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Enhancement of Heparin Cofactor II Anticoagulant Activity*

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Heparin cofactor II (HCII) is a serpin whose thrombin inhibition activity is accelerated by glycosaminoglycans. We describe the novel properties of a carboxyl-terminal histidine-tagged recombinant HCII (rHCII-$CHis_6$). Thrombin inhibition by rHCII-$CHis_6$ was increased >2-fold at ~5 μg/ml heparin compared with wild-type recombinant HCII (wt-rHCII) at 50–100 μg/ml heparin. Enhanced activity of rHCII-$CHis_6$ was reversed by treatment with carboxypeptidase A. We assessed the role of the HCII acidic domain by constructing amino-terminal deletion mutants (Δ1–52, Δ1–68, and Δ1–75) in wt-rHCII and rHCII-$CHis_6$. Without glycosaminoglycan, unlike wt-rHCII deletion mutants, the rHCII-$CHis_6$ deletion mutants were less active compared with full-length rHCII-$CHis_6$. With glycosaminoglycans, Δ1–68 and Δ1–75 rHCIIs were all less active. We assessed the character of the tag by comparing rHCII-$CHis_6$, rHCII-$CAla_6$, and rHCII-$CLys_6$ to wt-rHCII. Only rHCII-$CHis_6$ had increased activity with heparin, whereas all three mutants have increased heparin binding. We generated a carboxyl-terminal histidine-tagged recombinant antithrombin III to study the tag on another serpin. Interestingly, this mutant antithrombin III had reduced heparin cofactor activity compared with wild-type protein. In a plasma-based assay, the glycosaminoglycan-dependent inhibition of thrombin by rHCII-$CHis_6$ was significantly greater compared with wt-rHCII. Thus, HCII variants with increased function, such as rHCII-$CHis_6$, may offer novel reagents for clinical application.

Serine protease inhibitors (serpins)[1] are a class of highly conserved proteins whose prototypical member is α_1-proteinase inhibitor (1, 2). Serpins function primarily to inhibit serine proteases that are involved in many normal biological processes including coagulation, fibrinolysis, inflammation, wound healing, and tissue repair as well as some pathological processes such as atherosclerosis and cancer metastasis (2). Within the serpin superfamily is a subclass of glycosaminoglycan-binding serpins (1–3). This group includes antithrombin III (ATIII), heparin cofactor II (HCII), protein C inhibitor, protease nexin-1, and plasminogen activator inhibitor-1 (2). Glycosaminoglycans bound by these serpins include heparin, chondroitin sulfates, dermatan sulfate, and proteoglycans with these molecules as side chains.

Serpins inhibit their target proteases by acting as suicide substrates (1, 2). Serpins contain an exposed reactive site loop. Within the reactive site loop of the serpin is the P1–P1′ bond (4). The target protease will recognize this reactive site bond and attack it as a substrate. Once attacked, the serpin and protease are caught in a 1:1 covalent complex in which the protease is rendered inactive (5). The complex is then cleared via receptor-mediated endocytosis (6–8).

Heparin cofactor II is a 65.5-kDa glycoprotein whose inhibitory activity is directed toward thrombin and chymotrypsin (9, 10). Unlike the physiologic thrombin inhibitor ATIII (11, 12), HCII inhibition of thrombin is accelerated by both heparin and dermatan sulfate (13, 14). Maximal rates of thrombin inhibition by HCII are seen in the presence of dermatan sulfate. As many dermatan sulfate-containing proteoglycans are located extravascularly it has been speculated that HCII is an extravascular thrombin inhibitor (15–17).

Heparin cofactor II is unusual in that its reactive site bond is Leu-Ser (18, 19). In the presence of glycosaminoglycan, HCII inhibits thrombin through an unusual mechanism (2, 14, 20–25). Heparin cofactor II contains a unique amino-terminal region that is highly acidic and thus referred to as the "acidic domain." In the absence of glycosaminoglycan, the acidic domain is believed to interact with the D-helix region, which is highly basic. The D-helix region is involved in glycosaminoglycan binding. When glycosaminoglycan is present, it has been suggested that the acidic domain is displaced. The displaced acidic domain is then able to interact with the anion-binding exosite-1 of thrombin.

Standard procedures to purify HCII involve binding protein to heparin-Sepharose (26, 27). However, a further investigation of the HCII mechanism of action by mutagenesis of its glycosaminoglycan-binding region would disrupt purification of protein by heparin affinity. Therefore, we began to derive alternative purification protocols to avert this problem. Many researchers have used a sequence consisting of six histidine residues as an affinity ligand (28–30). By attaching this sequence to a protein, either the amino or carboxyl terminus, protein can be purified with a specialized Ni^{2+} matrix (31). This method had been used successfully as an affinity purification ligand (28–30). These data suggest that the histidine tag is a benign addition to proteins to which it was attached (28–30).

In this report we show the following: (*a*) carboxyl-terminal

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[1] The abbreviations used are: serpin, serine protease inhibitor; HCII, heparin cofactor II; ATIII, antithrombin III; wt, wild type; r, recombinant; $CHis_6$, carboxyl-terminal hexahistidine tag; $CHis_5Pro$, carboxyl-terminal pentahistidine-proline tag; $CAla_6$, carboxyl-terminal hexa-alanine tag; $CLys_6$, carboxyl-terminal hexalysine tag; Δ1–52, Δ1–68, and Δ1–75, deletion of residues 1–52, 1–68, and 1–75 in recombinant HCII, respectively; ABE-1, anion-binding exosite-1; BSA, bovine serum albumin; CPA, carboxypeptidase A; PEG, polyethylene glycol 8000; pB, Polybrene-hexadimethrine bromide; Gly-Pro-Arg-NA, tosyl-Gly-Pro-Arg-*p*-nitroanilide; Ala-Ala-Pro-Phe-NA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; REF, normal hemostasis reference plasma; DEF, human antithrombin III-deficient plasma; CPA, carboxypeptidase A.

hexahistidine-tagged recombinant HCII (rHCII-$CHis_6$) has enhanced progressive antithrombin and heparin cofactor activities and increased heparin-Sepharose binding compared with wild-type recombinant HCII (wt-rHCII); (*b*) a region within the amino terminus of HCII may interact with the carboxyl-terminal hexahistidine of rHCII-$CHis_6$; (*c*) carboxyl-terminal hexahistidine-tagged recombinant antithrombin III (rATIII-$CHis_6$) does not have these enhanced activities compared with wild-type recombinant ATIII (wt-rATIII); and (*d*) the enhanced heparin effect of rHCII-$CHis_6$ is maintained in a plasma-based thrombin inhibition assay. Collectively, these data suggest that rHCII-$CHis_6$ could be a novel anticoagulant therapy.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Recombinant Proteins—To facilitate our studies of HCII, site-directed mutagenesis (35) was performed on full-length HCII cDNA subcloned into the pBlueScript SK+ mutagenesis and cloning vector (Stratagene, La Jolla, CA) (36) at two sites to encode the identical amino acid sequence but with two nucleotide changes (at base pairs 595 and 1255) that create unique restriction sites (*Nhe*I and *Afl*II) in the cDNA. DNA sequencing using a Sequenase version 2.0 kit (Amersham Pharmacia Biotech) identified positive clones. Full-length HCII cDNA containing these unique restriction sites was then subcloned into the baculoviral transfer vector pVL1392 (PharMingen, La Jolla, CA) via flanking *Eco*RI sites as described previously (36).

Using this new HCII cDNA, a cassette of the cDNA was then subcloned into pBlueScript SK+ with *Xho*I and *Eco*RI. This cassette was then used to prepare rHCII-$CHis_6$, rHCII-$CAla_6$, and rHCII-$CLys_6$ by Kunkel's method (35) of oligonucleotide-directed mutagenesis using the primers 5′-GCCAACCCCAGCAGGTCC$(CAC)_6$TAGAGGTGGAGGTCT-AGG-3′, 5′-GCCA-ACCCCAGCAGGTCC$(GCC)_6$TAGAGGTGGAGG-3′, and 5′-GCCAACCCCAGCAGGTCC-$(AAG)_6$TAGAGGTGGAGG-3′, respectively. We also used this construct to create the truncation mutants Δ1–52-rHCII, Δ1–68-rHCII, and Δ1–75-rHCII, using the primers 5′-ACATCTGCGTGGGGTGAGGGGGAGGAGGAC-3′, 5′ACATCTGC-GTGGGGTGAAGACG-ACGACTAC-3′, and 5′-ACATCTGCGTGGGGT-ATCGTCGACAGTCTG-3′, respectively. By using the rHCII-$CHis_6$ construct, we created the truncation mutants Δ1–52-rHCII-$CHis_6$, Δ1–68-rHCII-$CHis_6$, and Δ1–75-rHCII-$CHis_6$ using the primers mentioned above, respectively. DNA sequencing identified positive clones. A cassette containing the carboxyl-terminal additions was then excised with *Afl*II and *Xba*I and subcloned into pVL1392 containing the full-length HCII cDNA cut with the same restriction enzymes. All truncation mutants were subcloned into pVL1392 using the restriction enzyme *Eco*RI and screened for proper orientation.

