Aspartic Acid Residues 72 and 75 and Tyrosine-sulfate 73 of Heparin Cofactor II Promote Intramolecular Interactions during Glycosaminoglycan Binding and Thrombin Inhibition*

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We used site-directed mutagenesis to investigate the role of Glu59, Asp60, Asp61, Tyr-sulfate 62, and Asp63 in the second acidic region (AR2) of the serpin heparin cofactor II (HCII) during formation of the thrombin/HCII complex with and without glycosaminoglycans. E69/Q/D70/N/D71/N recombinant (r)HCII, D72/N/Y73/F/D75/N rHCII, and E69/Q/D70/N/D71/N/D72/N/Y73/F/D75/N rHCII were prepared to localize acidic residues important for thrombin inhibition. Interestingly, D72/N/Y73/F/D75/N rHCII had significantly enhanced thrombin inhibition without glycosaminoglycan (4-fold greater) and with heparin (6-fold greater), showing maximal activity at 2 µg/ml heparin compared with wild-type recombinant HCII (wt-rHCII) with maximal activity at 20 µg/ml heparin. The other rHCII mutants had lesser-enhanced activities, but they all eluted from heparin-Sepharose at significantly higher ionic strengths compared with wt-rHCII. Neutralizing and reversing the charge of Asp72, Tyr-sulfate 73, and Asp75 were done to characterize their individual contribution to HCII activity. Only Y73K rHCII and D75K rHCII have significantly increased heparin cofactor activity compared with wt-rHCII; however, all of the individual rHCII mutants required substantially less glycosaminoglycan at maximal inhibition than did wt-rHCII. Inhibition of either α-thrombin/hirugen or γ-thrombin (both with an altered anion-binding exosite-1) by the AR2 rHCII mutants was similar to wt-rHCII. D72/N/Y73/F/D75/N rHCII and D75K rHCII were significantly more active than wt-rHCII in a plasma-based thrombin inhibition assay with glycosaminoglycans. These results indicate that improved thrombin inhibition in the AR2 HCII mutants is mediated by enhanced interactions between the acidic domain and anion-binding exosite-1 of thrombin and that AR2 may be a “molecular rheostat” to promote thrombin inhibition in the presence of glycosaminoglycans.

The serine protease thrombin is a critical component in coagulation, inflammation, and wound healing (1–9). Thrombin activity must be carefully regulated to maintain an appropriate balance within the vasculature. One mechanism of thrombin regulation is by serine protease inhibitors (serpins) such as antithrombin (ATIII) and heparin cofactor II (HCII) (10–14). Heparin cofactor II and ATIII belong to a sub-class of serpins whose activity is greatly enhanced upon binding to glycosaminoglycans like heparin and heparan sulfate (HCII and ATIII) and dermatan sulfate (HCII) (15–18). These glycosaminoglycans are found in vivo on cell surfaces and in extracellular matrix to support these inhibition reactions (19–22).

Heparin cofactor II has several features for thrombin inhibition and specificity that render it novel among heparin-binding serpins (24–26). Heparin cofactor II has an atypical Leu444 reactive center residue, whereas the more typical Arg is found in thrombin inhibitors like ATIII or protein C inhibitor (10, 12, 19). The inhibition of thrombin by HCII is enhanced by both heparin and dermatan sulfate, and the glycosaminoglycan binding site is primarily contained within the β-helix region of HCII (15, 27–34). Although HCII is ~30% identical in sequence to antithrombin and other serpins, it has a unique N-terminal extension of ~80 residues that contains a tandem repeat of negatively charged acidic residues (9 Asp, 5 Glu, and 2 Tyr) (25, 26). Interestingly, the acidic domain of HCII is homologous to the C terminus of the leech anticoagulant protein hirudin, which is a potent thrombin inhibitor. Within the acidic domain of HCII, the two regions are designated acidic region 1 (AR1) from residues 49–62 (4DWIGGEEDDYLD26) and acidic region 2 (AR2) from residues 63–75 (63LEKIFSEDDYID75). There is substantial evidence that the acidic domain of HCII is involved in facilitating thrombin inhibition, especially in the presence of glycosaminoglycans (27, 29, 30). Ragg et al. (29, 30) initially examined the role of the acidic domain in HCII by either deleting or neutralizing various acidic amino acids and found that AR1 and AR2 have separate functions for thrombin inhibition. Van Deerlin and Tollefsen (27) demonstrated that, by deleting portions of the acidic domain (AR1 and AR2), HCII was a less effective inhibitor of thrombin; interestingly, these mutants also had substantially enhanced binding to heparin compared with wild-type (wt) recombinant (r)-HCII. These studies suggested that the acidic domain of HCII both is involved in and is imperative for effective thrombin inhibition. The two current models to

