Arginine 200 of Heparin Cofactor II Promotes Intramolecular Interactions of the Acidic Domain

IMPLICATION FOR THROMBIN INHIBITION*

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Heparin cofactor II (HCII) is presumed to be a physiological inhibitor of the serine proteinase thrombin. The reaction between HCII and thrombin is quite unique, because it involves an unusual HCII-reactive site loop sequence of Leu444-Ser445, requires the presence of glycosaminoglycans for optimal activity and involves a protein-protein interaction besides the reactive site loop-active site interaction characteristic of serine proteinase inhibitor-serine proteinase pairs. Two mutations at a unique HCII residue, Arg200 → Ala or Glu, were generated by site-directed mutagenesis. The mutations did not alter either HCII binding to heparin-Sepharose or HCII inhibition of thrombin in the presence of heparin or dermatan sulfate, suggesting that Arg200 is not part of the glycosaminoglycan binding site of HCII. In the absence of glycosaminoglycan, there was a significant increase in α-thrombin inhibition by the Arg200 mutants as compared with wild type recombinant HCII (wt-rHCII), whereas inhibition rates with chymotrypsin were identical. Inhibition of γ2-thrombin, which lacks anion-binding exosite 1 (ABE-1), the region of α-thrombin that interacts with the acidic domain of HCII, was significantly reduced compared with α-thrombin, but the reduction was more dramatic for the Arg200-rHCII mutants. Hirugen, which binds to ABE-1 of α-thrombin, also diminished inhibition of α-thrombin by the Arg200-rHCII mutants to nearly wt-rHCII levels. Both Arg200-rHCII mutants had significantly increased kₐ values as compared with wt-rHCII, whereas the kₐ rates were unchanged. Collectively, these results suggest that the improved inhibitory activity of the Arg200-rHCII mutants is mediated by enhanced interactions between the acidic domain and ABE-1, resulting in an increased HCII-thrombin association rate.

Serine proteinase inhibitors (serpins)† are a superfamily of proteins whose primary function is to regulate the proteolytic activity of serine proteinases involved in such processes as coagulation, fibrinolysis, complement activation, inflammation, and tumor metastasis (Refs. 1 and 2 and reviewed in Ref. 3). Heparin cofactor II (HCII) belongs to a subfamily of serpins whose activity is greatly accelerated upon binding to glycosaminoglycans, such as heparin, heparan sulfate, and dermatan sulfate (4, 5). In vivo, glycosaminoglycan-containing proteoglycans found on cell surfaces and in extracellular matrix serve to accelerate this reaction (6–8). The physiological target of HCII is presumed to be thrombin, a pluripotent coagulation proteinase that participates in inflammation and wound healing processes based on its chemotactic, mitogenic, and cytokine-like action on vascular smooth muscle cells, monocytes, and fibroblasts (9–12). Thrombin activity generated during blood coagulation is regulated primarily by antithrombin, a heparin-binding serpin that inhibits most coagulation proteinases. However, extravascular thrombin activity associated with inflammation and wound healing processes is thought to be regulated by HCII, which exhibits remarkable specificity for thrombin (13, 14).

HCII possesses several characteristics for thrombin specificity that render it unique among heparin-binding serpins. HCII is the only heparin-binding serpin that binds dermatan sulfate to accelerate thrombin inhibition (15). The heparin and dermatan sulfate binding sites, which are distinct but overlapping, are localized primarily in the D-helix region (16–19). HCII is also unique because it has Leu444 at the P1 position, whereas most thrombin-inhibiting serpins (like antithrombin and protein C inhibitor) and typical thrombin substrates contain an Arg at the P1 site (20, 21). The P1 residue, which is located on an exposed loop that interacts with the active site of the proteinase, determines in large part the proteinase specificity of the serpin (for a review, see Ref. 3). The presence of a P1 Leu in HCII enables it to inhibit chymotrypsin, a nonphysiological target, more rapidly than thrombin in the absence of glycosaminoglycans (22). HCII with an Arg substituted for the P1 Leu no longer inhibits chymotrypsin (23). Interestingly, this mutant has an increased thrombin inhibition rate in the absence of glycosaminoglycans, but is also proteolytically inactivated by thrombin in the presence of heparin (23, 24).

