The Transferable Tail: Fusion of the N-Terminal Acidic Extension of Heparin Cofactor II to α_1-Proteinase Inhibitor M358R Specifically Increases the Rate of Thrombin Inhibition†

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ABSTRACT: The conversion of the reactive center bond of the serpin α_1-proteinase inhibitor (α_1-PI, also known as α_1-antitrypsin) from Met-Ser to Arg-Ser decreases the rate at which it inhibits neutrophil elastase and endows it with the ability to inhibit thrombin and activated protein C (APC). Another serpin, heparin cofactor II (HCII), contains a unique N-terminal extension that binds thrombin exosite 1. We fused residues 1−75 of HCII to the N-terminus of α_1-PI M358R, forming an HCII−α_1-PI chimera (HAPI M358R). It inhibited α-thrombin 21-fold faster than α_1-PI M358R, with second-order rate constants of 2.3×10^8 M^{-1} min^{-1} versus 1.1×10^7 M^{-1} min^{-1}, respectively. When γ_T-thrombin, which lacks an intact exosite 1, was substituted for α-thrombin, the kinetic advantage of HAPI M358R over α_1-PI M358R was reduced to 9-fold, whereas APC and trypsin, proteases lacking exosite 1-like regions, were inhibited only 1.3- and 2-fold more rapidly by HAPI M358R than by α_1-PI M358R, respectively. Maximal enhancement of α_1-PI M358R activity required the acidic residues found between HCII residues 55 and 75, because no enhancement was observed either by fusion of residues 1−54 alone or by fusion of a mutated HCII acidic extension in which all Glu and Asp residues between positions 55 and 75 were neutralized by mutation. Fusing residues 55−75 to α_1-PI M358R yielded a relative rate enhancement of only 6-fold, suggesting a need for the full tail region to achieve maximal enhancement. Our results suggest that transfer of the N-terminal acidic extension of HCII to α_1-PI M358R enhanced its inhibition of thrombin by conferring the ability to bind exosite 1 on HAPI M358R. This enhancement may aid in efforts to tailor this inhibitor for therapeutic use.

Regulation of the serine protease thrombin is critically important in maintaining hemostasis. Thrombin performs multiple roles in coagulation, including (1) cleavage of fibrinogen to fibrin, (2) activation of factors XIII and XI and cofactors V and VIII, (3) activation of platelets via protease-activated receptors, (4) conversion of protein C to APC,[1] and (5) activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (the two latter functions occurring when thrombin is bound to thrombomodulin) (reviewed in ref *1*). Less than optimal regulation of this versatile enzyme leads to thrombotic disease. In vivo, glycosaminoglycan-dependent serine protease inhibitors (serpins) such as antithrombin (AT) and heparin cofactor II (HCII) are important in modulating thrombin activity (*2*).

Serpins share a characteristic central β-sheet core structure (β-sheet A) and a protruding reactive center loop (RCL) (*2*) containing the scissile reactive center bond cleaved by serine proteases [termed P1−P1′ (*3*)]. Cleavage of this bond frees the RCL for insertion into β-sheet A, resulting in translocation of the protease from one pole of the serpin to the other (*4*) and formation of a stable covalent complex between the serpin and enzyme (*5*).

RCL structure is related to serpin activity and specificity, as shown by the dramatic example of α_1-proteinase inhibitor (α_1-PI) Pittsburgh (*6*), a naturally occurring variant in which the Met-Ser reactive center bond was replaced by Arg-Ser (i.e., M358R). This mutation converted α_1-PI into an efficient thrombin inhibitor and led to an ultimately lethal bleeding tendency (*6*). Clinical use of recombinant α_1-PI M358R as an antithrombotic agent was therefore contemplated (*7*). This strategy was complicated, however, when it was discovered that the enhanced activity of α_1-PI M358R was not limited to thrombin but instead extended to other factors (*8*), notably APC (*9*). This anti-APC activity may explain the failure of

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[1] Abbreviations: APC, activated protein C; α_1-PI, α_1-proteinase inhibitor, α_1-antitrypsin; α_1-PI M358R, α_1-PI with the substitution of Met358 by Arg; AT, antithrombin; γ_T-thrombin, proteolytic fragment of α-thrombin formed by digestion with trypsin; GPRP, Gly-Pro-Arg-Pro tetrapeptide; HAPI, fusion protein of residues 1−75 of heparin cofactor II and all of α_1-PI; HAPI M358R, HAPI with the M358R substitution; HCII, heparin cofactor II; k_2, second-order rate constant of inhibition; RCL, reactive center loop; serpin, serine protease inhibitor; P1−P1′, the reactive center peptide bond, where P1 is the amino acid N-terminal to cleavage and P1′ is the amino acid C-terminal to cleavage; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; SI, stoichiometry of inhibition; WT, wild type.

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α_1-PI M358R to improve outcomes in a baboon model of sepsis (*10*) since recombinant APC is clinically effective in septic patients (*11*).

We (*12*) and others (*13*, *14*) have attempted to increase the specificity of α_1-PI M358R for thrombin over APC by introducing additional mutations into its RCL. Hopkins et al. made these mutations based on the corresponding residues of antithrombin and in the best case decreased the anti-APC activity of α_1-PI M358R to levels similar to wild-type α_1-PI, while maintaining high anti-thrombin activity; however, the effects of these changes on the stoichiometry of inhibition (SI) were not investigated (*13*, *14*). Similarly, we used the corresponding residues of HCII and found a 70-fold decrease in the rate of APC inhibition by the most effective α_1-PI M358R variant (*12*); however, this decrease was accompanied by a 3-fold decrease in antithrombin activity and a substantial increase in stoichiometry. Before further refining the RCL mutagenesis approach, we considered the possibility of adding an extra-RCL thrombin binding site to α_1-PI M358R, one derived from HCII.

Alignment of HCII with other serpins shows that it contains a unique N-terminal extension of approximately 75 residues (*15*). This region (also referred to as a "tail", e.g., in ref *16*) contains two strongly acidic repeats that resemble the exosite 1 binding site of the leech protein hirudin (*17*). Binding of glycosaminoglycans to helix D of HCII conformationally activates this N-terminal acidic extension and makes it available for binding exosite 1 of thrombin (*18*−*24*). This interaction between exosite 1 and the liberated N-terminal extension of HCII transforms the serpin into an efficient thrombin inhibitor despite an unfavorable P1 Leu residue. Despite the solution of the crystal structures of both free HCII and an HCII−thrombin S195A complex, our knowledge of the role of N-terminal acidic extension remains incomplete, because only portions of this region could be resolved in either crystal (*16*). Before this study it was unknown if this extension could be productively transferred to another serpin or if it had to function in the context of the complete HCII polypeptide. We found that fusing the N-terminal extension of HCII to α_1-PI M358R substantially increased the rate of thrombin inhibition, reaching levels comparable to those reported for glycosaminoglycan-activated AT and HCII. The increased antithrombin activity of the fusion protein was accompanied neither by a substantial increase in APC inhibition rate nor by an increase in SI, demonstrated no requirement for glycosaminoglycans, and was maintained in a fibrin-rich environment.