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‡ The abbreviations used are: serpin, serine protease inhibitor; HCII, heparin cofactor II; ATIII, antithrombin III; wt, wild-type; r recombinant; E69/Q/D70/N/D71/N/D72/N/Y73/F/D75/N, QNNNFN; exosite-1, anion-binding exosite-1; BSA, bovine serum albumin; PEG, polyethylene glycol 8000; Gly-Pro-Arg-NH₂, tosyl-Gly-Pro-Arg-nitroanilide; Ala-Ala-Pro-Phe-NA, succinyl-Ala-Ala-Pro-Phe-p-nitroanilide; DEF, normal heparin cofactor II; NT, heparin cofactor II; HCII, antithrombin III; DEF, human antithrombin III-deficient plasma; DEF/REF, 1:1 mix of DEF and REF.
describe the mechanism for glycosaminoglycan-accelerated inhibition of thrombin by HClII are the “double-bridge” and the “allosteric” mechanisms (31, 35). In both models it is assumed that the acidic domain position is altered following glycosaminoglycan binding to the α-helix region of HClII, thereby allowing the N-terminal acidic domain to bind thrombin anion-binding exosite-1 (exosite-1) (27, 28, 31, 33, 34, 35, 37).

This paper investigates the role of six AR2 acidic residues in HClII during thrombin inhibition in the absence and presence of glycosaminoglycans and with thrombin derivatives with and without a functional exosite-1. The results indicate that Asp75, Tyr-sulfate, and Asp75 promote intramolecular interactions of the acidic domain with HClII in the absence of glycosaminoglycans, and altering the charge of these acidic residues substantially changes glycosaminoglycan-dependent activities of HClII.

EXPERIMENTAL PROCEDURES

Materials—Human plasma α-thrombin and thrombin derivatives were prepared and purified in our laboratory as previously described (38, 39). We prepared normal hemostasis reference plasma (catalog no. 258N) were purchased from Sigma Chemical Co. Tosyl-Gly-Pro-Arg-4-nitroanilide acetate (La Jolla, CA) and was nitrous acid-treated to remove contaminating synth (Oss, The Netherlands). Dermatan sulfate was from Calbiochem (Indianapolis, IN), and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sucAla-Ala-Pro-Phe-NA) was from Sigma. Hirugen and the reverse peptide to HClII’s acidic domain were synthesized and purified by the University of North Carolina at Chapel Hill Peptide Synthesis and Protein Core Facility.

Mutagenesis of Recombinant Proteins—The QuikChange site-directed mutagenesis kit from Stratagene was used to introduce the following mutations E69Q/D70N/D71N, D72N, Y73F, D75N, 5'-CCAGCTCGACGATGTTGATGAAGTTGTTGTTTTGACTGAATATCTTC-3' (forward, Y73K), 5'-CCTGGAGAAGATATTCAGTGAAAGACGACGACTACATCAAAACACAGCTTACAATCGTCGAC-3' (reverse, E69Q/D70N/D71N), 5'-TCGCAAGTGGCATTG AGTAAGTCTGGTTGTAGTAATCATCTCTCCAGGTCCTC-3' (reverse, E69Q/D70N/D71N), 5'-GGAACATTGCAATTCGAAAACACGAGCTCAGACGAGTTCGAC-3' (forward, D75N), 5'-GGAACATTGCAATTCGAAAACACGAGCTCAGACGAGTTCGAC-3' (forward, D75N), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, D72N/E75F/D75N), 5'-GAAGATATCTGCAATTCGAAAACACGAGCTCAGACGAGTTCGAC-3' (forward, D75N), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, D72N/E75F/D75N), 5'-CCTGGAGAAGATATTCAGTGAAAGACGACGACTACATCAAAACACAGCTTACAATCGTCGAC-3' (reverse, Y73K), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, Y73K), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, Y73K), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, Y73K), 5'-GGAAACTGACAGACTGTCGACGATGTTGATGAAGTCGTCGTCTTCATCGAATATC-3' (reverse, Y73K).