A full-length human ATIII cDNA was obtained from ATCC (catalog number 57224) in the vector pKT218. The ATIII-containing vector was digested with *Pst*I, and this insert contained 1.6 kilobase pairs of open reading frame, including the signal peptide sequence, and was subcloned into the baculoviral transfer vector pVL1392 cut with the same restriction enzyme.

Using this wt-ATIII cDNA, a cassette of the cDNA was subcloned into pBlueScript SK+ with *Sac*I and *Xba*I. This construct was then mutated (35) to form rATIII-cassette-$CHis_6$ with the primer 5′-G-GTGCAAAGAATAAGAACATTTTA$(GTG)_6$CTTAACACAAGGGTTGG-C-3′. DNA sequencing using Sequenase identified positive clones. The mutated cassette was excised from pBlueScript with the restriction enzymes *Nco*I and *Xba*I and subcloned into pVL1392 containing the full-length ATIII gene cut using the same restriction enzymes.

HCII- and ATIII-containing pVL1392 constructs were co-transfected with linearized *Autographica californica* nuclear polyhedrosis virus into *Spodoptera frugiperda* (*Sf* 9) insect cells (Invitrogen, Carlsbad, CA) in T25 flasks using BaculoGold Baculovirus Transfection Kits (PharMingen, La Jolla, CA) as detailed previously (34, 36). Media were collected from these cells 5 days post-transfection as recombinant viral stock. Production of rHCII or rATIII mutants and wild-type protein was verified by immunoblot analysis of whole cell lysates. Recombinant viral stocks were amplified and stored at −80 °C. *Sf*9 cells were maintained in Grace's medium supplemented with 10% fetal bovine serum, 0.3 g/liter L-glutamine, and 50 μg/ml gentamicin.

Protein Expression and Purification—Expression of both rHCII and rATIII was performed using HighFive™ insect cells (Invitrogen, Carlsbad, CA) maintained at 27 °C in Excel 405 medium (JRH Biosciences, Lenexa, KS). Most of the rHCII proteins were purified by sequential heparin-Sepharose and Q-Sepharose chromatography steps as described previously (34, 36). However, the cleared media of Δ1–68-rHCII, Δ1–75-rHCII, Δ1–68-rHCII-$CHis_6$, and Δ1–75-rHCII-$CHis_6$ were loaded on a 5-ml Hi-Trap heparin-Sepharose column using an FPLC System (Amersham Pharmacia Biotech) equilibrated with HNPN buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% PEG, 0.05% NaN_3) at pH 7.4, washed with 25 ml of HNPN, and then eluted with a linear gradient from 50 mM to 2 M NaCl, 20 mM Hepes, pH 7.4, 0.1% PEG, and 0.02% NaN_3.

The preparation and purification of the rATIII proteins began by infecting four T150 flasks of sub-confluent HighFive™ cells on day 1 with specific recombinant viral stocks. Two to three days post-infection, media were decanted from cells, and cell debris was spun out by centrifugation at 350 × *g* for 10 min in a Centra-8 centrifuge (International Equipment Co., Needham Heights, MA). The cleared medium (~100 ml) was diluted with an equal volume of a buffer made up of 20 mM Hepes, pH 6.5, 0.2% PEG, and 0.02% NaN_3. One ml of a 1:1 slurry of heparin-Sepharose in HNPN buffer was added to the diluted media and tumbled at 4 °C for 1 h. Heparin-Sepharose beads were then pelleted at 50 × *g* for 5 min and washed two times with 20 mM Hepes, pH 7.4, 750 mM NaCl, 0.1% PEG, and 0.02% NaN_3. The protein was eluted off of heparin-Sepharose with 20 mM Hepes, pH 7.4, 2.0 M NaCl, 0.1% PEG, and 0.02% NaN_3.

Recombinant Protein Immunodetection—Concentrations of rHCII and rATIII were determined by enzyme-linked immunosorbent assay using purified plasma proteins as the standard, and rabbit antihuman antithrombin III antibody was from Dako Corp. (Carpinteria, CA); monoclonal antibody 2-4-34 to human HCII was prepared in our laboratory, and a goat anti-human HCII IgG (catalog number GAHC2-IG) was from Enzyme Research Laboratories (South Bend, IN). Immunoblot analysis was carried out using a Phast System (Amersham Pharmacia Biotech) (34, 36).

Protease Inhibition—Protease inhibition rates were determined as described previously (14, 17, 34, 36). All assays were performed at room temperature in 96-well microtiter plates previously coated with 2 mg/ml BSA.

In the absence of glycosaminoglycan, 5–150 nM rHCII (wt, $CHis_6$, $CHis_5Pro$, $CAla_6$, $CLys_6$, Δ1–52, Δ1–68, Δ1–75, Δ1–52-$CHis_6$, Δ1–68-$CHis_6$, and Δ1–75-$CHis_6$) or rATIII (wt or $CHis_6$) was incubated with 0.5–1 nM α-thrombin, 1 nM γ_T-thrombin, 0.5 nM Factor Xa, 2 nM chymotrypsin, or 5 nM trypsin, in the presence of 1 mg/ml Polybrene (pB) (for thrombin and Factor Xa) and 2 mg/ml BSA in HNPN, pH 7.4.

In the absence of glycosaminoglycan either hirugen or a control peptide corresponding to the reverse sequence of the HCII acidic domain (residues 47–61) at 20 μM and rHCII (wt at 200 nM and $CHis_6$ at 100 nM) were incubated with 1 nM thrombin in the presence of 1 mg/ml pB and 2 mg/ml BSA in HNPN, pH 7.4 (37).

In the presence of either heparin (10 or 100 μg/ml) or dermatan sulfate (100 μg/ml), 0.5 nM thrombin was incubated with 20 μM hirugen peptide or control peptide and 10 nM wt-rHCII or rHCII-$CHis_6$ in the presence of 2 mg/ml BSA in HNPN, pH 7.4.

In the presence of glycosaminoglycans or sulfated polyanions, 5–10 nM rHCII (wt, $CHis_6$, $CHis_5Pro$, $CAla_6$, $CLys_6$, Δ1–52, Δ1–68, Δ1–75, Δ1–52-$CHis_6$, Δ1–68-$CHis_6$, and Δ1–75-$CHis_6$) was incubated with 0.5 nM thrombin, and 0–1 mg/ml heparin, 0–4 mg/ml dermatan sulfate, 0–6 mg/ml desmin (a gift from Dr. Egidio Marchi of Alfa Wassermann, S.p.A.), or 0–100 μg/ml fucoidan, or 10 nM rATIII (wt or $CHis_6$) was incubated with 1 nM thrombin or factor Xa and 0–1 mg/ml heparin in the presence of 2 mg/ml BSA in HNPN, pH 7.4.

Residual thrombin activity was measured with 150 μM Gly-Pro-Arg-NA and 1 mg/ml pB in the absence of glycosaminoglycan, 2 mg/ml pB in the presence of heparin or fucoidan, 4 mg/ml pB in the presence of dermatan sulfate, and 6 mg/ml pB in the presence of desmin. Residual factor Xa activity was measured with 500 μM Spectrozyme FXa and 2 mg/ml pB in the presence or absence of glycosaminoglycan. Residual chymotrypsin activity was measured with 150 μM Ala-Ala-Pro-Phe-NA, and residual trypsin activity was measured with 150 μM Gly-Pro-Arg-NA. Substrate cleavage was measured by color development at 405 nM on a V_{max} Kinetic Microplate Reader (Molecular Devices).