MATERIALS AND METHODS

Construction of pBAD-H_6-$HCII_{x-y}$-ELG_6-API M358R. We first modified the previously constructed plasmid pBAD-H_6-API M358R (*12*). The N-terminal hexahistidine tag was removed from the vector and replaced with an ELG_6 spacer by amplifying the cDNA of α_1-PI M358R with sense primer AB30299 (5′-GAT*GAGCTC*G GTGGAGGTGG AGGTGGAGAG GATCCCCAGG GAGATGCT-3′) and antisense pBADmychis-B-specific primer AB15674 (5′-AAATTCTGTTTTATCAGACC-3′). Digestion of the resulting PCR product with *Sac*I (italicized in the sense primer) and *Eco*RI (a unique restriction site at the 3′ end of the PCR product) permitted its insertion between these sites in plasmid pBADmychis-B (Invitrogen, La Jolla, CA). Like all constructs in this study, the resulting plasmid, pBAD-G_6-API M358R, was sequenced by the Institute for Molecular Biology and Biotechnology (McMaster University, Hamilton, Ontario, Canada) to verify the integrity of the coding sequence.

Plasmid pBAD-H_6-$HCII_{1-75}$-API M358R was provided with the nucleotides coding for the hexahistidine tag and the first 75 amino acids of HCII by PCR of plasmid pBAD-H_6-HCII, using the 5′ primer AB14986 (5′-GAT*CCATGG*G GTCTCATCAC CATCACCAT CACGGGAGCA AAGGCCCGCT GGATCAG-3′) and 3′ primer AB30298 (5′-GAT*GAGCTC*G TCGATGTAGT CGTCGTCTTC AC-3′). The product was restricted with *Nco*I and *Sac*I and ligated with *Nco*I/*Sac*I-digested pBAD-G_6-API M358R. The related vector, pBAD-H_6-$HCII_{1-75}$API, was constructed by digesting pBAD-H_6-API (*12*) with *Bst*XI and *Eco*RI and inserting this fragment in pBAD-H_6-$HCII_{1-75}$API M358R between these sites, reverting the M358R mutation to WT.

The plasmids pBAD-H_6-$HCII_{1-54}$-API M358R and pBAD-H_6-$HCII_{1-75NEUT}$API M358R [a plasmid in which codons Glu^{55}, Glu^{56}, Asp^{57}, Asp^{58}, Asp^{59}, Asp^{62}, Glu^{64}, Glu^{69}, Asp^{70}, Asp^{71}, Asp^{72}, and Asp^{75} of the acidic region of HCII were replaced with either neutral Asn (for Asp) or Gln (for Glu) residues] were constructed in a similar manner. For pBAD-H_6-$HCII_{1-54}$-API M358R, residues 1−54 of HCII were amplified using the sense primer AB14986 and antisense primer ML10938 (5′-GAT*GAGCTC*C CCCTCTGGAA TCCAGTCGTT GG-3′). For pBAD-H_6-$HCII_{1-75NEUT}$API M358R, residues 1−75 were amplified using the sense primer AB14986 and the antisense mutagenic primer ML10941 (5′-ACC*GAGCTC*G TTGATGTAGT TGTTGTTTTG ACTGAATATC TTCTGCAGGT TCAGATAGTT GTTGTTCTGC TGCCCCTCTG G-3′) that introduced the mutations in the acidic region of HCII. The amplified products were inserted into pBAD-G_6-API M358R as described above. The plasmid pBAD-H_6-$HCII_{55-75}$API M358R was made by inserting annealed oligonucleotides ML10939 (5′-CGTCGATGTA GTCGTCGTCT TCACTGAATA TCTTCTCCAG GTCCAGATAG TCGTCGTCCT CCTCGTGATG GTGATGGTGAT GAGACCC-3′) and ML10940 (5′-CGTCGATGTA GTCGTCGTCT TCACTGAATA TCTTCTCCAG GTCCAGATAG TCGTCGTCTT CTTCGTGATG GTGATGGTGA TGAGACCC-3′) with *Nco*I and *Sac*I compatible ends into the *Nco*I/*Sac*I-restricted pBAD-G_6-API M358R.

Expression and Purification of Variant Proteins. α_1-PI, α_1-PI M358R, and the various HCII-α_1-PI chimeric variants were expressed and purified as described previously (*12*, *25*, *26*), specifically using *Escherichia coli* TOP10 cells (Invitrogen) grown in LB medium supplemented with 100 μg/mL ampicillin, affinity chromatography using Ni-NTA agarose (Qiagen, Carlsbad, CA), and final purification using DEAE-Sepharose (GE Healthcare, Baie d'Urfé, Quebec, Canada). Protein concentrations were determined by ELISA using either affinity-purified sheep anti-human α_1-PI antibodies or goat anti-human HCII antibodies (Affinity Biologicals, Ancaster, Ontario, Canada) or by measuring the OD_{280} and using a molar extinction coefficient calculated by the method of Edelhoch (*27*, *28*); as both values were typically in good agreement, the former was used for consistency with our previously published work (*12*, *25*, *26*).

Gel-Based Analysis of Serpin−Enzyme Complexes. The ability of recombinant serpin variants to form SDS-stable complexes with α-thrombin was measured by incubating 1 μM serpin with 200 nM α-thrombin (Enzyme Research, South Bend, IN) at 37 °C, diluting aliquots at timed intervals with 2× SDS−PAGE loading buffer [0.16 M Tris-HCl, pH 6.8, 4.4% (v/v) SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.4 mg/mL Bromophenol Blue], and performing SDS−PAGE as described (*12*, *25*, *26*). In some experiments Coomassie Blue-stained gels were destained and dried, and their scanned images were analyzed using UN-Scan-It gel digitizing (Silk Scientific, Orem, UT) to determine band intensities.

Rates of Thrombin, APC, and Trypsin Inhibition by Recombinant Variants. The second-order rate constants (k_2) of α- and γ_T-thrombin (Haematologic Technologies, Essex Junction, VT), APC (Sigma-Aldrich, Mississauga, Ontario, Canada), and trypsin (Calbiochem, La Jolla, CA) inhibition by α_1-PI, α_1-PI M358R, and the chimeric variants were determined under pseudo-first-order conditions using a discontinuous assay. Briefly, recombinant serpins (70−1250 nM) were incubated with either α- or γ_T-thrombin (3.5−7 nM), APC (25 nM), or trypsin (5−10 nM) in PPNE buffer [20 mM Na_2HPO_4, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1% poly(ethylene glycol) 8000] for various times at room temperature. Residual protease activity was determined by diluting the reactions 1:10 in 150 μM S2238 (for thrombin), 400 μM S2366 (for APC), or 150 μM S2222 (for trypsin) and measuring the absorbance at 405 nm for 5 min using an EL808 plate reader (BioTek Instruments, Winooski, VT). From these data pseudo-first-order and second-order rate constants were derived as described (*26*).

Rate of Thrombin Inhibition by Serpins in the Presence of Soluble Fibrin. Soluble fibrin was prepared as described by Becker et al. (*29*). Briefly, 45 μM fibrinogen depleted of vWF, plasminogen, and fibronectin (Enzyme Research) was clotted with 5 nM thrombin at 37 °C. The fibrin clot was dialyzed against distilled water to remove fibrinopeptides A and B and then against 20 mM acetic acid prior to storage at −70 °C. Prior to use, soluble fibrin (SF) was neutralized with 40% (v/v) 1 M Tris-HCl, pH 7.4, and then supplemented with 5 mM GPRP-NH_2 peptide to prevent polymerization. Kinetic assays using either recombinant HCII (*26*), α_1-PI M358R, or H_{1-75}API M358R were performed as described above, except that 5 mM GPRP-NH_2, 3 μM SF, and 500 nM heparin were present. Reactions were terminated by 1:10 dilution in 150 μM S2238 supplemented with 10 mg/mL polybrene (Sigma, St. Louis, MO). Second-order rate constants were determined as described above.