Heparin-Sepharose Affinity Chromatography—We determined the relative heparin affinities by using 1–3 μg of HClII protein diluted in 20 mM Hepes, 50 mM NaCl, 0.1% PEG, 0.05% NaN₃, pH 7.4. We ran each protein on a 1-mL HiTrap heparin-Sepharose column equilibrated in 20 mM HEPS, 0.1% PEG, 50 mM NaCl, 0.05% NaN₃, pH 7.4, using a fast-protein liquid chromatography system (Amersham Biosciences, Inc.). After the sample was loaded onto the column, the protein was eluted with a 40-ml gradient of 20 mM Hepes, pH 7.4, from 50 to 1500 mM NaCl, and 0.5–ml fractions were collected. For each fraction, 100 μL was aliquoted onto a 96-well microtiter plate and an enzyme-linked immunosorbent assay was performed. The peak elution ion strength was determined by plotting 405-nm color development and NaCl concentration against the fraction number. All samples were run at least in triplicate using two or more recombinant protein preparations. Second order inhibition rate constants were calculated as described (32, 42, 43).

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Human Plasma Assays—The assay used to evaluate HClII in plasma was designed based on previously published methods (32, 42, 43). We performed this assay using human antithrombin-deficient plasma (REF), human antithrombin-deficient plasma (DEF), or a 50:50 mixture of both types of plasma (REF/DEF) (American Diagnostics) at room temperature in 96-well microtiter plates previously coated with 2 mg/ml BSA (32). We incubated 10 nM wt HClII or mutant HClII with either 1 μg/ml heparin or 0.5 μg/ml dermatan sulfate, in the presence of 2 mg/ml BSA in HNPN, pH 7.4, in the absence of Polysulfate.

In the presence of 1 μg/ml glycosaminoglycan, either hirugen or a control peptide corresponding to the reverse sequence of the acidic domain (residues 47–51) at 20 μg/ml HClII (30 nM) was incubated with 0.5 mM thrombin in the presence of 1 mg/ml Polysulfate and 2 mg/ml BSA in HNPN, pH 7.4. In the presence of either hirugen (5 or 20 μg/ml) or dermatan sulfate (20 or 200 μg/ml), 0.5 nM γC-thrombin was incubated with 10 nM HClII in the presence of 2 mg/ml BSA in HNPN, pH 7.4. Association time courses ranged from 1 to 240 min for α-thrombin, γC-thrombin, and chymotrypsin depending upon the assay conditions. Residual thrombin activity was measured with 150 μL TosGly-Pro-Arg-NA and 1 mg/ml Polysulfate in the presence of 2 mg/ml BSA in HNPN, pH 7.4. The production of HClII was verified by SDS-PAGE and Western blot analysis. Immunoblot and SDS-PAGE confirmed that each mutant HClII had the parental DNA was removed by digestion with DpnI endonuclease and the nicked vector DNA containing the desired mutation was transfected into Epicurian Coli XL1-Blue supercompetent cells (Stratagene). Full-length sequencing of each clone by Sequenase kit (version 2.0, Amersham Biosciences, Inc., Cleveland, OH) confirmed the incorporation of each of these mutations into the HClII-pVL1392 construct. Expression and Purification—HClII and wt HClII were cotransfected with linearized BaculoGold (BD PharMingen) Autographa californica nuclear polyhedrosis virus (AcNPV) into Spodoptera frugiperda (S/F9, Invitrogen) insect cells using established protocols in our laboratory (41). We expressed HClII in HighFive insect cells (Invitrogen, Carlsbad, CA) maintained at 25 °C in Excel 405 media (Invitrogen). Biosamples, Leucine Kinase (RS) as previously described. Three days post-baculovirus transfection, the media was collected and centrifuged to remove cell debris. The protein was purified essentially as described previously (41). Protein was then dialyzed against 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% PEG8000 and 0.02% NaN₃. Protein was aliquoted and stored at −80 °C. The production of HClII was verified by immunoblot analysis of whole cell lysates using a monoclonal antibody to HClII (mAb 2-4-34). The concentration of HClII was determined by enzyme-linked immunosorbent assay using purified plasma HClII as the standard as described previously (32). We obtained −70 μg of recombinant protein of each mutant from 100 mL of HighFive cells in a 200-ml shaker flask infected with the various recombinant viral stocks. Immunoblot and SDS-PAGE confirmed that each mutant HClII had the parental DNA was removed by digestion with DpnI endonuclease and the nicked vector DNA containing the desired mutation was transfected into Epicurian Coli XL1-Blue supercompetent cells (Stratagene). Full-length sequencing of each clone by Sequenase kit (version 2.0, Amersham Biosciences, Inc., Cleveland, OH) confirmed the incorporation of each of these mutations into the HClII-pVL1392 construct. Expression and Purification—HClII and wt HClII were cotransfected with linearized BaculoGold (BD PharMingen) Autographa californica nuclear polyhedrosis virus (AcNPV) into Spodoptera frugiperda (S/F9, Invitro
TABLE I

