases in the presence of heparin or heparan sulfate. Glycosaminoglycan-accelerated thrombin inhibition by AT requires the formation of a ternary complex in which heparin binds AT and thrombin simultaneously (10). Thrombin inhibition by AT in the presence of heparin is accelerated more than 1,000-fold. In contrast to AT, the target protease of HCII is believed to be only thrombin (11, 12), a pluripotent serine proteinase with additional biological roles in inflammation and wound healing. The P1-P1′ bond of HCII (Leu-Ser) is an unusual thrombin substrate sequence because all coagulation proteases prefer Arg at the P1 position (13, 14). Thus, in the absence of glycosaminoglycan, HCII is a poor thrombin inhibitor. However, HCII is an exceptional thrombin inhibitor in the presence of heparin or dermatan sulfate, either of which can accelerate the thrombin inhibition rate more than 9,000-fold (7, 15–17). Although heparin can simultaneously bind thrombin and HCII, ternary complex formation does not appear to be absolutely required for thrombin inhibition by HCII (18).

Mutations in the reactive site loop of AT (19), α1-antichymotrypsin (20, 21), and protein C inhibitor (22) have been shown to affect the inhibitor versus substrate properties of the serpin. In addition, exogenous factors such as heparin and ionic strength (23) and the addition of peptides that insert into the β-sheet of a serpin (24) can also change the serpin from an inhibitor to a substrate. An HCII mutant with an Arg substituted for Leu at the P1 position (L444R-rHCII) was previously engineered and characterized by Derechin et al. (25). Consistent with
thrombin preferring a P1 Arg residue, the thrombin inhibition rate of the mutant in the absence of glycosaminoglycan was about 100-fold higher than wild type recombinant HCII (wt-rHCII). Because a P1 Leu to Arg mutation in HCII caused increased thrombin inhibitory activity in the absence of glycosaminoglycans (25), we became interested in determining whether this mutation altered HCII’s inhibitor versus substrate properties in the absence or the presence of glycosaminoglycans. In this report we demonstrate that (a) although L444R-rHCII is a better thrombin inhibitor, it is also a better thrombin substrate, (b) heparin promotes L444R-rHCII inactivation by α-thrombin in a mechanism dependent upon ternary complex formation, (c) dermatan sulfate does not promote L444R-rHCII inactivation, and (d) the effect of heparin on inactivation is manifested only with L444R-rHCII and not with wt-rHCII.

EXPERIMENTAL PROCEDURES

Generation and Expression of wt-rHCII and L444R-rHCII—Human wild type recombinant HCII (cDNA kindly provided by Dr. Douglas M. Tollefsen, Washington University School of Medicine, St. Louis, MO) was overexpressed in the baculovirus expression system and characterized (17). To obtain L444R-rHCII, site-directed mutagenesis was performed by the method of Kunkel (26) on a XhoI-EcoRI HCII cDNA cassette transfected in the pBluescript SK+ mutation vector (Stratagene). Positive clones were identified by DNA sequencing (Sequenase®, version 2.0, U. S. Biochemical). A XhoI-EcoRI cassette containing the mutation was subcloned into full-length HCII cDNA in the p[ExK]-3 blue vector (Promega). Full-length HCII cDNA was then subcloned into the baculoviral transfer vector pVL1392 (PharMingen) via flanking EcoRI sites and co-transfected with linearized Autographica californica nuclear polyhedrosis virus (AcNPV) into Spodoptera frugiperda (SF9) cells (InVitrogen) insect cells (27). The infectious medium was collected 4 days post-transfection and used in plaque assays as described in PharMingen protocols. Individual plaques were isolated and amplified by infection of SF9 cells in 24-well plates. Production of rHCII was determined by immunoblot analysis of whole cell lysates from plaque-infected cells; positive clones were amplified and stored at 4 °C. SF9 cells were maintained in spinner flasks in Grace’s medium (JRH Scientific) supplemented with 10% fetal bovine serum (HiClone), 0.3 g/liter L-glutamine (LifeTechnologies, Inc.), and 50 μg/ml gentamicin (Life Technologies).h