Assays were performed at least in triplicate on two or more recombinant protein preparations. All inhibition studies were measured under pseudo-first order conditions where inhibitor [I] is at least 10-fold higher than enzyme (protease) [*E*], and second-order rate constants were calculated as described previously (34, 36).

Heparin-Sepharose Affinity Chromatography—To determine relative heparin affinities, 3–6 μg of rHCII protein was diluted in 20 mM Hepes, pH 7.4, and was run either on a 1-ml heparin-Sepharose or a 2-ml Hi-Trap heparin-Sepharose column (equilibrated in 20 mM Hepes, pH 7.4, and 50 mM NaCl) using an FPLC® System (Amersham Pharmacia Biotech). After the samples were loaded on the column, the proteins

TABLE I

HCII Inhibition of thrombin and chymotrypsin in the absence of glycosaminoglycans

Inhibition of proteases by rHCII mutants was measured in the absence of glycosaminoglycans as described under "Experimental Procedures." Values are expressed as means ± S.D.

Serpin	Thrombin $k_2 \times 10^{4a}$	Ratio (mutant/wt)	Chymotrypsin $k_2 \times 10^{4a}$	Ratio (mutant/wt)
	$M^{-1}min^{-1}$		$M^{-1}min^{-1}$	
wt-rHCII	1.3 ± 0.19		71 ± 12	
rHCII-CHis$_6$	1.9 ± 0.10[b]	1.5	61 ± 16[c]	0.86
rHCII-CAla$_6$	1.7 ± 0.41[d]	1.5	48 ± 7.6[e]	0.77
rHCII-CLys$_6$	1.5 ± 0.38[e]	1.4	56 ± 5.7[e]	0.90
Δ1–52-rHCII	1.4 ± 0.19[d]	1.3	61 ± 17[e]	0.98
Δ1–68-rHCII	1.0 ± 0.42[e]	0.90	102 ± 31[d]	1.6
Δ1–75-rHCII	1.2 ± 0.26[e]	1.1	86 ± 27[e]	1.4
Δ1–52-rHCII-CHis$_6$	0.95 ± 0.15[g]	0.53[f]	38 ± 9.1[g]	0.66[f]
Δ1–68-rHCII-CHis$_6$	0.56 ± 0.26[g]	0.31[f]	30 ± 2.3[g]	0.52[f]
Δ1–75-rHCII-CHis$_6$	0.71 ± 0.15[g]	0.39[f]	12 ± 7.0[g]	0.21[f]

[a] Rate constants are the mean values of 3–9 separate determinations with at least 3 different preparations of recombinant protein.
[b] $p \leq 0.003$ compared with wt-rHCII.
[c] Not statistically different from wt-rHCII.
[d] $p \leq 0.05$ compared with wt-rHCII (using the values $1.1 \pm .05 \times 10^4$ and $62 \pm 14 \times 10^4$ $M^{-1}min^{-1}$ for thrombin and chymotrypsin, respectively).
[e] Not significantly different from wt-rHCII (using the values $1.1 \pm .05 \times 10^4$ and $62 \pm 14 \times 10^4$ $M^{-1}min^{-1}$ for thrombin and chymotrypsin, respectively).
[f] Ratio calculated as mutant/rHCII-CHis$_6$
[g] $p \leq 0.05$ compared with rHCII-CHis$_6$ (using the values $1.8 \pm .05 \times 10^4$ and $58 \pm 18 \times 10^4$ M^{-1} min^{-1} for thrombin and chymotrypsin, respectively).

were eluted with a 40-ml gradient of 20 mM Hepes, pH 7.4, from 50 to 800 mM NaCl for heparin-Sepharose, and from 150 mM to 2 M NaCl for Hi-Trap heparin-Sepharose, and 1-ml fractions were collected. 100 μl of each fraction was aliquoted onto a 96-well microtiter plate, and enzyme-linked immunosorbent assay (protocol shown above) was performed. The peak elution ionic 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 protein preparations. Recombinant ATIII samples were run similarly to the HCII samples using heparin-Sepharose, except the elution gradient was run from 50 mM to 2 M NaCl.

Carboxypeptidase A Treatment—CPA stored in toluene was washed with distilled H_2O and then dialyzed into HNPN buffer at pH 7.4. 40 ng of CPA was combined with approximately 1 μg of recombinant protein and incubated at room temperature. Following a 2-h incubation at room temperature, 2 mM EDTA was added to quench CPA activity. Controls were run in which EDTA was added to the reaction prior to the addition of CPA. CPA-digested proteins were then assayed according to previously mentioned procedures.

Plasma Assays—Human antithrombin III-deficient plasma (catalog number 203) and normal hemostasis reference plasma (catalog number 258N) were purchased from American Diagnostica (Greenwich, CT). The assay to evaluate rHCII (wt and rHCII-CHis$_6$) in plasma was designed based on previously published methods (38, 39). All assays were performed at room temperature in 96-well microtiter plates previously coated with 2 mg/ml BSA. This assay was performed using normal hemostasis reference plasma (REF), human antithrombin III-deficient plasma (DEF), or a 50:50 mixture of these plasmas (REF/DEF). Within the assay, 10 nM rHCII or rHCII-CHis$_6$ was incubated for 15 s with 1 nM thrombin and 1 μg/ml heparin or 50 μg/ml dermatan sulfate, in the presence of a 1:100 dilution of plasma. Residual thrombin activity was measured with 300 μM GPA and 2 mg/ml pB. Substrate cleavage was measured by color development at 405 nM on a V_{max} Kinetic Microplate Reader. Second order rate constants were measured at least in triplicate on two recombinant protein preparations as described above.

Statistical Analysis—The statistical significance of the data in Tables I–IV was evaluated using Student's *t* tests; *p* values ≤0.05 were considered significant.

RESULTS

Carboxyl-terminal Histidine-Tagged rHCII—-Recombinant HCII proteins were generated using Kunkel's method of oligonucleotide-directed mutagenesis. More specifically, rHCII-CHis$_6$ was made by inserting six histidine codons directly before the TAG stop codon. By using a baculoviral expression system, we typically obtained ~60 μg of protein from four T150 flasks of HighFive™ cells infected with recombinant viral stock. After purification, immunoblot analysis showed that rHCII-CHis$_6$ was a single band that co-migrated with wt-rHCII.

We have compared the rates of inhibition of thrombin and chymotrypsin by rHCII-CHis$_6$ and wt-rHCII (Table I). In the absence of glycosaminoglycan, the rate of thrombin inhibition by rHCII-CHis$_6$ is significantly faster (1.5-fold) than that of wt-rHCII. However, when comparing the same proteins in their ability to inhibit chymotrypsin, we see that wt-rHCII and rHCII-CHis$_6$ are essentially the same.

The antithrombin properties of wt-HCII can be enhanced more than 10,000-fold by the addition of glycosaminoglycans such as heparin or dermatan sulfate. The carboxyl-terminal histidine-tagged HCII influences the heparin-accelerated antithrombin activity. As shown in the *top panel* of Fig. 1, the maximal rate of wt-rHCII inhibition of thrombin is $9.29 \pm 2.8 \times 10^8$ M^{-1} min^{-1} at 50–100 μg/ml heparin. However, we see over a 2-fold increase in the rate of thrombin inhibition by rHCII-CHis$_6$ at $2.23 \pm .43 \times 10^9$ M^{-1} min^{-1} at only 5 μg/ml heparin. Therefore, in addition to the increase in rate, we also see an approximate 20-fold decrease in the amount of heparin required for maximal activity. These results are summarized in Table II and indicate that the histidine tag augmented the ability of HCII to inhibit thrombin in the presence of heparin. Addition of an N^{α}-acetylated hexahistidine peptide at 1,000 molar excess to wt-rHCII had neither a positive nor a negative effect on the heparin cofactor activity of HCII (data not included). These data suggest that the rate of thrombin inhibition by rHCII-CHis$_6$ with heparin is increased over 100,000-fold and is comparable to rates obtained with the physiologic thrombin inhibitor ATIII with heparin.