HCII Inhibition of α-thrombin, γα-thrombin, and Chymotrypsin in the absence of glycosaminoglycans

The data summarized below are the rate constants for α-thrombin (1 nM), γα-thrombin (1 nM), or chymotrypsin (4 nM) inhibition by wild-type and mutant HCII derivatives (100 nM HCII for α-thrombin and γα-thrombin; 40 nM HCII for chymotrypsin). The values represent the mean ± S.D. of three to six separate determinations with at least three different protein preparations. All assays were performed as described under “Experimental Procedures.”

| rHCII                  | α-Thrombin | γα-Thrombin | Chymotrypsin |
|------------------------|------------|-------------|--------------|
|                        | $k_2 \times 10^4$ (μM$^{-1}$ min$^{-1}$) | $k_2 \times 10^4$ (μM$^{-1}$ min$^{-1}$) | $k_2 \times 10^4$ (μM$^{-1}$ min$^{-1}$) |
| Wild-type              | 0.9 ± 0.3  | 2.1 ± 0.7   | 24 ± 7.6     |
| E69Q/D70N/D71N         | 1.1 ± 0.1  | 2.8 ± 1.6   | 29 ± 16 (1.2)* |
| D72N/Y73F/D75N         | 3.8 ± 0.3 (4.2) | 1.0 ± 0.4 (0.5) | 27 ± 16 (1.1) |
| QNNNFN                 | 2.9 ± 0.9 (3.2) | ND (3.0) | 30 ± 5.4 (1.3) |
| D72K                   | 0.9 ± 0.3 (1.0) | 3.1 ± 0.9 (1.5) | 31 ± 5.4 (1.3) |
| Y73F                   | 1.2 ± 0.6 (1.3) | 2.2 ± 0.5 (1.0) | 15 ± 3.9 (0.6) |
| Y73K                   | 1.3 ± 0.5 (1.4) | 3.4 ± 0.7 (1.6) | 21 ± 7.7 (0.9) |
| D75N                   | 1.0 ± 0.4 (1.1) | 2.2 ± 0.7 (1.0) | 23 ± 12 (1.0) |
| D75K                   | 2.3 ± 0.4 (2.6) | 3.4 ± 1.1 (1.6) | 27 ± 7.7 (1.1) |

* Values in parentheses are the ratio calculated as mutant rHCII/wt-rHCII.
** p ≤ 0.05 compared with wt-rHCII.
ND, not determined.

thrombin was added to each well. After 15 s, 150 μl of Gly-Pro-Arg-NA was added, and the residual thrombin activity was measured by color development at 405 nm on a kinetic microplate reader.

Statistical Analysis—The statistical significance of the data was evaluated using Tukey’s Student $t$ test; $p$ values ≤ 0.05 were considered significant.

RESULTS

Inhibition of α-Thrombin, γα-Thrombin, and Chymotrypsin by the AR2 rHCII Derivatives in the Absence of Glycosaminoglycans—Wild-type rHCII inhibits α-thrombin at a rate of $\sim 1 \times 10^4$ μM$^{-1}$ min$^{-1}$ in the absence of glycosaminoglycans, whereas E69Q/D70N/D71N rHCII, D72N/Y73F/D75N rHCII, and QNNNFN rHCII inhibit α-thrombin 1.2-, 4.2-, and 3.2-fold faster than wt-rHCII, respectively (Table I). The results with these “shotgun” HCII mutants suggest that neutralizing Asp$^{72}$, Asp$^{75}$, and Tyr-sulfate$^{73}$ had the biggest influence on thrombin inhibition. Comparing the effect of these individual residues in HCII individually for thrombin inhibition (using neutral and reverse-charge mutations), we found only D75K had a significantly increased rate of α-thrombin inhibition (2.6-fold) when compared with wt-rHCII (Table I). γα-Thrombin (α-thrombin with an altered exosite-1) inhibition by wt-rHCII and all of the AR2 rHCII derivatives was $\sim 2–3 \times 10^4$ μM$^{-1}$ min$^{-1}$ (Table I), which further demonstrates the importance of exosite-1 in thrombin during HCII inhibition. Chymotrypsin inhibition by HCII requires neither the acidic domain nor glycosaminoglycan binding, and we found no significant difference for any of the AR2 rHCII derivatives compared with wt-rHCII (Table I). These results indicate that altering the charge of AR2 in HCII creates a better α-thrombin inhibitor in the absence of glycosaminoglycans, and that Asp$^{72}$, Tyr-sulfate$^{73}$, and Asp$^{75}$ contribute the most to this effect.