Protein Expression and Purification—High Five™ insect cells (InVitrogen) grown at 27 °C in serum free cell-ex Cult 405 medium with 0.05% l-glutamine (Life Technologies, Inc.), and 50 μg/ml gentamicin (Life Technologies) were infected with recombinant baculovirus carrying the L444R-rHCII cDNA. The infected cultures were maintained in spinner flasks and shaken at 200 rpm at 27 °C. Culture supernatants were collected after 60–72 h of expression and concentrated by centrifugation at 10,000 × g. The concentrated recombinant HCII was purified by size exclusion chromatography on a 5/100 column of Superdex 200 gel filtration media (Pharmacia) equilibrated in a buffer containing 20 mm Hepes, 0.10 mm EDTA, 0.10% polyethylene glycol 8000, 0.05% NaN3, and batch adsorbed with 0.5 ml of heparin-Sepharose eluate. The heparin-Sepharose eluate was diluted in HPN buffer to a final concentration of 50 μM NaCl, pH 7.4, and batch adsorbed with 0.5 ml of heparin-Sepharose beads (Pharmacia Biotech Inc.) for 1 h at 4 °C. rHCII was eluted from the heparin-Sepharose with 0.5 μM NaCl in HPN buffer, pH 7.4, after two washes in 75 mM NaCl in HPN, pH 6.5. The heparin-Sepharose eluate was diluted in HPN buffer to a final concentration of 50 μM NaCl, pH 7.8, and batch adsorbed with 0.5 ml of Q-Sepharose beads (Pharmacia) for 1 h at 4 °C. After two washes in 50 μM NaCl, the protein was eluted with 0.5 μM NaCl in HPN buffer, pH 7.8. The eluate was stored at −80 °C.

rHCII Immunodetection—A direct enzyme-linked immunosorbent assay using a mouse anti-HCII monoclonal antibody and a goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) was used as described previously to measure HCII concentrations (17). Human plasma HCII, purified as described previously (28) was used as a standard. Assays were performed in 96-well microtiter plates and color development was monitored at 405 nm on a V max microplate reader. Second order inhibition rates (k2, M−1 min−1) were obtained as follows: In the absence of glycosaminoglycan, 200 nm wt-rHCII or 25 nm L444R-rHCII were incubated with 1 nM thrombin in the presence of 50 μg/ml polybrene (Sigma) and 2 mg/ml bovine serum albumin in HPNP, pH 7.4 (20 mM Heps, 150 mM NaCl, 0.1% polystyrene glycol 8000, 0.05% NaN3) for a range of time periods. For glycosaminoglycan-accelerated thrombin inhibition, 5 mM HCII, and 0.5 mM thrombin were incubated with increasing amounts of heparin or dermatan sulfate for 20–80 s. Inhibition was quenched with 150 μM tosyl-Gly-Pro-Arg-p-nitroanilide. Second order inhibition constants were measured in triplicate on two to three different preparations of rHCII. The rates were obtained under pseudo-first order reaction conditions ([II] >> [E]) and were calculated using the equation k2 = (−ln[α]/[t]), where [α] is the residual protease activity, t is the association time, and [I] is the HCII concentration.

Visualisation of rHCII-Thrombin Complexes and rHCII Proteolysis—rHCII (10 nm) was incubated with thrombin (10 nm) in HPNP, pH 7.4, in the absence of glycosaminoglycan or in the presence of either 5 μg/ml heparin or 20 μg/ml dermatan sulfate for 1, 5, and 30 min at room temperature. The reaction was terminated by the addition of 5 mM D-Phe-Pro-Arg-chloromethyl ketone (Calbiochem), which inactivates uncomplexed thrombin, and Laemmli sample buffer. SDS-PAGE and immunoblotting with an anti-HCII monoclonal antibody were performed as described above.

Extended Time Course Studies—HCII (10 nm) and thrombin (1 nm) were incubated in HPNP, pH 7.4 in the absence of glycosaminoglycan or in the presence of 5 μg/ml heparin or 20 μg/ml dermatan sulfate for various time points up to 18 h, and the residual thrombin activity was measured as described above. To determine the effect of ternary complex formation on substrate versus inhibitor properties of HCII, 1 nM thrombin and 10 nM HCII were incubated for 18 h in the presence of heparin fragments (1.4 μM, 3.4–8.4 μg/ml; Stago Diagnostika, kindly provided by Dr. Michel M. Canton) ranging from 8 to 20 oligosaccharides in length (HF8, HF12, HF16, and HF20). A low evaporation lid coated with anti-fogging agent ( Molecular Devices) was used to minimize evaporation.