Stability of Recombinant Proteins. Ten micrograms of either purified His-tagged HCII prepared by arabinose-inducible bacterial expression as described (*25*) or HAPI M358R was separately diluted into 0.1 mL of citrated human plasma (pH 7.0) and heated at 37 °C for up to 24 h. At time 0, hourly intervals up to 6 h, and at the 24 h time point, aliquots were removed from the incubation and analyzed by SDS−PAGE and immunoblotting with an anti-hexahistidine antibody (Qiagen, Chatsworth, CA).

Stoichiometries of Inhibition. The number of molecules of recombinant α_1-PI required to inhibit one molecule of thrombin was determined as previously described (*12*, *25*, *26*). A constant amount of thrombin (100 nM) was incubated with different amounts of serpin in PPNE buffer for 4 h at room temperature. Residual thrombin activity was determined by quenching the reaction with 150 μM S2238 and recording the change in absorbance at 405 nm over 5 min. A plot of thrombin activity versus the ratio of serpin/thrombin was then used to determine the SI.

Visualization of Protein Structures. The Protein Data Base (PDB) files 1JMO and 2HNT, relating to crystal structures of the HCII−thrombin S195A encounter complex (*16*) and γ-thrombin (*30*), respectively, were manipulated using MDL Chime and Protein Explorer software (*31*) (http://proteinexplorer.org), including its "contacts" function.

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed on purified recombinant proteins provided in 25 mM ammonium bicarbonate to the Advanced Protein Technology Centre, Hospital for Sick Children, Toronto, Ontario, Canada.

RESULTS

Expression and Initial Characterization of α_1-PI and HCII−α_1-PI Chimeric Proteins. Previously, we and others have shown that both α_1-PI and HCII can be expressed in *E. coli*, in functional forms that inhibit target proteases, at rates highly similar to their plasma-derived counterparts (*12*, *20*, *25*, *26*, *32*). In this study, we also employed bacterially expressed hexahistidine-tagged serpins; as shown in Figure 1A, two previously described recombinant forms of α_1-PI (*12*) and five novel chimeric proteins containing all, part, or mutated versions of the N-terminal acidic tail of HCII were examined. Following nickel chelate affinity and ion-exchange chromatography, the seven recombinant serpins were obtained with purities ranging from 95.2% to 99.9%, as demonstrated by the SDS gel shown in Figure 1B and quantification of bands on its scanned image. Where detected, minor contaminating polypeptides were not substrates for thrombin cleavage (see Figure 2) and were therefore assumed to be inactive. Five of the purified recombinant serpins migrated with apparent molecular masses differing from their theoretical masses, given parenthetically, by no more than 6%: WT α_1-PI (45335), α_1-PI M358R (45360), $HCII_{55-75}$API M358R (H_{55-75}API M358R, 48610), $HCII_{1-54}$API M358R (H_{1-54}API M358R, 51961), and $HCII_{1-75NEUT}$API M358R ($H_{1-75NEUT}$API M358R, with Asp to Asn and Glu to Gln charge-neutralizing mutations between residues 55 and 75, 54528). In contrast, the theoretical masses of $HCII_{1-75}$API (HAPI, 54515) and $HCII_{1-75}$API M358R (HAPI M358R, 54540) differed from those calculated from their mobility by 17%. We sought clarification of this discrepancy by mass spectrometry. The predominant spectral peak in each preparation of the seven proteins gave a mass differing from that predicted by less than 0.04%. Taken together with the immunoreactivity of each recombinant protein with both anti-hexahistidine and anti-α_1-PI specific antibodies (data not shown), our observations indicated that all recombinant proteins were purified in intact form and that the atypical migration of HAPI and HAPI M358R likely arose due to diminished SDS binding to the heavily charged HCII 55−75 region in the context of the full tail. This purity and integrity of our preparations were further supported by the similarity between their concentrations determined by either anti-α_1-PI ELISA or total protein assays, including the Edelhoch method (*27*, *28*).

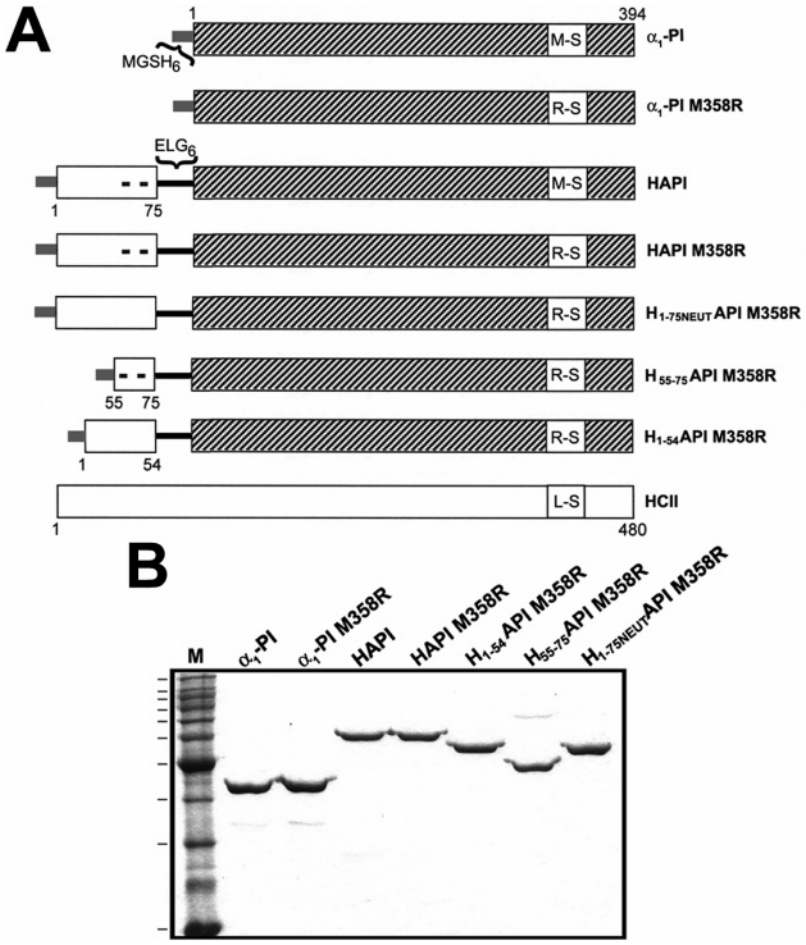

FIGURE 1: Schematic and electrophoretic representation of fusion proteins characterized in this study. The hexahistidine-tagged chimeric variants as well as wild-type α_1-PI and natural HCII are represented schematically in panel A. In each variant, an ELG_6 spacer (thin black bar) separated the HCII extension (open bar) from the body of α_1-PI (hatched bar), and an $MGSH_6$ hexahistidine tag (thicker gray bar) was present for nickel affinity chromatography. The negative symbols in the HCII extensions represent the two negatively charged hirudin-like repeats. The reactive center of each serpin is identified within the hatched areas. Numbers above the bar diagrams relate to α_1-PI and below them, to HCII. The electrophoretic mobility of the chimeric variants relative to wild-type α_1-PI and α_1-PI M358R is shown in panel B. For each variant, approximately 0.75 μg of protein was electrophoresed on a 10% SDS−polyacrylamide gel subsequently stained with Coomassie Blue. M, markers, refers to molecular mass markers of 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, and 20 kDa identified by tick marks at left.