Glycosaminoglycan-accelerated α-Thrombin Inhibition and Heparin-Sepharose Binding by the AR2 rHCII Derivatives—With heparin, each of the shotgun rHCII derivatives inhibit α-thrombin 3- to 6-fold faster than wt-rHCII (Fig. 1, top panel). Interestingly, less heparin was required for each rHCII mutant to reach maximal thrombin inhibition (1–2 μg/ml) compared with wt-rHCII (~20 μg/ml) (Table II). In the presence of dermatan sulfate, wt-rHCII inhibits thrombin at a rate of $3.3 \times 10^4$ μM$^{-1}$ min$^{-1}$ (Fig. 1, bottom panel). D72N/Y73F/D75N rHCII inhibits thrombin at a rate 3.3 times faster than that of wt-HCII whereas E69Q/D70N/D71N rHCII and QNNNFN rHCII inhibit thrombin similar to wt-rHCII (Fig. 1, lower panel and Table II). However, the amount of dermatan sulfate needed for maximal thrombin inhibitory activity is also significantly decreased for the shotgun AR2 rHCII derivatives (5–20 μg/ml) as compared with wt-rHCII (100–200 μg/ml) (Table II). The individual AR2 rHCII derivatives of Asp$^{72}$, Tyr-sulfate$^{73}$, and Asp$^{75}$ all required less heparin and dermatan sulfate to reach maximal thrombin inhibition compared with wt-rHCII (Fig. 2); however, only Y73K rHCII and D75K rHCII have significantly increased activity in the presence of heparin whereas none have significantly increased activity in the presence of dermatan sulfate (Table II and Fig. 1). Because the individual Asp$^{72}$, Tyr-sulfate$^{73}$, and Asp$^{75}$ rHCII mutants do not have increased glycosaminoglycan-accelerated thrombin inhibitory activity to the same extent, as does D72N/Y73F/D75N rHCII, this implies an additive influence of these AR2 residues to accelerate the HCII-glycosaminoglycan thrombin inhibition reaction.

Because the AR2 rHCII derivatives required substantially less glycosaminoglycan to reach maximal thrombin inhibition, we next addressed whether this implied a more accessible glycosaminoglycan-binding site. We used NaCl gradient elution from heparin-Sepharose to determine whether the AR2 rHCII mutations have altered apparent affinity for immobilized heparin (Table II). We found that the AR2 rHCII derivat-
derivatives (except D75N) required more NaCl to elute from heparin-Sepharose compared with wt-rHCII, ranging from 525–550 mM NaCl for wt-rHCII and D75N rHCII to 650–925 mM NaCl for the remainder of the AR2 rHCII derivatives. Collectively, the activity data and the heparin-Sepharose elution results suggest that altering the charge of AR2 allows heparin to bind with higher affinity to the D-helix region of HCII.

**α-Thrombin/Hirugen and γ-T-Thrombin Inhibition by the AR2 rHCII Derivatives**—We further studied the role of thrombin exosite-1 during AR2 rHCII inhibition (with heparin or dermatan sulfate). Consistent with past studies, hirugen drastically reduced the rate of α-thrombin inhibition with heparin or dermatan sulfate by both wt-rHCII and all of the AR2 rHCII derivatives (Fig. 3, shown for dermatan sulfate in the top panel). Although the inhibition rates for γ-α-thrombin are much reduced compared with α-thrombin, E69Q/D70N/D71N rHCII, D72N/Y73F/D75N rHCII, and D75K rHCII have slightly increased rates of γ-α-thrombin inhibition as compared with wt-rHCII with heparin or dermatan sulfate (Fig. 3, shown for dermatan sulfate in the bottom panel). Overall, these results imply that the process of thrombin recognition by all of the rHCII’s is similar whether using hirugen to sterically hinder access to thrombin exosite-1 or γ-α-thrombin where the β-loop of exosite-1 is absent.