Although HCII is ~30% identical in primary structure to antithrombin and other serpins (25), it has an unusual aminoterminal extension of approximately 80 residues (26, 27). The amino terminus contains a tandem repeat of two acidic stretches that are somewhat homologous to the carboxyl terminus of the leech thrombin inhibitor, hirudin (28). The acidic domains of both HCII and hirudin bind to anion-binding exosite-1 (ABE-1) of thrombin (29). The acidic domain of HCII is also thought to bind intramolecularly to the D-helix, the glycosaminoglycan binding site on HCII. Binding of glycosaminoglycans to the D-helix is thought to displace the acidic domain...
and promote its interaction with ABE-1 of thrombin (30, 31). The acidic domain interaction with ABE-1 appears to be the driving force for the rapid inhibition of thrombin by HCII in the presence of glycosaminoglycans and compensates for the unfavorable P1 Leu residue (19, 30, 32).

We are studying HCII to better understand the role of specific amino acid residues in this unique thrombin inhibition reaction. A comparison of serpin sequences shows that HCII is the only heparin-binding serpin with a basic residue at Arg200 (2). Arg200 of HCII is in strand 2 of β sheet A, adjacent to the dermatan sulfate-binding region of the D-helix; thus it may be poised to play a unique role in regulating HCII activity. In this study, we have found that Arg200 promotes the interaction of the acidic domain with HCII in the absence of glycosaminoglycans, but is not involved in glycosaminoglycan binding. We propose that the function of Arg200 is to keep HCII essentially “inactive” when it is circulating in the blood stream unbound to glycosaminoglycans.

**EXPERIMENTAL PROCEDURES**

*Generation and Expression of R200A-HCII and R200E-HCII—Human recombinant HCII (wt-HCII) (cDNA kindly provided by Dr. Douglas M. Tollefsen, Washington University School of Medicine, St. Louis, MO) was previously expressed in the baculovirus expression system and characterized (33). To generate R200A-HCII and R200E-HCII, site-directed mutagenesis was performed by the method of Kunkel on full-length human HCII cDNA subcloned via flanking EcoRI sites into the pBluescript SK+ mutagenesis and cloning vector (Stratagene) (34). A degenerate oligonucleotide was used to introduce two point mutations in the HCII cDNA (5′-CCG GGC CCG-3′), which caused substitutions of Ala or Glu, respectively, at residue Arg200. The mutations were identified by DNA sequencing (Sequenase® Version 2.0, U. S. Biochemical Corp.), and positive clones were sequenced in full to verify the absence of erroneously introduced mutations. Subcloning into baculoviral transfer vector pVL1392 (Pharmingen) and cotransfection with linearized BaculoGold™ (Pharmingen) Autographica californica nuclear polyhedrosis virus in Spodoptera frugiperda (Sf9, Invitrogen) insect cells was performed as described previously (33). The infectious medium was collected 4 days post-transfection and was further amplified in fresh Sf9 cells. Production of HCII was verified by immunoblot analysis of whole cell lysates from infected cells. Sf9 cells were maintained in spinner flasks of High-Five™ cells was performed as described previously (33). The virus was added to the High-Five™ media at a multiplicity of infection (M.O.I.) of 10 and the cell supernatant was collected after 2 days and centrifuged at 10,000 g for 5 min to remove cell debris. The medium was diluted with an equal volume of HPN buffer, pH 6.5 (20 mM HEPES (Boehringer Mannheim), 0.1% polyethylene glycol 8000 (Sigma), 0.05% NaN3) and batch-adsorbed with 0.5 ml of heparin-Sepharose beads (Pharmacia Biotech Inc.) for 1 h at 4°C. The HCII was eluted from the heparin-Sepharose with 0.5 M NaCl in HPN buffer, pH 7.4, after two washes in 75 mM NaCl in HPN, pH 6.5, buffer. The heparin-Sepharose eluate was diluted in HPN buffer to a final concentration of 50 mM NaCl, pH 7.8, and batch-adsorbed with 0.5 ml of Q-Sepharose (Sigma) for 1 h at 4°C. After two washes in 50 mM NaCl, the protein was eluted with 0.5 M NaCl in HPN, pH 7.8, buffer, aliquoted, and stored at -70°C.

*Quantification of HCII—A direct enzyme-linked immunosorbent assay using a mouse anti-HCII monoclonal antibody and a goat IgG-conjugated to alkaline phosphatase (Sigma) was used as described previously to measure HCII concentrations (33). Human plasma HCII, purified as described previously (35), was used for the standard curve. Assays were performed in 96-well microtiter plates and color development was monitored at 405 nm on a Microtek plate reader (Molecular Devices). The competition between substrate and inhibitor for thrombin can be described by the following scheme (39).