Demonstration of Denaturation-Resistant Complex Formation with α-Thrombin. Initially, we produced two chimeric proteins by fusing amino acids 1−75 of HCII, containing both negatively charged hirudin-like repeats followed by an ELG_6 spacer, to the N-terminus of either α_1-PI or α_1-PI M358R (see Figure 1A). The ability of the HCII−α_1PI chimeric variants to form a denaturation-resistant serpin−enzyme complex with α-thrombin was initially assessed qualitatively by SDS−PAGE and compared to that of their wild-type counterparts. Both wild-type α_1-PI and HAPI were able to form SDS-stable complexes with α-thrombin, although the extent of complex formation was greater for HAPI than α_1-PI (Figure 2A,B). In a similar assay shown in Figure 2C,D, despite the use of lower concentrations of serpin and enzyme, complex formation between thrombin and both α_1-PI M358R and HAPI M358R was maximal in only 1 min at 37 °C, raising the possibility that fusion of the HCII region enhanced the ability of both wild-type and M358R forms of α_1-PI to inhibit thrombin. Minor cleaved serpin products with thrombin-dependent increases in mobility were observed both for α_1-PI M358R and for HAPI M358R (see Figure 2C,D), suggesting both that the HCII tail fusion did not provoke a large shift to the substrate branch of the serpin reaction pathway (reviewed in ref *2*) and that the stoichiometry was greater than 1. Similar results with respect to complex formation were also obtained with chimeric proteins H_{55-75}API M358R, H_{1-54}API M358R, and $H_{1-75NEUT}$API M358R (data not shown).

Kinetic Characterization of HAPI and HAPI M358R Proteins. The second-order rate constants (k_2) of thrombin inhibition for both chimeric proteins were measured to assess quantitatively whether the N-terminal extensions to α_1-PI and α_1-PI M358R mediated an increase in the rate of thrombin inhibition. For HAPI, k_2 values were approximately 4 times greater than for wild-type α_1-PI (Table 1). For HAPI M358R, the measured mean k_2 was $(2.3 \pm 0.2) \times 10^8$ M^{-1} min^{-1}, which represented a 21-fold rate enhancement compared to recombinant α_1-PI M358R produced in the same system. The increase in reactivity observed for HAPI and HAPI M358R was not associated with an increase in their stoichiometries of inhibition (SI) relative to α_1-PI M358R, which ranged from mean values of 2.9 to 3.7 (Table 1). Additional kinetic analysis showed that the increase in reactivity observed for HAPI M358R was specific for thrombin. The variant HAPI M358R inhibited APC with a rate constant of $(1.8 \pm 0.1) \times 10^6$ M^{-1} min^{-1}, comparable to

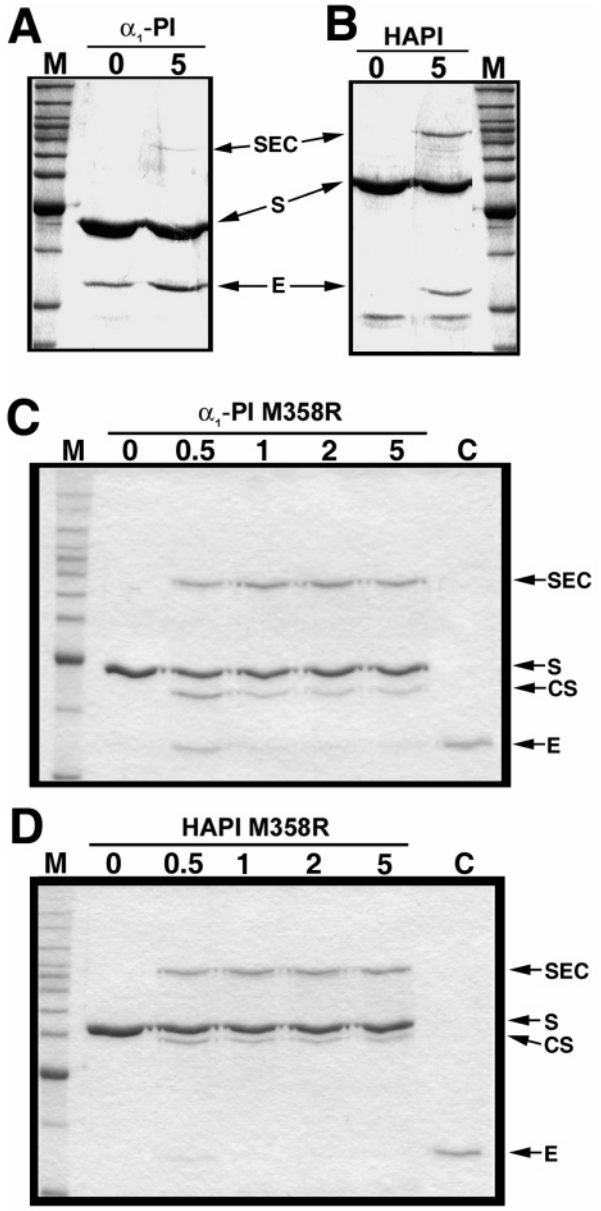

FIGURE 2: Formation of SDS-stable serpin−enzyme complexes between either α_1-PI (panel A), HAPI (panel B), α_1-PI M358R (panel C), or HAPI M358R (panel D) and thrombin. Reactions were carried out at 37 °C for the times (in minutes) specified above the lanes, at a serpin to thrombin ratio of 5:1. The products were electrophoresed on 10% SDS−polyacrylamide gels subsequently stained with Coomassie Blue. SEC represents the serpin−enzyme complex formed after time *t*; S, unreacted serpin; CS, cleaved serpin; and E (enzyme), unreacted thrombin (B chain). The marker (M) at the left of each panel corresponds to 160, 120, 100, 90, 80, 70, 60, 50 (darker), 40, and 30 kDa; control lane C contains thrombin alone.

that of $(1.4 \pm 0.1) \times 10^6$ M^{-1} min^{-1} determined for α_1-PI M358R, while for α_1-PI and HAPI, a k_2 against APC could not be determined, suggesting minimal anti-APC activity by these recombinant proteins. Similarly, the rate of trypsin inhibition by HAPI M358R was increased only 2-fold compared to that of α_1-PI M358R (Table 1). The specificity of the rate enhancement for thrombin suggested a mechanism dependent on a unique feature of thrombin, e.g., exosite 1.

We next turned to γ_T-thrombin inhibition kinetics to address the role of exosite 1 function in the activity of HAPI M358R. γ_T-thrombin is a product of a proteolytic digestion of α-thrombin by trypsin, in which cleavages at Arg^{67} and Arg^{77A} disrupt the β-loop of exosite 1, and an additional cleavage occurs in the autolysis loop (*33*, *34*); exosite 1 is diminished but not eliminated by these cleavages (*19*). The inhibitory enhancement associated with adding the HCII tail to α_1-PI M358R was reduced when γ_T-thrombin was substituted for α-thrombin in kinetic assays; γ_T-thrombin was inhibited only 9-fold more rapidly by HAPI M358R than by α_1-PI M358R (Table 1). These results supported the acquisition of exosite 1 binding as an important contributor to the enhanced inhibitory activity of HAPI M358R.