RESULTS

Generation, Expression, and Kinetic Analysis of L444R-rHCII—L444R-rHCII was generated, expressed, and purified as described under “Experimental Procedures.” In a typical preparation, 50–130 μg of protein were obtained from two T150 flasks of High Five cells after sequential heparin- and Q-Sepharose batch adsorption of the conditioned medium. Immunoblot analysis and SDS-PAGE showed that L444R-rHCII co-mi-
**TABLE I**

| Inhibition rate constant \(k_a, \text{m}^{-1} \text{s}^{-1}\) for wt-rHCII and L444R-rHCII with \(\alpha\)-thrombin, R93A, R97A, R101A-thrombin, and wild type recombinant thrombin are summarized below. For glycosaminoglycan-accelerated inhibition, template curves were performed in the presence of increasing concentrations of glycosaminoglycan, and the maximal inhibition rate from each individual curve was used in the calculation of the average inhibition rate. Assays were performed as described under “Experimental Procedures,” and the values are expressed as the means ± S.D. of two to six determinations. |
|---|---|---|
| | wt-rHCII | L444R-rHCII |
| | \(\alpha\)-Thrombin | R93A, R97A, R101A-thrombin | Wild type recombinant thrombin |
| GAG | 2.4 ± 0.3 \(\times\) 10^{-6} | 1.3 ± 0.3 \(\times\) 10^{-6} | 2.5 ± 0.1 \(\times\) 10^{-6} |
| + heparin | 5.6 ± 1.5 \(\times\) 10^{-6} | 2.6 ± 0.3 \(\times\) 10^{-6} | 2.7 ± 0.2 \(\times\) 10^{-6} |
| + DS | 1.2 ± 0.2 \(\times\) 10^{-6} | 1.1 ± 0.1 \(\times\) 10^{-6} | 1.1 ± 0.07 \(\times\) 10^{-6} |

\(a\) GAG, glycosaminoglycan; DS, dermatan sulfate.

\(n = 4.\)

\(n = 3.\)

\(n = 6.\)

\(n = 5.\)

\(n = 2.\)

A Stoichiometry of Inhibition in the Absence of Glycosaminoglycans—The SI, the number of serpin molecules consumed before an inactivated serpin-proteinase complex forms, reflects the tendency of a serpin to behave as a substrate. The titration curves for L444R-rHCII and wt-rHCII with \(\alpha\)-thrombin and R93A, R97A, R101A-thrombin are shown in Fig. 1. In the absence of glycosaminoglycan, approximately seven molecules of L444R-rHCII are required to inhibit \(\alpha\)-thrombin before a stable complex is formed. Dermatan sulfate decreased the SI value to 2.5, whereas heparin increased it to 14.5, suggesting that heparin promoted a more substrate-like behavior for L444R-rHCII. With R93A, R97A, R101A-thrombin, the SI values in the absence of glycosaminoglycan (SI = 7.5) and in the presence of dermatan sulfate (SI = 2.5) were comparable with those obtained with \(\alpha\)-thrombin. In contrast, the addition of heparin (SI = 3) to the R93A, R97A, R101A-thrombin inhibition reaction had the opposite effect: it did with \(\alpha\)-thrombin and mimicked the results obtained with dermatan sulfate. Wild type rHCII had an SI value ranging from 1.8 to 3 in either the presence of heparin or dermatan sulfate with either thrombin variant. SI assays were also performed with wild type recombinant thrombin, and the data agree with those obtained for \(\alpha\)-thrombin (data not shown).