We do not see any change in rates of thrombin inhibition in the presence of the glycosaminoglycan, dermatan sulfate. As shown in the *bottom panel* of Fig. 1, the maximal rate of thrombin inhibition is $1.52 \pm 0.31 \times 10^9$ M^{-1} min^{-1} for wt-rHCII, and $1.80 \pm 0.50 \times 10^9$ M^{-1} min^{-1} for rHCII-CHis$_6$ maximal inhibition is seen at approximately 500 μg/ml dermatan sulfate for both proteins. These data, which are also summarized in Table II, indicate that the addition of the carboxyl-terminal histidine tag does not affect the rate at which thrombin inhibition by HCII is accelerated by dermatan sulfate.[2]

[2] We determined the ability of two other sulfated polysaccharides to accelerate thrombin inhibition by HCII. In the presence of fucoidan, we

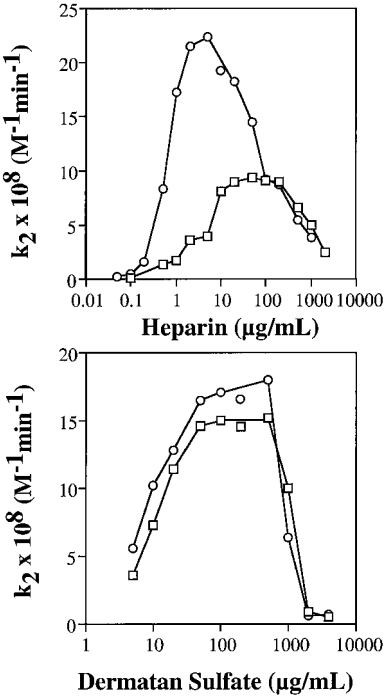

FIG. 1. **Inhibition of thrombin by wt-rHCII and rHCII-$CHis_6$ in the presence of heparin and dermatan sulfate.** Thrombin inhibition assays in the presence of glycosaminoglycan were performed as detailed under "Experimental Procedures" with α-thrombin and increasing amounts of heparin (*top panel*) and dermatan sulfate (*bottom panel*) comparing wt-rHCII (□) and rHCII-$CHis_6$ (○). The *curves* shown are averages of two or three recombinant protein preparations assayed two to three times each.

We further assessed the ability of these proteins to bind heparin-Sepharose. As shown in Table II, rHCII-$CHis_6$ eluted at almost two times the NaCl concentration as wt-rHCII, 575 mM *versus* 350 mM, respectively. Taken together with the enhanced heparin cofactor activity of rHCII-$CHis_6$, these data imply that the hexahistidine tag endows HCII with increased heparin binding.

To ensure that the enhanced activity of rHCII-$CHis_6$ could be attributed to the histidine tag, we attempted to remove the tag using the exopeptidase CPA. CPA removes amino acids from the carboxyl terminus of proteins; however, it is unable to cleave arginine, lysine, or proline. There is an arginine at the second to last position of the native HCII. Therefore, we assumed a CPA digest would remove the hexahistidine tag and the final serine residue of HCII stopping at the penultimate arginine. In the *top panel* of Fig. 2, we see that a rHCII-$CHis_6$ pre-CPA digest shows an increased rate of thrombin inhibition and a shift to a lower heparin requirement. When digested with CPA, the curve of thrombin inhibition shifts to lower rates of inhibition and the required heparin concentration increases. In contrast, the curves of thrombin inhibition by wt-rHCII do not drastically change before or after the CPA digest (Fig. 2, *middle panel*). Control experiments with EDTA added to rHCII-$CHis_6$ prior to the exopeptidase verified that the loss of rHCII-$CHis_6$ activity was due to the effect of active CPA.

To confirm that the function of CPA in reversing the enhanced activity was on the hexahistidine sequence, we expressed a mutant that had a His_5Pro carboxyl-terminal tag. rHCII-$CHis_5$Pro inhibits thrombin in the absence of glycosaminoglycans (3.2×10^4 M^{-1} min^{-1}) at rates ~2-fold higher than wt-rHCII and has increased heparin cofactor activity at a lower maximal heparin concentration ($1.87 \pm 0.37 \times 10^9$ M^{-1} min^{-1} at 10 μg/ml heparin; data not included). We hypothesized that this mutant should be resistant to CPA digestion because of the carboxyl-terminal proline residue. Pre-digested rHCII-$CHis_5$Pro has similar properties to that of rHCII-$CHis_6$ with an increased inhibition rate and a lower heparin requirement; however, as expected, its activity does not change appreciably after treatment with CPA (Fig. 2, *bottom panel*).

Thrombin inhibition in the presence of hirugen was performed to examine the potential role of thrombin anion-binding exosite-1 (ABE-1) in the enhanced activity of rHCII-$CHis_6$. Hirugen has a similar effect on antithrombin activity (without glycosaminoglycan) of rHCII-$CHis_6$ and wt-rHCII, with rates reduced >50% (Fig. 3). The effect of hirugen is specific since a control peptide did not significantly block the rHCII-thrombin reactions (Fig. 3). In the presence of either 100 μg/ml heparin (optimal for wt-rHCII) or dermatan sulfate (optimal for wt-rHCII and rHCII-$CHis_6$), both wt-rHCII and rHCII-$CHis_6$ lose >85% of their inhibitory activity in the presence of hirugen (Fig. 3). In the presence of 10 μg/ml heparin (optimal for rHCII-$CHis_6$) and hirugen, we see that wt-rHCII loses 90% of its inhibitory potential, whereas rHCII-$CHis_6$ loses 70% of its inhibitory activity (Fig. 3). These data imply that the hexahistidine tag does not alter the manner in which HCII interacts with ABE-1 of thrombin.[3]

Amino-terminal Deletion Mutants of wt-rHCII and rHCII-$CHis_6$—To assess the role of the acidic domain on HCII activity, we prepared amino-terminal deletions of rHCII with and without $CHis_6$. By using either a wt-rHCII or rHCII-$CHis_6$ single-stranded DNA template, we deleted amino acids 1–52, 1–68, or 1–75. From each 100-ml culture infected with recombinant baculoviral stock, we purified ~150 μg of protein. As expected, immunoblot analysis showed that purified Δ1–52-rHCII, Δ1–68-rHCII, and Δ1–75-rHCII (with and without $CHis_6$) were sequentially smaller than wt-rHCII.

In the absence of glycosaminoglycan, each of the six amino-terminal deletion mutations was compared with either wt-rHCII or rHCII-$CHis_6$ for thrombin and chymotrypsin inhibition (Table I). Deletion of the first 52 amino acids, but not for the 68 or 75 deletions, slightly but significantly increases the rate of thrombin inhibition in the untagged rHCII compared with wt-rHCII. Chymotrypsin inhibition by Δ1–68-rHCII is significantly faster than wt-rHCII; however, Δ1–52- and Δ1–75-rHCII show no significant differences in the rates of chymotrypsin inhibition (Table I). In contrast, the three deletions in the hexahistidine-tagged rHCII resulted in significant decreases in both thrombin and chymotrypsin inhibition activity compared with rHCII-$CHis_6$ (Table I). Deletion of the amino terminus, beginning with Δ1–52, drastically affects protease inhibition by $CHis_6$-tagged rHCII and may indicate that an interaction between the amino- and carboxyl-terminal regions of HCII is eliminated that leads to the loss of activity.

In the presence of heparin, Δ1–52-rHCII had similar activity

see no shift in the optimal concentration required to accelerate rHCII-$CHis_6$ compared with wt-rHCII (data not shown). The maximal rate of thrombin inhibition by wt-rHCII ($2.38 \pm 0.42 \times 10^7$ M^{-1} min^{-1}) is similar to that of rHCII-$CHis_6$ ($3.21 \pm 0.16 \times 10^7$ M^{-1} min^{-1}). In the presence of desmin, a low molecular weight dermatan sulfate, the optimal desmin concentrations for maximal inhibition of thrombin by either wt-rHCII (k_2 of $5.37 \pm 0.29 \times 10^8$ M^{-1} min^{-1}) or rHCII-$CHis_6$ ($\mathbf{k}_2$ of $2.57 \pm 0.33 \times 10^8$ M^{-1} min^{-1}) are similar.

[3] Inhibition of γ_T-thrombin by wt-rHCII and rHCII-$CHis_6$ was compared with further study of the role of ABE-1 of thrombin. In the absence of glycosaminoglycan, thrombin inhibition by rHCII-$CHis_6$ ($7.93 \pm 1.0 \times 10^3$ M^{-1} min^{-1}) is significantly faster (2-fold) than wt-rHCII ($3.81 \pm 0.30 \times 10^3$ M^{-1} min^{-1}). In the presence of heparin and dermatan sulfate, the rates of γ_T-thrombin inhibition by either wt-rHCII or rHCII-$CHis_6$ are greatly reduced in comparison to inhibition rates with α-thrombin (data not shown). These data agree with the currently accepted mechanism of thrombin inhibition by HCII.