Kinetic Characterization of Additional Chimeric Proteins. On the basis of the HCII literature, we hypothesized that the hirudin-like repeats were responsible for the enhanced activity of HAPI M358R. To test this idea, we expressed and characterized three additional HCII-α_1-PI chimeric variants (see Figure 1A). The first variant, H_{1-54}API M358R, consisted of the first 54 amino acids (lacking both hirudin-like repeats) of HCII fused to α_1-PI M358R. Another chimeric protein, H_{55-75}API M358R, consisted of the hirudin-like repeats of HCII (residues 55−75) appended to α_1-PI M358R. The last variant, $H_{1-75NEUT}$API M358R, was identical to HAPI M358R except that the negatively charged glutamic acid (Glu^{55}, Glu^{56}, Glu^{64}, and Glu^{69}) and aspartic acid residues (Asp^{57}, Asp^{58}, Asp^{59}, Asp^{62}, Asp^{70}, Asp^{71}, Asp^{72}, and Asp^{75}) of the acidic tail region of HCII were replaced with either neutral Asn (for Asp) or Gln (for Glu) residues. Kinetic analysis showed that the rate constant of α-thrombin inhibition by H_{1-54}API M358R was $(1.5 \pm 0.2) \times 10^7$ M^{-1} min^{-1}, a value comparable to that determined for α_1-PI M358R [$(1.1 \pm 0.05) \times 10^7$ M^{-1} min^{-1}; Table 1], suggesting little contribution of these tail residues to the inhibitory enhancement seen in HAPI M358R. Similarly, neutralizing the negative charges between residues 55−75 of the N-terminal tail of HAPI M358R eliminated the enhancement associated with fusion of the full tail. In contrast, appending residues 55−75 of HCII to α_1-PI M358R increased the rate of thrombin inhibition 5−6-fold relative to α_1-PI M358R (Table 1). Stoichiometries of inhibition of thrombin by H_{1-54}API M358R and H_{55-75}API M358R were similar to that of α_1-PI M358R (Table 1), although $H_{1-75NEUT}$API M358R exhibited a significantly elevated SI (by nonparametric ANOVA with Dunn's post-test versus HAPI M358R values). Our results showed that residues of the HCII acidic tail previously suggested to bind thrombin exosite 1, those between residues 55 and 75, were the most critical ones in the elevation of inhibitory activity demonstrated by HAPI M358R.

The rate of inhibition of γ_T-thrombin by these variants was also measured. As expected based on the results with α-thrombin, γ_T-thrombin inhibition by H_{1-54}API M358R and $H_{1-75NEUT}$API M358R occurred at a rate similar to that determined for α_1-PI M358R (Table 1). In contrast, H_{55-75}API M358R inhibited γ_T-thrombin inhibition approximately 2.5-fold more rapidly than α_1-PI M358R [with k_2 values of $(5.7 \pm 1.3) \times 10^6$ $M^{-1}min^{-1}$ versus $(1.4 \pm 0.1) \times 10^7$ M^{-1} min^{-1}]. As with HAPI M358R, the increase in γ_T-thrombin inhibition by H_{55-75}API M358R was not as substantial as the increase in α-thrombin inhibition observed with this variant, probably due to retention of only a part of exosite 1 in the proteolyzed enzyme and to less-than-optimal positioning of the truncated tail.

Table 1: Protease Inhibition by HCII−α_1-PI Fusion Proteins and Wild-Type Counterparts

	protease					
	α-thrombin		γ-thrombin	APC	trypsin	
serpin	k_2 [a] ($\times 10^6$ M^{-1} min^{-1})	SI[a]	k_2 [a] ($\times 10^6$ M^{-1} min^{-1})	k_2 [a] ($\times 10^6$ M^{-1} min^{-1})	k_2 [a] ($\times 10^6$ M^{-1} min^{-1})	α-thrombin/ APC ratio
α_1-PI	0.003 ± 0.0002	ND[b]	ND	ND	ND	ND
α_1-PI M358R	11.0 ± 0.5	3.2 ± 0.3	5.7 ± 1.3	1.4 ± 0.1	2700 ± 200	7.9
HAPI	0.013 ± 0.0005	3.7 ± 0.2	ND	not reactive	ND	ND
HAPI M358R	230 ± 22	2.9 ± 0.1	63 ± 6	1.8 ± 0.1	5500 ± 200	128
H_{1-54}API M358R	15 ± 2	3.4 ± 0.8	3.1 ± 0.2	0.62 ± 0.06	ND	24
H_{55-75}API M358R	68 ± 4	3.4 ± 0.7	14 ± 1	0.52 ± 0.04	ND	131
$H_{1-75NEUT}$API M358R	6.5 ± 0.9	5.9 ± 0.8	2.2 ± 0.1	1.2 ± 0.1	ND	5.4

[a] The mean of at least three determinations ± the standard error of the mean (SEM) is shown. [b] ND, not determined.

Thrombin Inhibition in the Presence of Heparin and Soluble Fibrin. Because thrombin binds to fibrin via exosite 1 *(1, 29)*, we questioned whether the tail-dependent enhancement of thrombin inhibition of HAPI M358R would be abrogated in a fibrin-rich environment. In the presence of heparin, HCII inhibits clot-bound thrombin less effectively than free thrombin, probably due to the ternary thrombin−heparin−fibrin complex in which fibrin binding is mediated by exosite 1 and heparin binding by exosite 2 (*29*). As shown in Figure 3, we therefore compared thrombin inhibition by HCII, HAPI M358R, and α_1-PI M358R in the presence of soluble fibrin with and without the further addition of heparin. Thrombin inhibition by HCII was reduced 41-fold in the presence of both heparin and soluble fibrin but only 2-fold by fibrin alone. In contrast, soluble fibrin reduced the rate of thrombin inhibition by α_1-PI M358R or HAPI M358R by 3.9−6-fold whether or not heparin was present. Thus, even in the presence of soluble fibrin and heparin, HAPI M358R remained a more effective thrombin inhibitor than α_1-PI M358R, with measured rate constants of $(1.5 \pm 0.1) \times 10^7$ M^{-1} min^{-1} and $(4.5 \pm 0.5) \times 10^5$ M^{-1} min^{-1}, respectively. It should be noted that control k_2 values shown in Figure 3 do not reach the levels reported in Table 1 for HAPI M358R due to the minor inhibitory effects of peptide GPRP, required to inhibit fibrin−fibrin interactions in the soluble fibrin portion of these experiments, or of heparin.

Stability of the HAPI M358R Chimera. Unlike in HCII, in HAPI M358R the acidic tail was fully available for thrombin interaction without the need for heparin activation. This finding raised the possibility that in HAPI M358R the transferred tail was in an exposed position. To determine if the position of the tail in HAPI M358R rendered it prone to proteolytic attack, we conducted a stability experiment in citrated plasma. Dilution of equal amounts of either HAPI M358R or H_6-HCII into citrated plasma, followed by incubation at 37 °C for up to 24 h, had no discernible effect on the stability of either bacterially expressed recombinant serpin (data not shown).