**Stoichiometry of Inhibition in the Absence and the Presence of Glycosaminoglycans—**The SI, the number of serpin molecules consumed before an inactivated serpin-proteinase complex forms, reflects the tendency of a serpin to behave as a substrate. The titration curves for L444R-rHCII and wt-rHCII with \(\alpha\)-thrombin and R93A, R97A, R101A-thrombin are shown in Fig. 1. In the absence of glycosaminoglycan, approximately seven molecules of L444R-rHCII are required to inhibit \(\alpha\)-thrombin before a stable complex is formed. Dermatan sulfate decreased the SI value to 2.5, whereas heparin increased it to 14.5, suggesting that heparin promoted a more substrate-like behavior for L444R-rHCII. With R93A, R97A, R101A-thrombin, the SI values in the absence of glycosaminoglycan (SI = 7.5) and in the presence of dermatan sulfate (SI = 2.5) were comparable with those obtained with \(\alpha\)-thrombin. In contrast, the addition of heparin (SI = 3) to the R93A, R97A, R101A-thrombin inhibition reaction had the opposite effect: it did with \(\alpha\)-thrombin and mimicked the results obtained with dermatan sulfate. Wild type rHCII had an SI value ranging from 1.8 to 3 in either the presence of heparin or dermatan sulfate with either thrombin variant. SI assays were also performed with wild type recombinant thrombin, and the data agree with those obtained for \(\alpha\)-thrombin (data not shown).

**Immunoblot Analysis of Thrombin-HCII Reactions—**The substrate activity of wt-rHCII and L444R-rHCII in the absence and the presence of glycosaminoglycan was visualized by SDS-PAGE and immunoblot analysis (Fig. 2). Incubation of wt-rHCII with \(\alpha\)-thrombin or R93A, R97A, R101A-thrombin in the presence of heparin or dermatan sulfate resulted in a stable bimolecular complex with no apparent proteolysis of the inhibitor (Fig. 2). Although heparin and dermatan sulfate accelerated complex formation between wt-rHCII and either thrombin variant, they had no effect on substrate activity. In the absence of glycosaminoglycan, neither complex formation nor proteolysis of wt-rHCII was visualized. In contrast, L444R-rHCII had more moderate substrate activity with \(\alpha\)-thrombin in the absence of glycosaminoglycan. After 30 min of incubation, approximately 10–25% of L444R-rHCII converted to a lower molecular weight form, consistent with proteolysis at the reactive site loop. Heparin accelerated the conversion of L444R-rHCII to a lower molecular weight form, with approximately 90% of the L444R-rHCII being cleaved by \(\alpha\)-thrombin after 30 min of incubation. Interestingly, dermatan sulfate prevented L444R-rHCII cleavage by \(\alpha\)-thrombin. Incubation of R93A, R97A, R101A-thrombin with L444R-rHCII in the absence or the presence of dermatan sulfate yielded results identical to those observed with \(\alpha\)-thrombin. However, heparin failed to facilitate the proteolytic
Heparin Promotes Inactivation of a Reactive Site Mutant

inactivation of L444R-rHCII by R93A,R97A,R101A-thrombin as it did with α-thrombin. Longer exposures of the autoradiograph revealed SDS-PAGE-stable complexes between L444R-rHCII and thrombin for all combinations except L444R-rHCII/α-thrombin/heparin (data not shown).3

Extended Time Course of HCII-Thrombin Reactions—The HCII and thrombin variants were incubated for various time periods up to 18 h in order to determine the time course and stability of thrombin inhibition. Shown in Fig. 3 is a representative plot of thrombin inhibition by L444R-rHCII in the absence and the presence of glycosaminoglycan. Inhibition of R93A,R97A,R101A-thrombin by L444R-rHCII occurs rapidly and is sustained over a 18-h period. Likewise, the inhibition of α-thrombin by L444R-rHCII in the absence of glycosaminoglycan and in the presence of dermatan sulfate is also sustained during the course of the experiment. In the presence of heparin, α-thrombin is rapidly inhibited during the first 30–60 min, but activity is slowly regained thereafter. Within 6 h of incubation, approximately 75–80% of α-thrombin activity is regained and is stable for at least 18 h. The instability of α-thrombin inhibition is due to the dissociation of L444R-rHCII/α-thrombin complexes promoted by heparin. There was no remaining thrombin activity at the 18-h time point for wt-rHCII with either thrombin derivative in the presence of heparin (data not included).