TABLE II

HCII and ATIII inhibition of serine proteases in the presence of glycosaminoglycans

Inhibition of serine proteases by rHCII and rATIII mutants was measured in the presence of increasing concentrations of either heparin or dermatan sulfate (DSO_4). The maximal inhibition of each curve was used in the calculation of the average inhibition rate. Values are expressed as means ± S.D. The number in parentheses that follows indicates the average glycosaminoglycan concentration at which the maximal rate was measured. The final column indicates the peak NaCl concentration at which each variant eluted from heparin-Sepharose. All assays were performed as described under "Experimental Procedures."

Serpin	Thrombin + heparin[a] $k_2 \times 10^8$	Ratio (mutant/wt)	Thrombin + DSO_4[a] $k_2 \times 10^8$	Ratio (mutant/wt)	Factor Xa + heparin[a] $k_2 \times 10^8$	Ratio (mutant/wt)	Heparin-Sepharose [NaCl]
	$M^{-1}min^{-1}$		$M^{-1}min^{-1}$		$M^{-1}min^{-1}$		mM
wt-rHCII	9.29 ± 2.8 (50)[b]		15.2 ± 3.1 (500)				350
rHCII-CHis$_6$	22.3 ± 4.3[c] (5)	2.4	18.0 ± 5.0[d] (500)	1.2			575
wt-rATIII	5.26 ± .68 (1)				1.41 ± 0.39 (10)		975
rATIII-CHis$_6$	2.87 ± .54[e] (2)	.55			0.785 ± 0.28[e] (10)	0.56	975

[a] Rate constants are the mean values of 3–6 separate determinations on at least 3 different protein preparations.
[b] Values in parentheses indicate optimal heparin concentration.
[c] $p \leq 0.029$ compared with wt-rHCII.
[d] Not statistically different from wt-rHCII.
[e] $p \leq 0.020$ compared with wt-rATIII.

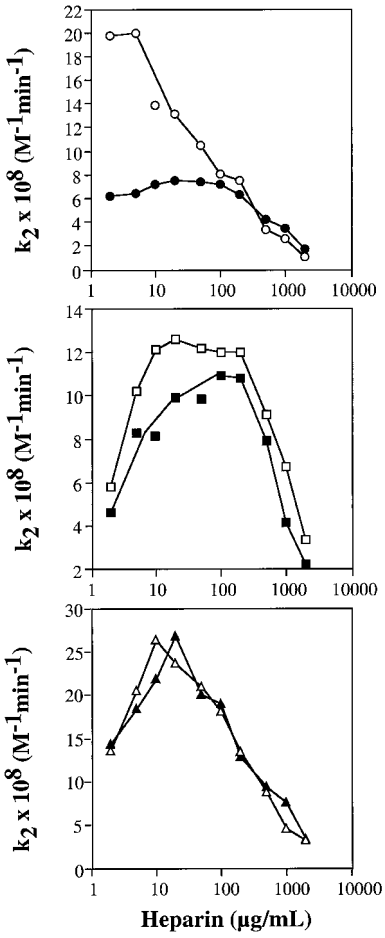

FIG. 2. **Carboxypeptidase A reversibility of rHCII mutants.** Thrombin inhibition assays in the presence of heparin were performed as detailed under "Experimental Procedures" with α-thrombin and increasing amounts of heparin. The *top panel* shows the curves for rHCII-CHis$_6$ pre- (○) and post-CPA (●) digest. The *middle panel* shows the curves for wt-rHCII pre- (□) and post-CPA (■) digest. The *bottom panel* shows the curves for rHCII-CHis$_5$Pro pre- (△) and post-CPA (▲) digest. The *curves* shown are representative data.

to wt-rHCII to accelerate thrombin inhibition (Table III). However, the 68 and 75 deletions have substantially decreased thrombin inhibition in the presence of heparin by almost 200–250-fold in comparison to full-length wt-rHCII (Table III). The deletions that contained the hexahistidine tag all showed differences compared with rHCII-CHis$_6$ (Table III). Unlike Δ1–52-rHCII, Δ1–52-rHCII-CHis$_6$ had a significantly decreased thrombin inhibition with heparin (Table III). Likewise, the 68 and 75 hexahistidine-tagged deletions have even greater decreases in heparin-accelerated thrombin inhibition compared with full-length rHCII-CHis$_6$ (Table III). As noted previously (22), deletion of the acidic domain of rHCII leads both to a successive reduction in the peak heparin concentration at which maximal thrombin inhibition occurs and to increased NaCl elution from a Hi-Trap Heparin-Sepharose matrix, whether containing wt-rHCII or rHCII-CHis$_6$ (Table III). In the presence of dermatan sulfate, a similar pattern was found for both wt-rHCII and rHCII-CHis$_6$ deletion constructs (Table III). Overall, these data agree with previous work concerning the masking of glycosaminoglycan binding properties of HCII by its amino-terminal acid domain region (22). It is notable that the results for Δ1–52-rHCII-CHis$_6$ may suggest a potential interaction between this portion of the amino terminus of HCII with its own carboxyl terminus.

Carboxyl-terminal Alanine- and Lysine-tagged rHCII—To assess the character of the carboxyl-terminal tag on HCII activity, we inserted six alanine or lysine codons directly before the TAG stop codon and made rHCII-CAla$_6$ and rHCII-CLys$_6$. We typically obtained ~125 μg of protein from a 100-ml culture infected with recombinant baculoviral stock. Immunoblot analysis showed that purified rHCII-CAla$_6$ and rHCII-CLys$_6$ comigrated with wt-rHCII.

In the absence of glycosaminoglycan, the rate of thrombin inhibition by rHCII-CHis$_6$ and rHCII-CAla$_6$, but not by rHCII-CLys$_6$, was significantly faster than wt-rHCII (Table I). In contrast, the carboxyl-terminal hexapeptide-tagged rHCIIs (His$_6$, Ala$_6$, or Lys$_6$) did not have rates of chymotrypsin that differed significantly from wt-rHCII (Table I). Thus, comparing chymotrypsin to thrombin inhibition, the data imply that the carboxyl-terminal tags do not drastically affect the conformation of the reactive site loop of full-length rHCII.

In the presence of heparin, we found the alanine and lysine tags had different effects on the rate of thrombin inhibition compared with rHCII-CHis$_6$ (Fig. 4 and Table III). Although rHCII-CLys$_6$ has a similar inhibitory rate to wt-rHCII, it does demonstrate the shift of the inhibition maximum to a lower heparin concentration (20 μg/ml) similar to but not the same as rHCII-CHis$_6$ (10 μg/ml). Recombinant HCII-CAla$_6$ was reduced in activity but has the similar heparin maximum as wt-rHCII. In the presence of dermatan sulfate, thrombin inhibition by rHCII-CHis$_6$ is similar to wt-rHCII, but the rates for both rHCII-CAla$_6$ and rHCII-CLys$_6$ are significantly reduced with a similar maximal dermatan sulfate concentration ranging from 100 to 200 μg/ml (Fig. 4 and Table III). Both rHCII-CHis$_6$ and rHCII-CLys$_6$ eluted at a significantly higher ionic strength than wt-rHCII by Hi-Trap Heparin-Sepharose chromatogra-