**Scheme 1**

where E, S, P, and I represent the enzyme (thrombin), substrate (S-Val-Leu-Arg-p-nitroanilide), product (p-nitroaniline), and inhibitor (HCII), respectively. \( K_e \) and \( k_{cat} \) are the Michaelis and catalytic constants for the enzyme-substrate reaction, respectively, and \( k_4 \) and \( k_5 \) are the association and dissociation rate constants for the thrombin-HCII reaction. This kinetic scheme assumes reversible binding, which the HCII-thrombin interaction appears to follow under the conditions established in this assay, since \( v_s \) was significant at all concentrations of serpins. The progress curve of product formation for this mechanism is given by Equation 1 (40),

\[
P = v_s + \left[ (v_e - v_s)/k_e^\prime \right](1 - e^{-k_4 t})
\]

(Eq. 1)

with a linear 1.0 ml/min salt gradient of 50 mM to 0.5 M NaCl. 1.0 µg of recombinant HCII was loaded, and 20 × 1 ml fractions were collected. The fractions were analyzed by thrombin inhibition assays in the presence of 10 µM/ml heparin, as described below.

*Protease Inhibition Assays—Human α-thrombin was isolated and prepared as described previously (36), and bovine chymotrypsin was purchased from Sigma. γT-Thrombin was prepared by limited proteolysis of plasma-derived α-thrombin with t-1-toyamido-2-phenylbenzyl chloromethyl ketone-treated trypsin (Cooper Biomedical) (I37) as modified in (32). The activity of γT-thrombin was then verified by chromogenic substrate cleavage and fibrin clotting assays. Chromogenic substrates were tosyl-Gly-Pro-Arg-p-nitroanilide (150 µM; Boehringer Mannheim) for thrombin and N-sucinyl-Ala-Ala-Pro-Phe-p-nitroanilide (500 µM; Sigma) for chymotrypsin. Hirugen (residues 53–64) was from Multiple Peptide Systems. A control peptide corresponding to the reverse sequence of the HCII acidic domain (residues 47–61) was synthesized on a Synergy™ peptide synthesizer (Applied Biosystems). Peptide inhibition assays for wt-HCII and the R200-HCII mutants were performed in 96-well enzyme-linked immunosorbent assay plates (previously coated with 2 mg/ml bovine serum albumin) at room temperature in HPN, pH 7.4, buffer containing 2 mg/ml bovine serum albumin. In the absence of glycosaminoglycan, 100 nM HCII and 1 nM thrombin were incubated together for 30–180 min in the presence of 50 µg/ml polybrene. The thrombin-HCII association time was 90 min for assays performed in the presence of hirugen. For the heparin (Biosynthetic, Osaka, The Netherlands) and dermatan sulfate (Calbiochem; nitroic acid-treated to remove contaminating heparin and heparan sulfate) template curves, 5 nM HCII, and 0.5 nM thrombin were incubated together for 20 s. The reactions were quenched by the addition of a chromogenic substrate solution containing 3 mg/ml polybrene, and color development was monitored at 405 nm on a Vmax microplate reader. Second order inhibition rate constants (k2, M−1 min−1) were measured in triplicate on two to four different preparations of rHCII. The rates were obtained under pseudo-first order reaction conditions as described previously and were calculated using the equation: $k_2 = (-a/\alpha t)/[I]$ where $\alpha$ is the residual protease activity, $t$ is the time, and [I] is the HCII concentration (38).

*Slow Binding Kinetics—*Slow binding kinetic assays were performed in 96-well bovine serum albumin-coated enzyme-linked immunosorbent assay plates at room temperature in HPN, pH 7.4, buffer containing 2 mg/ml bovine serum albumin. A low-evaporation lid coated with anti-fogging agent (Molecular Devices) was used to minimize evaporation. Chromogenic substrate was S-2266 (s-Val-Leu-Arg-p-nitroanilide; Kabi Pharmacia). S-2266 was selected from several chromogenic substrates tested because its high K2 (262 µM; Ref. 39) permitted adequate inhibition of thrombin by HCII under the experimental conditions. Color development was monitored as described above. The reaction was started by the addition of 0.5 nM thrombin to wells containing varying concentrations of HCII (25–400 µM range) and 500 µM S-2266, and readings were taken at 10-min intervals for 8 h. Data points were excluded from the analysis when the level of substrate utilization exceeded 10%. Control assays indicated that the thrombin was stable during the course of the experiment.