**Physiological-based Thrombin Inhibition Studies by the AR2 rHCII Derivatives**—Our results presented so far show that some AR2 rHCII mutants are more active than wt-rHCII. We next assessed the potential of three AR2 rHCII derivatives.
Role of Second Acidic Region of Heparin Cofactor II

Even with T-thrombin or T-thrombin (containing a mixture of plasma and buffer with added glycosaminoglycans), some AR2 mutants were more active than wt-rHCII yet this inhibition rate was still lower when compared with D72N/Y73F/D75N rHCII. The increased inhibition activity appears to be caused by an enhanced interaction between the HCII acidic domain and exosite-1 of thrombin, because inhibition rates of both γ₁-thrombin and α-thrombin in the presence of hirugen were all reduced to rates similar to wt-rHCII. Finally, the comparable inhibition rates of wt-rHCII and the AR2 rHCII mutants with chymotrypsin (which requires neither the HCII acidic domain nor glycosaminoglycans for inhibition) indicate that the reactive site loop has not been altered to an “activated” conformation. The results further implicate a role for the acidic domain due to altered HCII intramolecular interactions that increased activity to α-thrombin.

We initially hypothesized that the AR2 rHCII mutants would have significant changes related both to glycosaminoglycan binding by HCII and to glycosaminoglycan-mediated inhibitory activity of HCII with thrombin. This hypothesis is due to the unique role postulated for AR2 to be an intramolecular mimic of a glycosaminoglycan (27, 29, 30). The majority of the AR2 rHCII mutations did affect both heparin- and dermatan sulfate-accelerated thrombin inhibition. Interestingly, the glycosaminoglycan-dependent inhibition curves for most of the AR2 rHCII mutants were “left-shifted” when compared with wt-rHCII. The optimal glycosaminoglycan concentration, which is the concentration corresponding to the maximum inhibition rate, is expected to be a function of the affinity of HCII for glycosaminoglycan because α-thrombin was used for all experiments and all other variables were constant. We found that AR2 mutants, like D72N/Y73F/D75N rHCII and Y73K rHCII, had maximum heparin and dermatan sulfate optima clearly reduced from wt-rHCII, in some cases as much as 10- to 20-fold less glycosaminoglycan was required for rates that were increased 2-to 6-fold compared with that for wt-rHCII. Additionally, the inhibition values were still enhanced for the AR2 rHCII mutants compared with wt-rHCII when tested with a mixture of plasma and buffer with added glycosaminoglycans. Even with γ₁-thrombin or α-thrombin/hirugen in the presence of glycosaminoglycans, some AR2 mutants were more active than wt-rHCII (although all of these rates were drastically reduced compared with α-thrombin), most likely because the acidic domain binding site on exosite-1 is neither totally removed in γ₁-thrombin nor completely blocked by hirugen in α-thrombin (for instance, with hirugen (20 μM) and heparin (10 μg/ml) the inhibition rates (kₘ, µ⁻¹ min⁻¹) for D72N/Y73F/D75N rHCII and D75K rHCII were reduced to 2.0 ± 0.5 × 10⁻⁸ and 1.0 ± 0.3 × 10⁻⁸, respectively, as compared with 0.4 ± 0.1 ×
Asp75 support crucial intramolecular interactions that hinder the molecule, where the negative charges were removed from the intact HCII molecular form to elute from heparin-Sepharose. These elution differences with wt-rHCII. What is remarkable about these AR2 rHCII mutants required from 1.4- to 1.7-fold higher NaCl concentrations to elute the proteins from heparin-Sepharose is a measurement of the acidic domain (residues 1-275) of HCII inhibited thrombin cleavage of fibrinogen but not thrombin chromogenic substrate activity (48). Because proteolysis of fibrinogen requires binding between exosite-1 and fibrinogen, this suggested that the HCII acidic peptide interacts with exosite-1. Deletion of HCII residues 1-52 by recombinant DNA technology did not affect thrombin inhibition in either the absence or presence of glycosaminoglycans (27). By contrast, deletion of residues 1-67 (first acidic region) greatly decreased HCII inhibition of thrombin in the presence of glycosaminoglycans but only slightly reduced it in the absence of glycosaminoglycan, suggesting that the first acidic domain interacted with thrombin (27). Interestingly, deletion of the entire acidic domain (residues 1-74) caused no further decreases in activity, suggesting that only the first acidic domain interacts directly with thrombin. Our laboratory (28, 32, 34) and others (27, 33) showed that glycosaminoglycan-accelerated HCII inhibition of γ-thrombin (in which exosite-1 has been removed by limited proteolysis) was reduced by greater than 1000-fold as compared with α-thrombin, whereas inhibition by antithrombin was relatively unaffected by the absence of exosite-1. Site-directed mutants of the basic residues in exosite-1 showed specificity similar to where hirugen binds exosite-1, and large decreases in activity with HCII-glycosaminoglycans were seen for some of the mutants (31, 35, 37).