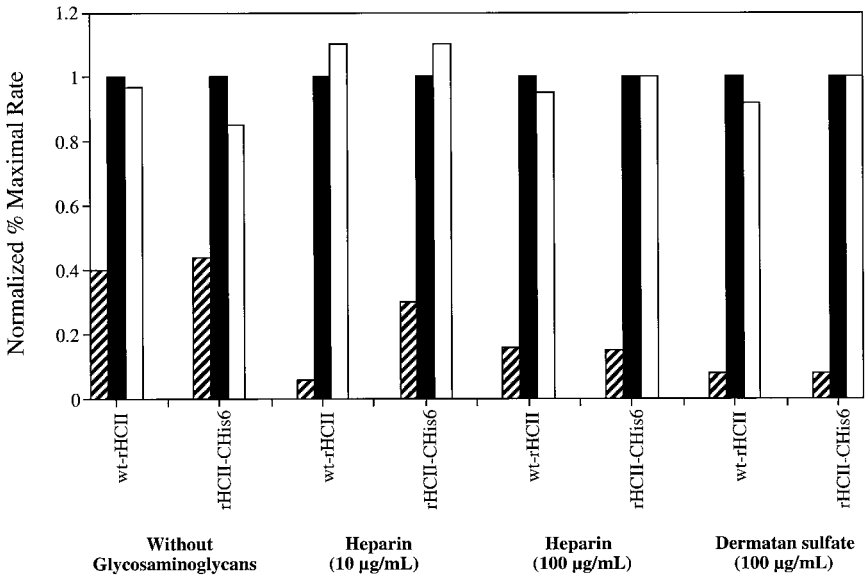

FIG. 3. **Effect of hirugen on the rate of thrombin inhibition by wt-rHCII and rHCII-CHis$_6$.** Wild-type-rHCII and rHCII-CHis$_6$ were incubated with α-thrombin in the absence and presence of glycosaminoglycan in the presence of hirugen (▨), in the absence of hirugen (■), or in the presence of control peptide (□). Residual thrombin activity was determined as described under "Experimental Procedures." The data are presented as normalized percent maximal rate of inhibition using the following rate constants of inhibition (k_2) in the absence of peptide: with no glycosaminoglycan, wt-rHCII is 1.52×10^4 M^{-1} min^{-1} and rHCII-CHis$_6$ is 1.86×10^4 M^{-1} min^{-1}; in the presence of 10 μg/ml heparin, wt-rHCII is 1.90×10^8 M^{-1} min^{-1} and rHCII-CHis$_6$ is 2.40×10^8 M^{-1} min^{-1}; in the presence of 100 μg/ml heparin, wt-rHCII is 2.20×10^8 M^{-1} min^{-1} and rHCII-CHis$_6$ is 2.20×10^8 M^{-1} min^{-1}; in the presence of 100 μg/ml dermatan sulfate, wt-rHCII is 2.50×10^8 M^{-1} min^{-1} and rHCII-CHis$_6$ is 1.80×10^8 M^{-1} min^{-1}. The above data represent the means of two determinations in triplicate.

TABLE III
HCII Inhibition of thrombin in the presence of glycosaminoglycans

Inhibition of thrombin by rHCII mutants was measured in the presence of increasing concentrations of either heparin or dermatan sulfate. The maximal inhibition of each curve was used in the calculation of the average inhibition rate. Values are expressed as means ± S.D. The number in parentheses that follows indicates the average glycosaminoglycan concentration at which the maximal rate was measured. The final column indicates the peak NaCl concentration at which each variant eluted from Hi-Trap heparin-Sepharose. All assays were performed as described under "Experimental Procedures."

Serpin	+Heparin[a] $k_2 \times 10^7$	Ratio (mutant/wt)	+Dermatan Sulfate[a] $k_2 \times 10^7$	Ratio (mutant/wt)	Hi-Trap heparin-Sepharose [NaCl]
	$M^{-1}min^{-1}$		$M^{-1}min^{-1}$		mM
wt-rHCII	63 ± 10 (100)		87 ± 9.1 (200)		540
rHCII-CHis$_6$	149 ± 46 (10)[b]	2.4	69 ± 16 (200)[c]	0.79	740
rHCII-CAla$_6$	35 ± 11 (100)[b]	0.56	48 ± 20 (200)[b]	0.55	610
rHCII-CLys$_6$	51 ± 11 (20)[c]	0.81	32 ± 5.6 (100)[b]	0.38	740
Δ1–52-rHCII	78 ± 7.4 (20)[b]	1.2	69 ± 7.7 (200)[c]	0.79	600
Δ1–68-rHCII	0.39 ± 0.16 (2)[b]	0.0062	0.044 ± 0.019 (20)[b]	0.00051	800
Δ1–75-rHCII	0.30 ± 0.061 (2)[b]	0.0048	0.018 ± 0.0008 (20)[b]	0.00021	1200
Δ1–52-rHCII-CHis$_6$	51 ± 4.5 (10)[d]	0.34[e]	21 ± 1.4 (100)[d]	0.30[e]	600
Δ1–68-rHCII-CHis$_6$	0.64 ± 0.093 (2)[d]	0.0043[e]	0.030 ± 0.0056 (20)[d]	0.00043[e]	1100
Δ1–75-rHCI-CHis$_6$	0.29 ± 0.13 (2)[d]	0.0019[e]	0.010 ± 0.0044 (20)[d]	0.00014[e]	1200

[a] Rate constants are mean values of 3–6 determinations with at least 3 preparations of recombinant protein preparations.
[b] $p \leq 0.05$ compared with wt-rHCII.
[c] Not significantly different from wt-rHCII.
[d] $p \leq 0.05$ compared with rHCII-CHis$_6$.
[e] Ratio calculated as mutant/rHCII-CHis$_6$

phy, whereas rHCII-CAla$_6$ eluted at a slightly higher NaCl concentration than wt-rHCII (Table III). These results suggest that a unique interaction may occur between hexahistidine and HCII, which is not manifest by either hexa-alanine or hexalysine attached to the carboxyl terminus of rHCII.

Carboxyl-terminal Histidine-Tagged rATIII—To examine whether augmentation of activity was a general phenomenon of other glycosaminoglycan-binding serpins, we added a hexahistidine carboxyl tag to recombinant wild-type ATIII. Again we used Kunkel's method to insert six histidine codons directly before the TAA stop codon. We used a baculoviral expression system, and 60–150 μg of protein was obtained from four T150 flasks of HighFive™ cells infected with recombinant viral stock. Immunoblot analysis showed that purified rATIII-CHis$_6$ co-migrated with wt-rATIII as a single band.

As a control to evaluate recombinant ATIII proteins, we obtained inhibition rates of $1.32 \pm 0.22 \times 10^5$ M^{-1} min^{-1} and $9.15 \pm 0.44 \times 10^4$ M^{-1} min^{-1} for thrombin and Factor Xa with human plasma-derived ATIII in the absence of glycosaminoglycan, respectively. The rates of thrombin ($8.81 \pm 1.3 \times 10^4$ M^{-1} min^{-1}) and trypsin ($180 \pm 91 \times 10^5$ M^{-1} min^{-1}) inhibition by rATIII-CHis$_6$ are essentially unchanged as compared with wt-rATIII ($10.4 \pm 2.3 \times 10^4$ M^{-1} min^{-1} and $184 \pm 57 \times 10^4$ M^{-1} min^{-1} for thrombin and trypsin, respectively). However, the rate of Factor Xa inhibition by rATIII-CHis$_6$ is $9.87 \pm 0.57 \times 10^4$ M^{-1} min^{-1}, which is significantly lower than wt-rATIII ($16.4 \pm 1.2 \times 10^4$ M^{-1} min^{-1}).

Fig. 5 shows the heparin-catalyzed ATIII inhibition of thrombin (*top panel*) and Factor Xa (*bottom panel*). We see that there is an almost 2-fold slower rate of thrombin inhibition by rATIII-

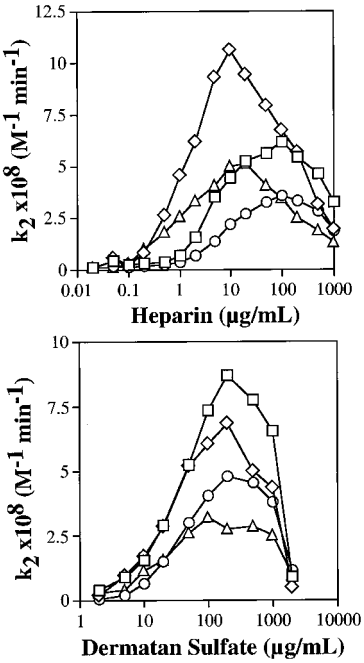

FIG. 4. **Inhibition of thrombin by wt-HCII, rHCII-CHis$_6$, rHCII-CAla$_6$, and rHCII-CLys$_6$ in the presence of glycosaminoglycans.** Thrombin inhibition assays in the presence of glycosaminoglycans were performed with α-thrombin and increasing amounts of heparin (*top panel*) or dermatan sulfate (*bottom panel*) comparing wt-rHCII (□), rHCII-CHis$_6$ (◇), rHCII-CAla$_6$ (○), and rHCII-CLys$_6$ (△). The *curves* shown are the averages of two recombinant protein preparations assayed three times each.