The competition between substrate and inhibitor for thrombin can be described by the following scheme (39).

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Role of Arg^{200} in Heparin Cofactor II

RESULTS

Generation and Expression of R200A-rHCII and R200E-rHCII—R200A-rHCII and R200E-rHCII were engineered, expressed, and purified as described under “Experimental Procedures.” The protein yield from a typical preparation was essentially the same for wt-rHCII (50–100 μg purified from two T150 flasks). Immunoblot analysis and SDS-PAGE demonstrated the mutants had the same electrophoretic mobility as wt-rHCII (data not shown).

Heparin-Sepharose Affinity Chromatography of wt-rHCII and the Arg^{200} Mutants—Gradient salt elution from heparin-Sepharose was performed to determine whether the mutations altered the apparent affinity of HCII for immobilized heparin. Since the intermolecular interactions between heparin and HCII are thought to be primarily ionic, the concentration of NaCl required to elute the proteins is a measure of their relative affinity (41). There were no significant differences in the NaCl concentrations (mM) required to elute the proteins: 270 ± 40 for wt-rHCII (n = 3), 260 ± 20 for R200A-rHCII (n = 4), and 270 ± 40 for R200E-rHCII (n = 3).

The optimal glycosaminoglycan (GAG) concentration is shown in parentheses below the inhibition rate.

Inhibition rate constants (k_2, M^{-1} min^{-1}) for wt-rHCII, R200A-rHCII, and R200E-rHCII with α-thrombin, γ_T-thrombin, and chymotrypsin are summarized below. For glycosaminoglycan-accelerated inhibition, template curves were performed in the presence of increasing concentrations of glycosaminoglycan, and the maximal inhibition rates were taken from an average of the curves. Assays were performed as described under “Experimental Procedures,” and values are expressed as the mean ± S.D. of four to seven determinations.

TABLE I

| Glycosaminoglycan-accelerated α-Thrombin Inhibition—| wt-rHCII | R200A-rHCII | R200E-rHCII |
|---------------------------------------------------|----------|-------------|-------------|
| α-Thrombin                                        | 2.7 ± 1.1 × 10^6 | 9.5 ± 3.2 × 10^4 | 15.4 ± 3.8 × 10^4 |
| - Heparin                                         | 4.1 ± 0.4 × 10^6 | 5.5 ± 1.4 × 10^6 | 4.6 ± 0.8 × 10^6 |
| (200 μg/ml)                                       | (500 μg/ml) | (500 μg/ml) | (500 μg/ml) |
| + Dermatan sulfate                                 | 1.2 ± 0.2 × 10^6 | 1.3 ± 0.3 × 10^6 | 1.1 ± 0.2 × 10^6 |
| (1000 μg/ml)                                      | (1000 μg/ml) | (1000 μg/ml) | (1000 μg/ml) |
| γ_T-Thrombin                                      | 1.4 ± 0.5 × 10^6 | 3.3 ± 1.2 × 10^6 | 3.2 ± 0.9 × 10^6 |
| Chymotrypsin                                      | 1.6 ± 0.2 × 10^6 | 1.7 ± 0.9 × 10^6 | 1.7 ± 1.0 × 10^6 |

* p < 0.05 compared with wt-rHCII.
* The optimal glycosaminoglycan (GAG) concentration is shown in parentheses below the inhibition rate.
* a b c

Fig. 1. Glycosaminoglycan-accelerated α-thrombin inhibition by wt-rHCII and the Arg^{200} mutants. α-Thrombin (0.5 nm) was incubated with 5 nm wt-rHCII (●), R200A-rHCII (△), or R200E-rHCII (□) in the presence of increasing concentration of heparin (A) or dermatan (B) sulfate. The inhibition rate constant (k_2) is plotted on the y axis versus the glycosaminoglycan concentration on the x axis. The above curves represent the mean values of four to seven determinations.

where P is the amount of product at time t, k’ is an apparent first-order rate constant, and v_i and v_o are the initial and steady-state velocities, respectively. A plot of k’ versus inhibitor concentration (Equation 2) yields a slope equal to k’a, the apparent association rate, and a y intercept equal to k_d (39).

k’ = k_d + k’a · I

The k’a is related to the true rate constant by Equation 3.

k_d = k’a (1 + (S/K_m))

v_o was determined from a linear fit to the first 10 measurements of the thrombin alone control for each row on the microplate, and this value was then used in Equation 1. The data from the progress curves were fitted to Equation 1 by nonlinear regression using software written for SlideWrite. The values for k’ at each HCII concentration were then used to determine k’a and k_d by a linear fit of the data to Equation 2. The true k_d was then determined from Equation 3.