Role of Heparin and Heparin Fragments on the Substrate Activity of L444R-rHCII—To determine whether heparin bridging of α-thrombin to L444R-rHCII was a factor in the substrate-like behavior of L444R-rHCII, thrombin inhibition assays with heparin fragments of 8–20 polysaccharides in length (HF8, HF12, HF16, and HF20) were performed. The minimal heparin length required for ternary complex formation is thought to be between 18 and 26 polysaccharides (16, 33–35).4 Thus, we postulated that the heparin-mediated inactivation of L444R-rHCII by α-thrombin would decrease with decreasing heparin fragment length. We chose an 18-h incubation time to ensure that the reaction process reached completion with regards to either proteinase inhibition or serpin inactivation with release of thrombin. Both α-thrombin and R93A,R97A,R101A-thrombin were essentially completely inhibited after 18 h of incubation in the presence of wt-rHCII and HF12, HF16, HF20, or heparin; approximately 5–10% of activity remained with the HF8, consistent with the lower inhibition rate of wt-rHCII with a short heparin fragment (Fig. 4). The same results were obtained for L444R-rHCII with R93A,R97A,R101A-thrombin. In contrast, approximately 30 and 46% of α-thrombin activity remained when incubated with L444R-rHCII and HF16 or HF20, respectively, as compared with approximately 5% remaining activity with HF8 or HF12. Control experiments verified that all of the heparin fragments accelerated thrombin inhibition by L444R-rHCII as found previously with other heparin-binding serpins (16). However, unlike HF8 and HF12, which supported complete α-thrombin inhibition by L444R-rHCII, HF16- and HF20-accelerated α-thrombin inhibition leveled off after 30 min of incubation, which would be consistent with inactivation of L444R-rHCII (data not included).

3 The rapid heparin-accelerated inhibition of α-thrombin by L444R-rHCII would lead one to predict the formation of SDS-PAGE-stable complexes. However, there are multiple examples in the literature of serpin-proteinase pairs that form highly stable complexes under native but not under denaturing conditions. Under different experimental conditions where higher concentration of L444R-rHCII and α-thrombin were used, SDS-PAGE-stable complexes were visualized in the presence of heparin.

4 Heparin-accelerated thrombin inhibition by HCII decreases linearly with decreasing heparin size (16, 35), but because ternary complex formation is not required for the inhibition reaction, the minimal heparin length required for bridging is difficult to estimate from these studies. Because ternary complex formation between AT/thrombin/heparin is required for the inhibition reaction, it is possible to deduce a minimal size requirement for heparin bridging AT to thrombin. Based upon the sequence homology between AT and HCII, upon localization of their heparin binding sites to the D helix region, and from our comparison of molecular models of HCII-thrombin and AT-thrombin complexes in the presence of heparin, we would predict that AT-thrombin and HCII-thrombin complexes require heparin molecules of a similar length to successfully bridge the serpin and thrombin together.
Heparin Promotes Inactivation of a Reactive Site Mutant

**DISCUSSION**

Mutations in the reactive site loop of numerous serpins have altered their proteinase specificity and inhibitory activity (Refs. 19–22, 36, 37, for a review see Ref. 38). The regions studied most intensively have been the P1-P1' residues and the residues of the hinge region (P9-P15), where the reactive site loop turns and joins the A β-sheet as strand 5A. A common prediction from these studies would be that increased inhibitory activity (measured by $k_2$ values) would give lower SI values. Furthermore, increased substrate activity would be correlated with reduced inhibitory activity. In 1990, Derechin et al. (25) prepared an HCII mutant with a P1 residue substitution of Leu to Arg with the expectation that this form of rHCII would be a superb thrombin inhibitor (measured by $k_2$ values). In the absence of glycosaminoglycans, their mutant was about 100-fold more active than wild type recombinant HCII. However, the maximal rate enhancement of thrombin inhibition by glycosaminoglycans was lower with L444R-rHCII than with wt-rHCII.5 We became interested in further exploring the mechanism of action of this 'ideal' thrombin inhibitor in the absence and the presence of glycosaminoglycans. The present study shows that substitution of an Arg at the P1 Leu position of rHCII increases its thrombin inhibitory activity in the absence of glycosaminoglycan but also increases its substrate activity. Although heparin and dermatan sulfate both accelerate α-thrombin inhibition by L444R-rHCII to levels slightly lower than with wt-rHCII, heparin, but not dermatan sulfate, promotes the proteolytic inactivation of L444R-rHCII by α-thrombin.