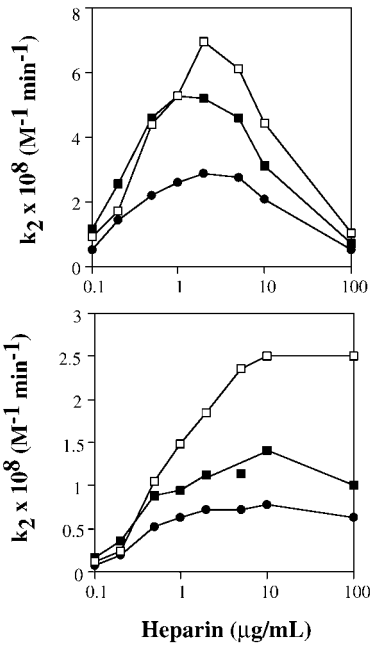

FIG. 5. **Inhibition of thrombin and Factor Xa by rATIII mutants in the presence of heparin.** Thrombin or Factor Xa assays in the presence of heparin were performed as detailed under "Experimental Procedures" with α-thrombin (*top panel*) or Factor Xa (*bottom panel*) and increasing amounts of heparin comparing plasma purified ATIII (□), wt-rATIII (■), and rATIII-CHis$_6$ (●). The *curves* are the averages of two or three protein preparations assayed two to three times each.

CHis$_6$ (2.87 ± 0.54 × 10^8 M^{-1} min^{-1}) as compared with wt-rATIII (5.26 ± 0.68 × 10^8 M^{-1} min^{-1}). The rates of Factor Xa inhibition in the presence of heparin show the same trend. Proteolysis of rATIII-CHis$_6$ with CPA should, theoretically, remove the entire histidine tag leaving the intact native protein since the final amino acid of ATIII is lysine. Rates of thrombin inhibition by rATIII-CHis$_6$ with 10 μg/ml heparin increased 40% after treatment with CPA, compared with wt-rATIII (data not shown). To compare the recombinant ATIII proteins to plasma-derived ATIII with heparin, we obtained maximal rates of inhibition of 6.94 ± 0.25 × 10^8 M^{-1} min^{-1} and 2.51 ± 0.083 × 10^8 M^{-1} min^{-1}, for thrombin and Factor Xa, respectively (Fig. 5). The *curves* in Fig. 5 illustrate that the amount of heparin required for maximal inhibition of either thrombin or Factor Xa by these ATIII derivatives does not change significantly when the histidine tag is added. The data are summarized in Table II.

We then assessed the ability of the rATIII molecules to bind heparin-Sepharose. As shown in Table II, rATIII-CHis$_6$ eluted at the same NaCl concentration as wt-rATIII (975 mM). These data support the previous data showing no shift in the amount of heparin required for antithrombin or anti-Factor Xa activity.

Histidine-tagged rHCII in Plasma-based Thrombin Inhibition Assays—The data that have been presented to this point indicate that rHCII-CHis$_6$ is an excellent thrombin inhibitor and is now comparable to the physiologic inhibitor ATIII in its rates and heparin requirements. The next set of experiments was performed to assess the potential of rHCII-CHis$_6$ as a therapeutic agent in a more physiologic based setting (*i.e.* plasma).

The results comparing rHCII-CHis$_6$ to wt-rHCII are summarized in Table IV. At each plasma condition the rates of thrombin inhibition were measured in the presence of 1 μg/ml heparin. The thrombin inhibitory capabilities of rHCII-CHis$_6$ with each plasma condition are significantly greater than those of wt-rHCII performed using the same conditions. Thrombin inhibition in REF plasma, which would contain both HCII and ATIII, gave thrombin inhibition rates that are increased 1.5-fold for rHCII-CHis$_6$ compared with wt-rHCII. Using DEF plasma, which is totally deficient in ATIII, the enhancement of thrombin inhibition by rHCII-CHis$_6$ over wt-rHCII was more apparent with a 4.6-fold increased rate. In a 50:50 mixture of REF/DEF, which mimics a heterozygous ATIII deficiency, rHCII-CHis$_6$ inhibition of thrombin was increased 1.5-fold compared with wt-rHCII. Furthermore, the rates of inhibition in the presence of 50 μg/ml of dermatan sulfate are also significantly greater with rHCII-CHis$_6$ than with wt-rHCII for each of the plasma conditions tested, with rates increased about 1.2–1.3-fold (Table IV). These data suggest that rHCII-CHis$_6$ is a significantly better thrombin inhibitor than is wt-rHCII in the presence of glycosaminoglycans in a more complex assay setting.

DISCUSSION

We have "serpendipitously" constructed an HCII mutant that is a significantly better inhibitor of thrombin than the wild-type molecule. This mutant, rHCII-CHis$_6$, is a carboxyl-terminal hexahistidine-tagged heparin cofactor II. In the absence of glycosaminoglycan we see a small increase in rates of thrombin inhibition. In the presence of heparin, rHCII-CHis$_6$ has antithrombotic activity that reaches rates comparable to those of the physiologic thrombin inhibitor ATIII. Addition of the hexahistidine tag to HCII also increases the affinity of this molecule for heparin. In contrast, the enhanced activity of rHCII-CHis$_6$ is not seen with other sulfated polysaccharides like dermatan sulfate, desmin, or fucoidan. Our results demonstrate that the activity is solely a result of the addition of the carboxyl-terminal histidine tag and that rHCII-CHis$_6$ functions to inhibit thrombin through the same mechanism as wt-rHCII, which is highly dependent on ABE-1 of thrombin. Augmentation of heparin cofactor activity in rHCII-CHis$_6$ is reversible by CPA proteolysis. We also presented another mu-

TABLE IV
Inhibition of thrombin activity in plasma

Inhibition of thrombin activity (1 nM) in the presence of wt-rHCII and rHCII-$CHis_6$ (10 nM) and glycosaminoglycan in normal human reference plasma (REF), antithrombin III-deficient human plasma (DEF) or a 50:50 mixture of these plasmas (REF/DEF). Inhibition rates are given as the average inhibition rate ± S.D. These assays were performed as described under "Experimental Procedures."

Plasma addition	Inhibition rate[a] $k_2 \times 10^8$			
	Heparin (1 μg/ml)		Dermatan sulfate (50 μg/ml)	
	wt-rHCII	rHCII-$CHis_6$	wt-rHCII	rHCII-$CHis_6$
	$M^{-1}min^{-1}$			
Normal reference (REF)	2.2 ± 0.17	3.3 ± 0.37[b]	5.7 ± 0.45	7.2 ± 0.55[c]
ATIII-deficient (DEF)	0.26 ± 0.050	1.2 ± 0.10[b]	6.5 ± 0.50	7.5 ± 0.59[c]
50:50 mixture (REF/DEF)	1.9 ± 0.050	2.8 ± 0.18[b]	5.8 ± 0.24	7.0 ± 0.48[c]

[a] Rate constants are the mean values of 3–6 determinations with 3 different preparations of recombinant proteins.
[b] $p \leq 0.001$ compared with wt-rHCII.
[c] $p \leq 0.01$ compared with wt-rHCII.

tant, rHCII-$CHis_5$Pro, which retains the enhanced activity but is resistant to CPA. We also showed that the addition of a carboxyl-terminal hexahistidine tag to ATIII actually interferes with the ability of ATIII to inhibit two of its target serine proteases, thrombin and Factor Xa, and has no influence on the heparin binding of the molecule. This contrast in activity between HCII and ATIII with a carboxyl-terminal hexahistidine tag is especially notable since their reactive site loops are very similar in sequence and in length (1, 27). Therefore, the increase in antithrombin activity is not a general phenomenon for glycosaminoglycan-binding serpins.

Previous work has indicated that when the hexahistidine tag is left attached, it rarely affects the properties of the native protein (28–30). However, we have not found any examples of heparin-binding proteins being hexahistidine-tagged anywhere in the literature. This was the point at which we made our serendipitous finding.