FIG. 1. Glycosaminoglycan-accelerated α-thrombin inhibition by wt-rHCII and the Arg^{200} mutants.
wt-rHCII (p < 0.05), they are both now only 2.3-fold higher than wt-rHCII (Table I).

The Effect of Hirugen on Inhibition of α-Thrombin in the Absence of Glycosaminoglycan—To further examine the role of ABE-1 in the enhanced activity of the Arg200 mutants, the rate of α-thrombin inhibition in the presence of hirugen was determined. By binding directly to ABE-1, hirugen interferes with acidic domain-ABE-1 interactions and reduces the rate of α-thrombin inhibition by HCII (33). Increasing amounts of hirugen resulted in a dose-dependent blockage of the HCII-thrombin reaction, with a maximal response at 100 μM for all three HCII proteins (data not shown). Hirugen has a greater effect on the ability of the Arg200 mutants to inhibit α-thrombin than it does on wt-rHCII. At 100 μM hirugen, the α-thrombin inhibition rates (k₂, M⁻¹ min⁻¹) for R200A-rHCII and R200E-rHCII were reduced to 2.2 ± 0.8 × 10⁴ and 2.8 ± 0.6 × 10⁴, respectively, as compared with 1.0 ± 0.4 × 10⁴ for wt-rHCII (Fig. 2). This effect is specific since a control peptide that was highly negatively charged could not block the HCII-thrombin reactions (Fig. 2).

Slow Binding Kinetics for the Inhibition of α-Thrombin—To determine whether the enhanced activity of the mutants is due to an increased HCII-thrombin association rate (kₐ) or to a decreased HCII-thrombin dissociation rate (k₉), a slow binding kinetics assay was developed for HCII inhibition of thrombin. In slow binding kinetics, the inhibitor and substrate compete for binding to the proteinase. Shown in Fig. 3 are representative time courses of α-thrombin inhibition at different HCII concentrations. Analysis of the raw data yielded a good fit to Equation 1, typically with correlation coefficients (r²) of 0.999. The values obtained for k' were used to estimate kₐ and k₉, as described under “Experimental Procedures.” A representative plot of k' versus HCII concentration, shown in Fig. 4, demonstrates the significantly increased kₐ of the Arg200 mutants as compared with wt-rHCII. The kₐ values (Table II) for R200A-rHCII and R200E-rHCII are 5- and 9-fold higher, respectively, than wt-rHCII. Interestingly, the calculated k₉ for all the HCII variants are not significantly different (p > 0.05).

DISCUSSION

We initially hypothesized that Arg²⁰⁰ of HCII would be important for the glycosaminoglycan-mediated inhibitory activity of HCII with thrombin because no other heparin-binding serpin has a basic residue at the analogous position. Furthermore, Arg²⁰⁰ is adjacent to the D-helix region of HCII, previously established to be important for activity. We expected that changing this positively charged residue to an uncharged alanine or a negatively charged glutamic acid would decrease both heparin-Sepharose affinity and glycosaminoglycan-mediated thrombin inhibition. However, mutations at Arg²⁰⁰ did not affect either heparin binding or heparin- and dermatan sulfate-accelerated thrombin inhibition. Although slightly higher concentrations of heparin were required to optimally stimulate inhibition of thrombin by the mutants, the maximal inhibition rate was nearly identical to wt-HCII. Mutation of critical D-helix residues in previous studies has led to both significantly decreased glycosaminoglycan-accelerated thrombin inhibition and reduced heparin-Sepharose affinity (16–18). The
hirugen, the mutants are still more active than wt-rHCII, most indicating that the acidic domain still interacts with the remaining inhibition rates with between the HCII acidic domain and ABE-1 of thrombin, since mutants appears to be caused by an enhanced interaction be-

significantly increased. The increased inhibitory activity of the mutants was accelerated by hirugen. We have shown previously that dermatan sulfate accelerates the presence of both heparin and dermatan sulfate and that conformation, further implicating a role for the acidic domain domain of HCII or glycosaminoglycans for inhibition) indicate increased activity of the mutants to almost the same level as that of Arg200 mutants. The values represent the mean ± S.D. of four to five determinations.