Heparin-induced proteolytic inactivation of a related serpin, AT, was described previously by Olson (23). AT was shown to be an inhibitor in the presence of heparin at physiological ionic strength, but at lower ionic strength, heparin promoted the substrate pathway of AT with thrombin (23). Low ionic strength presumably increased heparin binding to an anti-thrombin-thrombin intermediate complex, which normally exhibits low heparin affinity (39–42). Furthermore, only heparin molecules long enough to bridge thrombin and AT promoted the substrate pathway. This study showed that the formation of cleaved AT occurred through a substrate pathway different than that of the slow spontaneous dissociation of stable anti-thrombin-thrombin complexes (43). Our results suggest that unlike AT, heparin affects both the proteolytic inactivation of excess L444R-rHCII by thrombin and accelerates the slow dissociation of L444R-rHCII-thrombin complexes at physiological ionic strength. The result of extended time course incubations of L444R-rHCII with α-thrombin in the presence of heparin reflects the slow release of active thrombin from ternary complexes composed of L444R-rHCII, thrombin, and heparin. One plausible explanation is that the heparin-promoted ternary complex places L444R-rHCII in a favorable position for cleavage by α-thrombin; thus, initially, inactivation of L444R-rHCII and inhibition of thrombin are occurring simultaneously and over time, the remaining L444R-rHCII that is complexed with thrombin is slowly cleaved, releasing active thrombin.

AT requires ternary complex formation not only for heparin-accelerated thrombin inhibition but also for heparin-accelerated inactivation by thrombin (10, 23, 33). With L444R-rHCII, ternary complex formation mediated by heparin appears necessary only for the proteolytic inactivation by α-thrombin. With R93A,R97A,R101A-thrombin, which is defective in glycosaminoglycan binding and ternary complex formation, heparin accelerates its inhibition by L444R-rHCII but not L444R-rHCII inactivation. Dermatan sulfate, which cannot mediate ternary complex formation (18), does not promote inactivation of L444R-rHCII, regardless of which proteinase is used. The requirement of ternary complex formation for L444R-rHCII inactivation is further supported by the data with small heparin oligosaccharides of 8 or 12 units. These heparin fragments are unable to bridge both HCII and thrombin, and they accelerate α-thrombin inhibition by L444R-rHCII (and wt-rHCII) without promoting L444R-rHCII inactivation. L444R-rHCII inactivation by α-thrombin is promoted by heparin oligosaccharides ≥ 16 units, close to the putative minimal length capable of bridging HCII to thrombin (34, 35).4 The detrimental inactivating action of the longer heparin oligosaccharides on L444R-rHCII was absent with R93A,R97A,R101A-thrombin. Thus, ternary complex formation between heparin, thrombin, and L444R-rHCII, rather than heparin binding to L444R-rHCII per se, appears to cause the substrate-like activity of L444R-rHCII.

The differences observed between this study and Olson’s study (23) are not surprising because HCII and AT appear to follow different mechanisms of heparin-accelerated thrombin inhibition. Ternary complex formation is absolutely required for the heparin-accelerated inhibition of thrombin by AT (10). Binding of heparin to AT also results in a conformational change in the reactive site loop that slightly increases the rate of thrombin inhibition (44–46). Glycosaminoglycan-accelerated thrombin inhibition by HCII occurs through an allosteric mechanism, whereby binding of the glycosaminoglycan to HCII facilitates the interaction of a hirudin-like amino-terminal do-

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5 The original description comparing L444R-rHCII with wt-rHCII had a heparin-accelerated α-thrombin inhibition rate for wt-rHCII that was ~100-fold lower than expected, making this comparison difficult to interpret (25). A point mutation (H159R) was subsequently found from a polymerase chain reaction artifact in the HCII cDNA used in the original study which affected heparin-accelerated but not dermatan sulfate-accelerated thrombin inhibition by rHCII (51).
main of HCII with anion-binding exosite 1 of thrombin (18). Termary complex formation occurs in the presence of heparin but makes only a minor contribution in HCII inhibition of thrombin. Dermatan sulfate, which binds thrombin only weakly and thus does not participate in ternary complex formation, is as effective as heparin in accelerating thrombin clotting. We also thank Dougald M. Monroe and Susannah J. Parker, K. A., and Tollefsen, D. M. (1985) J. Biol. Chem. 260, 3561–3565

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