The report by Baglin et al. (49) of the determination of the structures of native HCII and S195A thrombin-complexed HCII revealed some of the expected features based on the above discussion; however, the location of the acidic domain in HCII was unequivocal. The resolved native structure of HCII was a dimer formed by anti-parallel β-sheet interaction between strands 1C of each monomer with the acidic domain and the N terminus undefined. Recently, Liaw et al. (33) employed the exact opposite strategy to our study, where they replaced the basic residues in the β-helix region of HCII with neutral residues. They found an increase in progressive antithrombin activity of more than 100-fold compared with wt-rHCII (33). As expected, thrombin exosite-1 was essential for inhibition by these rHCII mutants (33). More recently, Brinkmeyer et al. (36) characterized an rHCII mutant with a re-formable disulfide bond between P52C and F195C. The oxidized form of this mutant was unable to form stable HCII-thrombin complexes; however, after reduction with β-mercaptoethanol the reduced HCII mutant was an active thrombin inhibitor in the presence of glycosaminoglycans.

### Table III

| Plasma       | Heparin (1 µg/ml) | Dermatan Sulfate (5 µg/ml) | k2 × 10^6 s⁻¹ min⁻¹ |
|--------------|-------------------|---------------------------|----------------------|
|              | Wild-type rHCII   | E69Q/D70N/D71N rHCII      |                      |
|              |                   | E69Q/D70N/D71N rHCII      |                      |
| Normal       | 2.9 ± 1.4         | 6.6 ± 1.3                 | 4.2 ± 0.2            |
| Reference    | 2.3               | 4.9 ± 1.0                 | 2.7 ± 0.2            |
| Antithrombin | 1.3 ± 0.4         | 3.2 ± 0.2                 |                      |
| III-deficient| 1.5 ± 0.8         | 2.8 ± 0.3                 | 4.1 ± 0.7            |
| 50:50 mix    | 2.6 ± 1.1         | 7.4 ± 0.8                 | 4.1 ± 0.1            |

* Values in parentheses are the ratio calculated as mutant rHCII/wt-rHCII.

10^8 for wt-rHCII. We have shown previously that dermatan sulfate accelerates γ-thrombin inhibition by HCII by 30-fold, indicating that the acidic domain still interacts with the remaining portions of exosite-1 in a productive manner. These results imply that for wild-type HCII, Asp72, Tyr-sulfate73, and Asp75 help maintain critical intramolecular interactions that attenuate thrombin inhibition activity until glycosaminoglycans bind, which expels the acidic domain to then contact exosite-1 of thrombin.

To complement the above results, the optimum glycosaminoglycan concentration was inversely related to apparent heparin affinity for each AR2 rHCII mutant as found using heparin-Sepharose elution profiles. Because the intermolecular interactions between glycosaminoglycans and HCII are thought to be primarily ionic, the concentration of NaCl required to elute the proteins from heparin-Sepharose is a measure of their relative affinity. We found that some of the AR2 rHCII mutants required from 1.4- to 1.7-fold higher NaCl concentrations to elute from heparin-Sepharose when compared with wt-rHCII. What is remarkable about these AR2 rHCII mutants is that the majority of the negatively charged acidic domain is present while still requiring increased NaCl concentrations to elute from heparin-Sepharose. These elution differences are somewhat analogous to N-terminal deletions when the negative charges were removed from the intact HCII molecule, where Δ1-67 rHCII and Δ1-74 rHCII eluted from heparin-Sepharose at 1.4- and 2.1-fold higher NaCl concentrations, respectively, compared with wt-rHCII (27, 32). We found previously that a synthetic peptide corresponding to the glycosaminoglycan binding site in HCII (from residues 183 to 200) bound with 2.6-fold greater affinity to heparin-Sepharose when compared with native HCII, indicating that the glycosaminoglycan-binding site was either masked or in an altered conformation compared with the peptide (47). It is intriguing to speculate that both Y73K and D75K (which had the highest increase in NaCl elution from heparin-Sepharose) might be directly contributing to glycosaminoglycan binding and be juxtaposed to the β-helix glycosaminoglycan region of HCII. Compared with wt-rHCII, the AR2 rHCII mutants are generally more permissive to glycosaminoglycan binding. These results indicate that for wild-type HCII, Asp72, Tyr-sulfate73, and Asp75 support crucial intramolecular interactions that hinder glycosaminoglycan binding.