Table II

| k_a (μM) | k_d (μM) | k_a/k_d ratio |
|----------|----------|---------------|
| wt-rHCII | 1.3 ± 0.3 x 10^4 | 1.5 ± 0.4 x 10^3 |
| R200A-rHCII | 6.5 ± 1.8 x 10^4 | 1.6 ± 0.8 x 10^3 |
| R200E-rHCII | 11.4 ± 1.7 x 10^4 | 1.6 ± 1.0 x 10^3 |

a p < 0.05 compared with wt-rHCII.
b Not statistically different from wt-rHCII.

finding that R200A-rHCII and R200E-rHCII are fully active in the presence of both heparin and dermatan sulfate and that their heparin-Sepharose elution profiles are nearly identical indicates that Arg200 is not critical for glycosaminoglycan binding.

Interestingly, the thrombin inhibition rates of R200A-rHCII and R200E-rHCII in the absence of glycosaminoglycans were significantly increased. The increased inhibitory activity of the mutants appears to be caused by an enhanced interaction between the HCII acidic domain and ABE-1 of thrombin, since inhibition rates with γ2-thrombin are more significantly reduced for the mutants than for wt-rHCII. Furthermore, the addition of hirugen to the HCII-thrombin reaction reduces the activity of the mutants to almost the same level as that of wt-rHCII. However, even with γ2-thrombin or α-thrombin/hirugen, the mutants are still more active than wt-rHCII, most likely because the acidic domain binding site on ABE-1 is not totally removed in γ2-thrombin nor completely blocked by hirugen. We have shown previously that dermatan sulfate accelerates γ2-thrombin inhibition by HCII by 30-fold, indicating that the acidic domain still interacts with the remaining portions of ABE-1 in a productive manner (32). Finally, the identical inhibition rates of wt-rHCII and the Arg200 mutants with chymotrypsin (which does not require either the acidic domain of HCII or glycosaminoglycans for inhibition) indicate that the reactive site loop has not been altered to an "activated" conformation, further implicating a role for the acidic domain in the increased activity of the mutants.

To determine whether the enhanced interaction between the acidic domain of the Arg200 mutants and ABE-1 of thrombin resulted in an increased association rate (k_a) or a decreased dissociation rate (k_d), a slow binding kinetics assay, previously used to characterize other serpin-proteinase reactions, was adapted for HCII-thrombin. These studies revealed that the increased inhibitory activity of the mutants is due to an increased association rate (k_a) with thrombin. This suggests that the acidic domain is more involved in the initial "handshake" between HCII and thrombin than in the stabilization of the bimolecular serpin-proteinase complex. These results imply that for wild type HCII, Arg200 helps to maintain acidic domain-HCII intramolecular interactions, thus attenuating thrombin inhibition in the absence of glycosaminoglycans.

Previous studies have led to the hypothesis that the D-helix is the intramolecular binding site for the acidic domain in HCII. A study instrumental in developing this hypothesis showed that amino-terminal truncation mutants of HCII that are missing one (Δ67-rHCII) or both (Δγ4-rHCII) acidic repeats have greatly increased heparin-Sepharose affinity (30). A D-helix double mutant (R184Q/K185Q-rHCII) generated by site-directed mutagenesis was shown to have significantly decreased heparin-Sepharose affinity, enhanced ability to form SDS-PAGE-stable complexes with thrombin in the absence of glycosaminoglycans, but reduced ability to form complexes in the presence of glycosaminoglycans (19). This study suggested that the increased formation of R184Q/K185Q-rHCII-thrombin complexes in the absence of glycosaminoglycans was due to a disruption in acidic domain interactions with R184 and K185, resulting in increased acidic domain interaction with ABE-1.

Our results imply that in the absence of glycosaminoglycan, the presence of an Arg at residue 200 either promotes acidic domain interactions with the D-helix of HCII or binds the acidic domain directly. Changing Arg200 to an Ala or Glu would alter the "equilibrium" and favor the interaction of the acidic domain and ABE-1, resulting in increased thrombin inhibition. These results are also consistent with the hypothesis that the mechanism of acidic domain binding to ABE-1 is part of the HCII-thrombin association reaction. Finally, this interaction would further contribute to maintaining blood plasma HCII in a conformation that would be a poor thrombin inhibitor in the absence of glycosaminoglycans.

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