As described in the Introduction, HCII is believed to inhibit thrombin through an unusual mechanism in the presence of glycosaminoglycan (2, 14, 20–25). Since the enhanced activity of the hexahistidine tag was only seen with the glycosaminoglycan-binding serpin HCII but not ATIII, the data presented support the concept that the D-helix region-acidic domain interaction is altered. The increase in antithrombin activity of rHCII-$CHis_6$ suggests that the acidic domain may be in an altered conformation to more easily encounter thrombin ABE-1. Data presented describing inhibition in the presence of hirugen, a peptide of the carboxyl-terminal region of hirudin, indicate that ABE-1 of thrombin is still very important in the mechanism of thrombin inhibition by rHCII-$CHis_6$. This is further supported by inhibition of γ_T-thrombin, a proteolyzed form of thrombin defective at ABE-1. However, the slight residual increased activity of rHCII-$CHis_6$ toward γ_T-thrombin might imply that other residues of ABE-1 not perturbed by proteolysis (or blocked by hirugen in α-thrombin) might be involved in this inhibition reaction. The increased binding of rHCII-$CHis_6$ to heparin-Sepharose compared with wt-rHCII also lends support to the notion that part of the D-helix region is more accessible to heparin interaction. The reduced heparin concentration needed for peak activity for rHCII-$CHis_6$ is related to heparin-Sepharose affinity and is consistent with that seen previously for heparin binding characteristics and activity for heparin-binding serpins (3, 42). The change in heparin but not dermatan sulfate binding of rHCII-$CHis_6$ further implies that the glycosaminoglycan-binding site of HCII has both distinct and overlapping structural elements for heparin and dermatan sulfate interactions and agrees with previous variants/mutants of HCII altered in the D-helix region (23, 24). The comparable inhibition rates of wt-rHCII and rHCII-$CHis_6$ with chymotrypsin (which does not use 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 an alteration between the D-helix/acidic domain regions in the increased activity of rHCII-$CHis_6$.

It is also possible that these results could be due to the addition/exposure of a secondary heparin-binding site or some other effect of the hexahistidine on the conformation of HCII. Morgan and co-workers (43–45) have published extensively on the function of histidine-proline-rich glycoprotein as a heparin-binding molecule. This protein is notable for its tandem histidine-rich repeats that bind heparin either in the presence of divalent cations or at sub-physiologic pH values. All of our experiments were carried out at physiologic pH and in the absence of added divalent cations. Trace metal contamination is an unlikely source of our results since the control CPA-digested proteins are treated with EDTA and still maintain enhanced activity and heparin binding. The lack of effect on the heparin binding ability of ATIII also argues against the addition of a new heparin-binding site in either ATIII or HCII due to the hexahistidine tag itself. To address further the mechanism of hexahistidine and HCII, we then focused our work both on the composition of the tag and the influence of the amino-terminal acidic domain region of HCII.

We explored the contribution of the amino-terminal acidic domain of HCII to the enhanced activity of rHCII-$CHis_6$ by sequentially deleting the first 52, 68, or 75 amino acids from either wt-rHCII or rHCII-$CHis_6$. In 1991, van Deerlin and Tollefsen (22) described similar amino-terminal deletion mutants of HCII. Our data for deletions of wt-rHCII are in agreement with their results. No function has been assigned to the first 52 amino acids. However, within residues 53 and 75 there are 13 acidic amino acids (Asp, Glu, and sulfated-Tyr). These residues are grouped in two distinct clusters called "acidic region 1" and "acidic region 2" (AR-1 and AR-2). When glycosaminoglycans bind the D-helix of HCII it is believed that AR-2 is displaced, which allows AR-1 to be more accessible to bind ABE-1 of thrombin (22). The removal of amino acids 1–52 should be relatively benign based on this model of HCII. However, the removal of amino acids 1–68 or 1–75 should influence the interaction of HCII with thrombin, especially in the presence of glycosaminoglycans.

In the absence of glycosaminoglycan, the deletion mutants of wt-rHCII had no major loss of protease inhibition activity. Based on the results, the amino-terminal region of wt-HCII is not significantly involved in the inhibition of chymotrypsin or thrombin. In contrast, all three deletions in rHCII-$CHis_6$ lead to significant losses of both thrombin and chymotrypsin inhibition. The losses found in inhibition must be due to the presence of the hexahistidine tag in rHCII-$CHis_6$. Most likely the tag in the deletion mutants causes a change in the reactive site loop region of HCII since inhibition is dependent on this structure. These results suggest that either the amino-terminal

acidic domain shields the reactive site loop from the hexahistidine tag or it interacts with the hexahistidine tag to then keep the tag from perturbing the reactive site loop.

In the presence of glycosaminoglycan, the thrombin inhibition rates are only slightly affected by deletion of the first 52 amino acids of wt-rHCII. In the presence of heparin or dermatan sulfate, the loss of residues 1–68 or 1–75 leads to decreased antithrombotic activity, in agreement with the accepted model of HCII. However, the rates of thrombin inhibition by the rHCII-$CHis_6$ deletions are somewhat different. The enhanced heparin cofactor activity of rHCII-$CHis_6$ is lost with the deletion of the first 52 amino acids. As expected, the 1–68 and 1–75 deletions caused a large loss of activity with both heparin and dermatan sulfate. The progressive loss of activity indicates that the protective effect the amino-terminal region of HCII imparts on the hexahistidine tag is partially mediated between residues 52 and 75. We believe these data provide evidence for the importance and specificity of an HCII carboxyl terminus (hexahistidine tag) and amino-terminal acidic domain interaction. Since there is no crystal structure of HCII, the data imply that the amino terminus may be in close proximity to the carboxyl terminus.

We compared rHCII-$CHis_6$, rHCII-$CAla_6$, and rHCII-$CLys_6$ to provide information about the character of the carboxyl-terminal hexapeptide tag. We hypothesized that if the enhanced activity of rHCII-$CHis_6$ was a result either of the extra length or of partial positive charge on the tag, then a hexa-alanine or a hexalysine tag could be used to probe this phenomenon further. We found that only the hexahistidine or hexa-alanine tag increased the rate of thrombin inhibition in the absence of glycosaminoglycan. The inhibition of another serine protease, chymotrypsin, is not affected by the addition of each tag. These experiments provide evidence that the rate increases seen with the hexahistidine tag are not fully a result of charge on the tag, but changes in thrombin inhibition do further suggest that the acidic domain-D-helix interaction is perturbed.

In the presence of heparin we see a large increase in the rate of thrombin inhibition by rHCII-$CHis_6$ and a shift to a lower heparin requirement. In contrast, with rHCII-$CAla_6$ and rHCII-$CLys_6$, we see either a loss or no change in activity. In the presence of dermatan sulfate we do not observe increased rates of thrombin inhibition when comparing rHCII-$CHis_6$ to wt-rHCII. The hexa-alanine and hexalysine tags actually are detrimental to the inhibition of thrombin in the presence of dermatan sulfate. The increase in both dermatan sulfate and heparin binding by rHCII-$CLys_6$ implies that this protein may have less specific glycosaminoglycan binding abilities than the altered binding properties of rHCII-$CHis_6$. These data indicate that neither the positive charge nor the addition of six amino acids to the carboxyl terminus of HCII is solely responsible for increased heparin binding. However, these results do suggest that the increase in rates of rHCII inhibition with heparin seems to be specific to the hexahistidine tag.

Current antithrombotic therapies include heparin, low molecular weight heparin, and other heparinoids, oral anticoagulants such as warfarin, synthetic molecules such as Argatroban, and naturally occurring peptides isolated from hematophagous parasites, most notably hirudin (46–48). Engineered protease inhibitors and proteases are being investigated as anticoagulant therapies (49–55). Chimeric anticoagulants or serpins with specifically engineered reactive site loops have been described (51–55). Recombinant HCII-$CHis_6$ may offer some unique advantages over currently available treatments. In the presence of heparin, the activity of rHCII-$CHis_6$ is comparable to that of the physiologic inhibitor ATIII. The histidine-tagged HCII functions optimally at a significantly lower heparin concentration than does wild-type HCII. In a plasma-based assay, we also see increased antithrombotic activity of rHCII-$CHis_6$ with both heparin and dermatan sulfate. Unlike ATIII, HCII is a very specific anticoagulant with its activity being targeted to thrombin in the coagulation cascade. Carboxypeptidase A activity has been reported in plasma (56), and rHCII-$CHis_6$ is susceptible to CPA digestion. This could allow for the eventual degradation of rHCII-$CHis_6$ to a less active protein, thus acting as a "temporary" thrombin inhibitor. In addition, we found rHCII-$CHis_5$Pro has increased antithrombin rates at low heparin concentrations but is resistant to CPA proteolysis. This HCII mutant could be a "longer acting" version of the same anticoagulant therapy. Recombinant HCII-$CHis_6$ derivatives could offer a novel alternative to existing anticoagulant therapy.

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