The mechanism of glycosaminoglycan-accelerated thrombin inhibition by HCII is unique among thrombin-inhibiting serpins (15, 16, 47). HCII appears to employ an allosteric mechanism whereby binding of glycosaminoglycans to the β-helix region is thought to alter the acidic domain, which then serves as a “tethered-ligand” for exosite-1 of thrombin (27, 28, 31-37). The acidic domain/exosite-1 interaction is a critical component of the inhibition mechanism in the presence of glycosaminoglycans, whereas ternary complex formation bridging HCII, glycosaminoglycan, and thrombin appears to play a more minor role in the inhibition mechanism. The proposed interaction between the acidic domain and exosite-1 is well supported by a variety of data. A synthetic peptide corresponding to residues 54-75 of HCII inhibited thrombin cleavage of fibrinogen but not thrombin chromogenic substrate activity (48). Because proteolysis of fibrinogen requires binding between exosite-1 and fibrinogen, this suggested that the HCII acidic peptide interacts with exosite-1. Deletion of HCII residues 1-52 by recombinant DNA technology did not affect thrombin inhibition in either the absence or presence of glycosaminoglycans (27). By contrast, deletion of residues 1-67 (first acidic region) greatly decreased HCII inhibition of thrombin in the presence of glycosaminoglycans but only slightly reduced it in the absence of glycosaminoglycan, suggesting that the first acidic domain interacted with thrombin (27). Interestingly, deletion of the entire acidic domain (residues 1-74) caused no further decreases in activity, suggesting that only the first acidic domain interacts directly with thrombin. Our laboratory (28, 32, 34) and others (27, 33) showed that glycosaminoglycan-accelerated HCII inhibition of γ-thrombin (in which exosite-1 has been removed by limited proteolysis) was reduced by greater than 1000-fold as compared with α-thrombin, whereas inhibition by antithrombin was relatively unaffected by the absence of exosite-1. Site-directed mutants of the basic residues in exosite-1 showed specificity similar to where hirugen binds exosite-1, and large decreases in activity with HCII-glycosaminoglycans were seen for some of the mutants (31, 35, 37).

The report by Baglin et al. (49) of the determination of the structures of native HCII and S195A thrombin-complexed HCII revealed some of the expected features based on the above discussion; however, the location of the acidic domain in HCII was unequivocal. The resolved native structure of HCII was a dimer formed by anti-parallel β-sheet interaction between strands 1C of each monomer with the acidic domain and the N terminus undefined. Recently, Liaw et al. (33) employed the exact opposite strategy to our study, where they replaced the basic residues in the β-helix region of HCII with neutral residues. They found an increase in progressive antithrombin activity of more than 100-fold compared with wt-rHCII (33). As expected, thrombin exosite-1 was essential for inhibition by these rHCII mutants (33). More recently, Brinkmeyer et al. (36) characterized an rHCII mutant with a re-formable disulfide bond between P52C and F195C. The oxidized form of this mutant was unable to form stable HCII-thrombin complexes; however, after reduction with β-mercaptoethanol the reduced HCII mutant was an active thrombin inhibitor in the presence of glycosaminoglycans.
Collectively, these data allow us to provide an updated proposal of the mechanism of thrombin inhibition by HCII (Fig. 4). The historical depiction of HCII is given Fig. 4A, and this shows the acidic domain docked to the β-helix region (27, 29, 30, 32, 36). The mutants and data described by Liaw et al. (33) suggest that the acidic domain is no longer bound to the β-helix, and its location is altered and may resemble that shown in Fig. 4B. Following glycosaminoglycan binding, the acidic domain is fully extended and interaction with exosite-1 in thrombin is favored (Fig. 4C). The structural data from Baglin et al. (49) show that the HCII-S195A thrombin complex resembles that predicted with the acidic domain and exosite-1 in direct contact with the expected interaction between the reactive center of HCII and active site of thrombin (Fig. 4D). The AR2 rHCII mutants described here would appear to be in equilibrium between conformations A, B, and C above, which, when in the presence of glycosaminoglycan, the encounter between the active site of thrombin and the reactive center of HCII is enhanced and stabilized by acidic domain and exosite-1 orientation and interaction (drawing is based on a schematic by D. M. Tollefsen).

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