DISCUSSION

The unique N-terminal acidic tail enhances the rate of thrombin inhibition by HCII by binding thrombin exosite 1. Maximal exosite 1−tail interactions require allosteric activation of HCII mediated by helix D, whose conformation either directly or indirectly controls the accessibility of the tail to thrombin (*16, 20, 21*). Glycosaminoglycans such as heparin or dermatan sulfate serve as the ligands for allosteric activation, binding on or near helix D (*35−37*). In this study we sought to determine if the tail would function in the context of α_1-PI M358R, a thrombin-inhibiting serpin different from HCII, one lacking affinity for glycosaminoglycans (*38*). We found that the tail functioned efficiently in this context, needing no added glycosaminoglycan to enhance α_1-PI M358R activity, despite the attainment by HAPI M358R of rates of thrombin inhibition equivalent to those reported for recombinant wild-type HCII or AT in the presence of glycosaminoglycans (*20−24*). Indeed, heparin had no effect

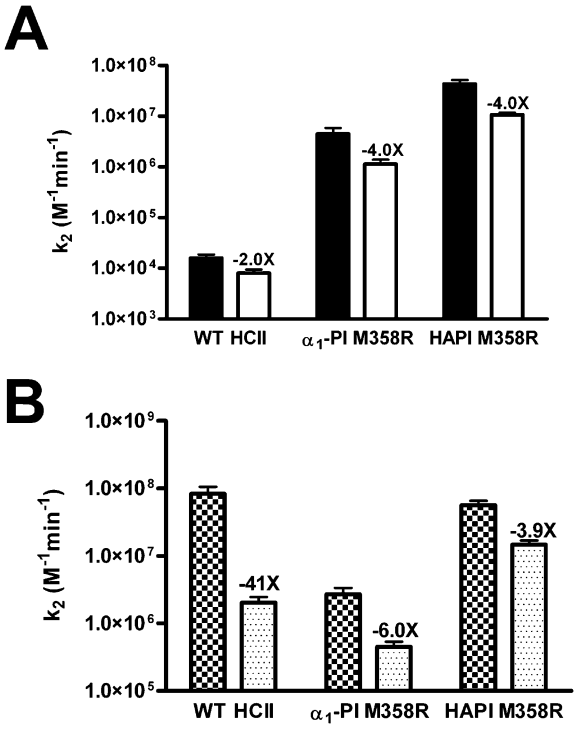

FIGURE 3: Fibrin-bound thrombin inhibition by HCII, α_1-PI M358R, and HAPI M358R in the absence or presence of heparin. The mean second-order rate constants for the inactivation of thrombin by the recombinant proteins identified below each bar were measured under pseudo-first-order conditions in the presence of 3 μM soluble fibrin (open bars) or in its absence (solid bars) but in the presence of all other additives including peptide GPRP, as shown in panel A. Panel B, as in panel A but with the addition of 0.5 μM heparin to both reactions lacking SF (checked bars) and those containing it (dotted bars). As in panel A, the fold reduction in the rate of thrombin inhibition in the presence of heparin due to SF is indicated above the dotted bars. For each different condition, the rate of thrombin inhibition was determined independently at least three times; the average was calculated and then used to measure the fold reductions shown on this plot; error bars show the SE of the mean.

on the rate of thrombin inhibition by HAPI M358R (compare HAPI M358R k_2 values in panel A vs panel B of Figure 3). Thus, unlike in its natural setting, the acidic tail of HCII does not assume a cryptic or partially cryptic conformation when appended to α_1-PI M358R but is instead readily available for thrombin binding. The position of the full tail in native HCII is not known (*16*), and it may be somewhat mobile because it is possible to link residue 52, 54, or 68 to residue 195 if these residues are mutated to Cys, although the engineered disulfide bonds must be reduced to restore function (*39*). We nevertheless found that the transferred tail is not unduly exposed, being no more vulnerable to proteolysis in HAPI M358R than in its natural setting in heparin-free HCII in plasma in vitro.

The enhanced inhibition of thrombin by the chimeric protein HAPI M358R correlated with its acquisition of exosite 1 binding capacity. Several lines of evidence support this conclusion. First, the enhanced inhibition was specific for thrombin; minimal enhancement was observed with APC or trypsin, proteases lacking structures analogous to thrombin exosite 1, at least as defined by hirudin interaction. Second, maximal enhancement was dependent on the presence of Asp and Glu residues found between positions 55 and 75 in HCII, the location of the two hirudin-like repeats; similarly, a nonspecific stabilization of α_1-PI M358R by the addition of *any* N-terminal residues could be dismissed, because of the lack of enhancement associated with fusion of either residues 1−54 or a mutant 1−75 extension lacking these charged residues. Third, the enhanced inhibition mediated by the chimeric proteins was diminished when γ_T-thrombin was substituted for α-thrombin, consistent with the partial disruption of exosite 1 that takes place in this proteolytic derivative of α-thrombin.

In γ_T-thrombin, cleavages at Arg^{67} and Arg^{77A} disrupt the β-loop of exosite 1 and lead to disorder in the Lys^{70}−Glu^{80} loop region (*30*) and a greatly reduced ability to cleave fibrinogen (*34*). The region between Arg^{67} and Glu^{80} in α-thrombin figures prominently in the HCII tail−S195A thrombin interface, with the majority of the contacts being hydrophobic, involving Phe^{34}, Leu^{65}, Arg^{67}, Tyr^{76}, Ile^{82}, and Met^{84} in exosite 1 and an amphipathic helix in HCII, located between the hirudin-like repeats and involving Leu^{61}, Leu^{63}, Ile^{66}, and Phe^{67} (*16*). As depicted in Figure 4A, inspection of the crystal structure for contacts between HCII Gly^{54} and Asp^{75} and S195A thrombin revealed both β-loop contacts and additional contacts such as those with atoms in Lys^{36}, Lys^{110}, and Ser^{153}. Although no equivalent γ-thrombin−HCII complex exists, inspection of the portion of crystallized γ-thrombin that corresponds to the portion of α-thrombin S195A in close contact with HCII revealed that Ile^{82}, Met^{84}, Lys^{36}, Lys^{110}, and Ser^{153} residues in γ-thrombin closely approximate their positions in the encounter complex formed between α-thrombin S195A and HCII (Figure 4B). This partial maintenance of the possible tail−thrombin contact interface in γ-thrombin is consistent with previous suggestions that γ_T-thrombin must retain a partial exosite 1, because it undergoes dermatan sulfate-accelerated inhibition by HCII (*19*) and because HCII mutants in which intramolecular tail-HCII body interactions are disrupted exhibit enhanced inhibition of γ_T-thrombin (*24*). These concepts support our interpretation that the vestigial exosite 1 is involved in the enhanced inhibition of γ_T-thrombin by HAPI M358R and H_{55-75}API M358R.

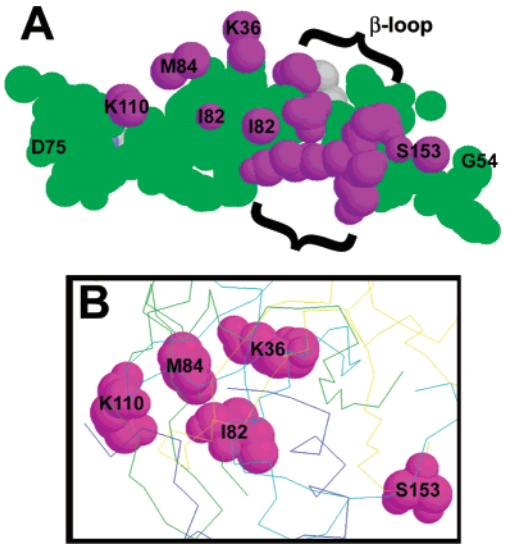

FIGURE 4: Structural diagrams of the HCII−α-thrombin interface and the corresponding region of γ-thrombin. In panel A, PDB file 1JMO was visualized using Protein Explorer software (see Materials and Methods). A space-filling view of the region of the HCII acidic tail between Asp^{75} and Gly^{54} (from left to right) is presented (green), oriented such that S195A thrombin is above HCII. Only those atoms of S195A thrombin that come within 4 Å of HCII residues are shown (purple); gray and blue atoms represent carbon and nitrogen atoms of sulfotyrosine residues 60 and 73. Thrombin residues found between the braces are from the β-loop while those outside the β-loop that make contacts with HCII are identified individually. These residues (all atoms) are shown in an equivalently oriented space-filling view of PDB file 2HNT (γ-thrombin) in inset panel B; thin lines depict the backbone of the four chains in this proteolyzed form of thrombin.

Formation of a ternary thrombin−heparin−fibrin complex has been shown to lead to protection of thrombin from inhibition by HCII, while α_1-PI M358R is essentially unperturbed in carrying out its inhibitory function in the presence of both heparin and soluble fibrin (*29*). In this respect, HAPI M358R resembled α_1-PI M358R, in that under conditions in which the rate of HCII inhibition of thrombin was reduced 41-fold, the rate of inhibition of the chimeric protein was reduced less than 4-fold. This modest decrease in rate was the same in the presence of either saturating soluble fibrin alone or fibrin and heparin in combination. Because both HAPI M358R and fibrin bind to exosite 1, these findings imply that the chimeric protein is capable of displacing thrombin from fibrin. This deduction is supported by the finding that dermatan sulfate-activated HCII, in which the acidic tail is available for interaction with exosite 1, effectively displaces thrombin from immobilized fibrin monomer (*40*). Moreover, the affinity of the hirudin 54−65 peptide, a plausible surrogate for the mobilized acidic tail of HCII, for thrombin is almost 3 orders of magnitude greater than that of fibrin for thrombin [as per K_d values of 9.8×10^{-8} (*41*) versus 1.5×10^{-6} M (*40*)]. These considerations suggest that in vivo use of HAPI M358R to control thrombin might not suffer some of the limitations of heparin with respect to difficulties in catalyzing the inhibition of clot-bound thrombin (*41*, *42*).

Previous efforts (*12*−*14*) to increase the activity and specificity of α_1-PI M358R as a thrombin inhibitor have focused on further altering its RCL by substitution of residues from AT (*13*, *14*) or HCII L444R (*12*). Although not determined in the AT-related approach, in the HCII-mimicking strategy, the increase in selectivity for thrombin over APC came at

the cost of increasing the reaction stoichiometry (*12*). In contrast, the approach taken in the present study was notable in that it did not have this undesirable effect. HCII mutant D, in which charged residues Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, and Arg^{193} in the heparin binding site were neutralized by mutagenesis, had been shown by Liaw et al. (*18*) to be partially activated for glycosaminoglycan-independent inhibition of thrombin; however, we recently showed that this enhancement came at the cost of an elevation of the SI to 9.8 (*26*). This less-than-optimal outcome presumably arose because of misalignments between the partially liberated acidic tail, thrombin exosite 1, the RCL, and the active site of the protease. We appear to have avoided this complication in the present approach. Moreover, Hopkins et al. argued that previously engineered α_1-PI M358R RCL variants with improved selectivity for thrombin were 1−2 orders of magnitude insufficiently active to consider for therapeutic use (*13*); we have arguably closed this "activity gap" by HCII tail transfer.

By fusing the unique 75 amino acid N-terminal tail of HCII to α_1-PI M358R, we increased the rate of thrombin inhibition, but not that of APC, by approximately 21-fold without elevating the SI. Combining this extra-RCL and other intra-RCL approaches (*12*−*14*) could lead to the development of a novel antithrombotic therapeutic protein, while shedding further light on the nature of the HCII N-terminal acidic extension, an intriguing thrombin binding motif. Our use of regions of two human plasma proteins might lead to an acceptably low immunogenicity of HAPI M358R clinically, but as is true for all recombinant therapeutic proteins, this potential problem cannot be discounted a priori. As a final point, it should be stressed that much of the evidence for tail−exosite 1 interaction, save for the S195A thrombin−HCII crystal structure (*16*), is indirect, relying upon the loss of inhibition by proteolyzed or mutated forms of thrombin or HCII, and this also applies to HAPI M358R. Demonstrating a direct interaction between the transferred tail of HCII and thrombin exosite 1 would be a desirable addition to our characterization of this chimeric serpin. Examining this interaction would require distinguishing it from that of the RCL and thrombin active site, but this goal could presumably be achieved using either PPACK- or S195A thrombin in either immobilized (ELISA-type) or free (gel filtration) formats or by inactivating the RCL by mutation.

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REFERENCES

1. Lane, D. A., Philippou, H., and Huntington, J. A. (2005) Directing thrombin, *Blood 106*, 2605−2612.
2. Gettins, P. G. (2002) Serpin structure, mechanism, and function, *Chem. Rev. 102*, 4751−4804.
3. Schechter, I., and Berger, A. (1967) On the size of the active site in proteases. I. Papain, *Biochem. Biophys. Res. Commun. 27*, 157−162.
4. Stratikos, E., and Gettins, P. G. (1999) Formation of the covalent serpin-proteinase complex involves translocation of the proteinase by more than 70 Å and full insertion of the reactive center loop into beta-sheet A, *Proc. Natl. Acad. Sci. U.S.A. 96*, 4808−4813.
5. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J. O., and Shore, J. D. (1995) Serpin-protease complexes are trapped as stable acyl-enzyme intermediates, *J. Biol. Chem. 270*, 25309−25312.
6. Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) Mutation of antitrypsin to antithrombin. alpha 1-antitrypsin Pittsburgh (358 Met leads to Arg), a fatal bleeding disorder, *N. Engl. J. Med. 309*, 694−698.
7. Travis, J., Owen, M., George, P., Carrell, R., Rosenberg, S., Hallewell, R. A., and Barr, P. J. (1985) Isolation and properties of recombinant DNA produced variants of human alpha 1-proteinase inhibitor, *J. Biol. Chem. 260*, 4384−4389.
8. Scott, C. F., Carrell, R. W., Glaser, C. B., Kueppers, F., Lewis, J. H., and Colman, R. W. (1986) Alpha-1-antitrypsin-Pittsburgh. A potent inhibitor of human plasma factor XIa, kallikrein, and factor XIIf, *J. Clin. Invest. 77*, 631−634.
9. Heeb, M. J., Bischoff, R., Courtney, M., and Griffin, J. H. (1990) Inhibition of activated protein C by recombinant alpha 1-antitrypsin variants with substitution of arginine or leucine for methionine358, *J. Biol. Chem. 265*, 2365−2369.
10. Harper, P. L., Taylor, F. B., DeLa Cadena, R. A., Courtney, M., Colman, R. W., and Carrell, R. W. (1998) Recombinant antitrypsin Pittsburgh undergoes proteolytic cleavage during E. coli sepsis and fails to prevent the associated coagulopathy in a primate model, *Thromb. Haemostasis 80*, 816−821.
11. Bernard, G. R., Vincent, J. L., Laterre, P. F., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steingrub, J. S., Garber, G. E., Helterbrand, J. D., Ely, E. W., and Fisher, C. J., Jr. (2001) Efficacy and safety of recombinant human activated protein C for severe sepsis, *N. Engl. J. Med. 344*, 699−709.
12. Filion, M. L., Bhakta, V., Nguyen, L. H., Liaw, P. S., and Sheffield, W. P. (2004) Full or partial substitution of the reactive center loop of alpha-1-proteinase inhibitor by that of heparin cofactor II: P1 Arg is required for maximal thrombin inhibition, *Biochemistry 43*, 14864−14872.
13. Hopkins, P. C., Crowther, D. C., Carrell, R. W., and Stone, S. R. (1995) Development of a novel recombinant serpin with potential antithrombotic properties, *J. Biol. Chem. 270*, 11866−11871.
14. Hopkins, P. C., Pike, R. N., and Stone, S. R. (2000) Evolution of serpin specificity: cooperative interactions in the reactive-site loop sequence of antithrombin specifically restrict the inhibition of activated protein C, *J. Mol. Evol. 51*, 507−515.
15. Huber, R., and Carrell, R. W. (1989) Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins, *Biochemistry 28*, 8951−8966.
16. Baglin, T. P., Carrell, R. W., Church, F. C., Esmon, C. T., and Huntington, J. A. (2002) Crystal structures of native and thrombin-complexed heparin cofactor II reveal a multistep allosteric mechanism, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11079−11084.
17. Hortin, G. L., Tollefsen, D. M., and Benutto, B. M. (1989) Antithrombin activity of a peptide corresponding to residues 54−75 of heparin cofactor II, *J. Biol. Chem. 264*, 13979−13982.
18. Liaw, P. C., Austin, R. C., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) Comparison of heparin- and dermatan sulfate-mediated catalysis of thrombin inactivation by heparin cofactor II, *J. Biol. Chem. 274*, 27597−27604.
19. Rogers, S. J., Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) Role of thrombin exosites in inhibition by heparin cofactor II, *J. Biol. Chem. 267*, 3613−3617.
20. Van Deerlin, V. M., and Tollefsen, D. M. (1991) The N-terminal acidic domain of heparin cofactor II mediates the inhibition of alpha-thrombin in the presence of glycosaminoglycans, *J. Biol. Chem. 266*, 20223−20231.
21. Ragg, H., Ulshofer, T., and Gerewitz, J. (1990) On the activation of human leuserpin-2, a thrombin inhibitor, by glycosaminoglycans, *J. Biol. Chem. 265*, 5211−5218.
22. Sheehan, J. P., Wu, Q., Tollefsen, D. M., and Sadler, J. E. (1993) Mutagenesis of thrombin selectively modulates inhibition by serpins heparin cofactor II and antithrombin III. Interaction with the anion-binding exosite determines heparin cofactor II specificity, *J. Biol. Chem. 268*, 3639−3645.

23. Myles, T., Church, F. C., Whinna, H. C., Monard, D., and Stone, S. R. (1998) Role of thrombin anion-binding exosite-I in the formation of thrombin- serpin complexes, *J. Biol. Chem. 273*, 31203−31208.
24. Mitchell, J. W., and Church, F. C. (2002) Aspartic acid residues 72 and 75 and tyrosine-sulfate 73 of heparin cofactor II promote intramolecular interactions during glycosaminoglycan binding and thrombin inhibition, *J. Biol. Chem. 277*, 19823−19830.
25. Cunningham, M. A., Bhakta, V., and Sheffield, W. P. (2002) Altering heparin cofactor II at VAL439 (P6) either impairs inhibition of thrombin or confers elastase resistance, *Thromb. Haemostasis 88*, 89−97.
26. Sutherland, J. S., Bhakta, V., and Sheffield, W. P. (2006) Investigating serpin-enzyme complex formation and stability via single and multiple residue reactive centre loop substitutions in heparin cofactor II, *Thromb. Res. 117*, 447−461.
27. Edelhoch, H. (1967) Spectroscopic determination of tryptophan and tyrosine in proteins, *Biochemistry 6*, 1948−1954.
28. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein, *Protein Sci. 4*, 2411−2423.
29. Becker, D. L., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) Exosites 1 and 2 are essential for protection of fibrin-bound thrombin from heparin-catalyzed inhibition by antithrombin and heparin cofactor II, *J. Biol. Chem. 274*, 6226−6233.
30. Rydel, T. J., Yin, M., Padmanabhan, K. P., Blankenship, D. T., Cardin, A. D., Correa, P. E., Fenton, J. W., II, and Tulinsky, A. (1994) Crystallographic structure of human gamma-thrombin, *J. Biol. Chem. 269*, 22000−22006.
31. Martz, E. (2002) Protein Explorer: easy yet powerful macromolecular visualization, *Trends Biochem. Sci. 27*, 107−109.
32. Hopkins, P. C., Carrell, R. W., and Stone, S. R. (1993) Effects of mutations in the hinge region of serpins, *Biochemistry 32*, 7650−7657.
33. Stone, S. R., Braun, P. J., and Hofsteenge, J. (1987) Identification of regions of alpha-thrombin involved in its interaction with hirudin, *Biochemistry 26*, 4617−4624.
34. Hofsteenge, J., Braun, P. J., and Stone, S. R. (1988) Enzymatic properties of proteolytic derivatives of human alpha-thrombin, *Biochemistry 27*, 2144−2151.
35. Fortenberry, Y. M., Whinna, H. C., Gentry, H. R., Myles, T., Leung, L. L., and Church, F. C. (2004) Molecular mapping of the thrombin-heparin cofactor II complex, *J. Biol. Chem. 279*, 43237−43244.
36. Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., and Church, F. C. (1991) Role of lysine 173 in heparin binding to heparin cofactor II, *J. Biol. Chem. 266*, 8129−8135.
37. Blinder, M. A., Marasa, J. C., Reynolds, C. H., Deaven, L. L., and Tollefsen, D. M. (1988) Heparin cofactor II: cDNA sequence, chromosome localization, restriction fragment length polymorphism, and expression in *Escherichia coli*, *Biochemistry 27*, 752−759.
38. Frommherz, K. J., Faller, B., and Bieth, J. G. (1991) Heparin strongly decreases the rate of inhibition of neutrophil elastase by alpha 1-proteinase inhibitor, *J. Biol. Chem. 266*, 15356−15362.
39. Brinkmeyer, S., Eckert, R., and Ragg, H. (2004) Reformable intramolecular cross-linking of the N-terminal domain of heparin cofactor II: effects on enzyme inhibition, *Eur. J. Biochem. 271*, 4275−4283.
40. Liaw, P. C., Becker, D. L., Stafford, A. R., Fredenburgh, J. C., and Weitz, J. I. (2001) Molecular basis for the susceptibility of fibrin-bound thrombin to inactivation by heparin cofactor ii in the presence of dermatan sulfate but not heparin, *J. Biol. Chem. 276*, 20959−20965.
41. Liu, L. W., Ye, J., Johnson, A. E., and Esmon, C. T. (1991) Proteolytic formation of either of the two prothrombin activation intermediates results in formation of a hirugen-binding site, *J. Biol. Chem. 266*, 23633−23636.
42. Hogg, P. J., and Jackson, C. M. (1989) Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: implications for heparin efficacy, *Proc. Natl. Acad. Sci. U.S.A. 86*, 3619−3623.

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