Full or Partial Substitution of the Reactive Center Loop of α-1-Proteinase Inhibitor by that of Heparin Cofactor II: P1 Arg Is Required for Maximal Thrombin Inhibition

Marc L. Filion,[‡] Varsha Bhakta,[§] Laurie H. Nguyen,[‡] Peter S. Liaw,[‡] and William P. Sheffield*[,‡,§]

Department of Pathology and Molecular Medicine, McMaster University, HSC-4N66, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada, and Canadian Blood Services, Research and Development, Hamilton, Ontario, Canada

Received June 7, 2004; Revised Manuscript Received September 6, 2004

ABSTRACT: The abundant plasma protein α_1-proteinase inhibitor (α_1-PI) physiologically inhibits neutrophil elastase (NE) and factor XIa and belongs to the serine protease inhibitor (serpin) protein superfamily. Inhibitory serpins possess a surface peptide domain called the reactive center loop (RCL), which contains the P1−P1′ scissile peptide bond. Conversion of this bond in α_1-PI from Met-Ser to Arg-Ser in α_1-PI Pittsburgh (M358R) redirects α_1-PI from inhibiting NE to inhibiting thrombin (IIa), activated protein C (APC), and other proteases. In contrast to either the wild-type or M358R α_1-PI, heparin cofactor II (HCII) is a IIa-specific inhibitor with an atypical Leu-Ser reactive center. We examined the effects of replacement of all or part of the RCL of α_1-PI with the corresponding parts of the HCII RCL on the activity and specificity of the resulting chimeric inhibitors. A series of 12 N-terminally His-tagged α_1-PI proteins differing only in their RCL residues were expressed as soluble proteins in *Escherichia coli*. Substitution of the P16−P3′ loop of α_1-PI with that of HCII increased the low intrinsic antithrombin activity of α_1-PI to near that of heparin-free HCII, while analogous substitution of the P2′−P3′ dipeptide surpassed this level. However, gel-based complexing and quantitative kinetic assays showed that all mutant proteins inhibited thrombin at less than 2% of the rate of α_1-PI (M358R) unless the P1 residue was also mutated to Arg. An α_1-PI (P16−P3′ HCII/M358R) variant was only 3-fold less active than M358R against IIa but 70-fold less active against APC. The reduction in anti-APC activity is desired in an antithrombotic agent, but the improvement in inhibitory profile came at the cost of a 3.5-fold increase in the stoichiometry of inhibition. Our results suggest that, while P1 Arg is essential for maximal antithrombin activity in engineered α_1-PI proteins, substitution of the corresponding HCII residues can enhance thrombin specificity.

The serine protease inhibitors (serpins)[1] are a superfamily of proteins characterized by sequence identity of 25−30% and a similar core structure, one dominated by a large five-stranded β sheet and an extended surface loop (*1*). The superfamily contains both inhibitory and noninhibitory members, with the former subclass including many of the plasma protease inhibitors responsible for the control of blood coagulation, fibrinolysis, inflammation, and complement activation in humans and other mammals (*2*). The approximately 20-residue, extended surface loop contains a reactive center P1−P1′ peptide bond that is cleaved in inhibitory serpins and used as a numbering benchmark, with residues N terminal to the scissile bond being designated P1, P2, P3, etc. and those C terminal to the reactive center, P1′, P2′, P3′, etc. (*3*). Although this reactive center loop (RCL) does not exhibit a high degree of sequence conservation among serpins, current models of the mechanism of serpin inhibition predict a common pathway for all serpin−protease pairings (*1*). The relationship between loop structure and serpin efficacy and specificity is only partially understood.

The metastable nature of serpins, which exist in a stressed conformation, is tightly linked to their mechanism of action (*4*). The stress is relieved by RCL cleavage by the incoming protease and accompanied by a profound conformational change that results in insertion of the RCL as a sixth strand in β-sheet A and translocation of the covalently attached protease to the opposite pole of the serpin (*5*, *6*). The rapid deformation of the active site of the protease is thought to prevent completion of the proteolysis reaction, resulting in the formation of a denaturation-resistant, stable acyl−enzyme complex in which cleaved serpin and inactive protease are linked (*7*).

The serpin α_1-proteinase inhibitor (α_1-PI, also known as α_1-antitrypsin) is the most abundant protease inhibitor in

* To whom correspondence should be addressed. Telephone: (905) 525-9140 Ext. 22701. Fax: (905) 777-7856. E-mail: sheffiel@mcmaster.ca.

[‡] McMaster University.

[§] Canadian Blood Services.

[1] Abbreviations: APC, activated protein C; α_1-PI, α_1-proteinase inhibitor and α_1-antitrypsin; HCII, heparin cofactor II; HCII−IIa, HCII−thrombin complex; k_2, second-order rate constant of inhibition; M358L and M358R, substitution of Met358 by Leu and Arg, respectively; NE, neutrophil elastase; RCL, reactive center loop; serpin, serine protease inhibitor; IIa, thrombin; P1−P1′; the reactive center peptide bond, where P1 is the amino acid N-terminal to cleavage and P1′ is C-terminal to cleavage; P16−P3′, mutant form of α_1-PI in which the P16−P3′ residues have been replaced by the corresponding residues of HCII; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SI, stoichiometry of inhibition; WT, wild type.

10.1021/bi048833f CCC: $27.50
Published on Web 10/26/2004

human plasma, circulating at mean resting concentrations of approximately 11 μM (*8*), which can further increase during an acute phase response (*9*). It is among the smallest of the serpins, and this relatively uncomplicated structure has combined with its abundance and clinical relevance to render it one of the most studied serpins. The 1984 crystallization of cleaved α_1-PI provided the first serpin crystal structure and revealed an RCL-cleaved, loop-inserted form (*10*). Genetic deficiency of α_1-PI has been associated with pulmonary emphysema for 40 years (*11*), an association better understood following the identification of neutrophil elastase (NE) as the primary physiological target of α_1-PI (*12*). Finally, the naturally occurring Pittsburgh mutation of the α_1-PI P1 residue from Met to Arg resulted in a severe bleeding disorder because of an alteration of the α_1-PI target protease specificity away from NE and toward thrombin (IIa) (*13*).

The redirection of α_1-PI (M358R) activity prompted its consideration as an antithrombotic therapeutic protein. However, the mutant α_1-PI had also acquired enhanced inhibitory properties against factors XIa and XIIa (*14*) and activated protein C (APC) (*15*). At doses of α_1-PI (M358R) calculated to deliver 10 μM plasma concentrations, it worsened the outcome in a baboon model of sepsis, possibly because of its anti-APC activity (*16*). Extensive mutagenesis studies, involving the complete or partial substitution of the P7−P3′ sector of the RCL of antithrombin into α_1-PI but in all cases bearing a P1 Arg, showed that it was difficult to minimize anti-APC activity without reducing antithrombin activity and did not address the issue of possible substrate behavior of the chimeric inhibitors (*17*, *18*).

Heparin cofactor II (HCII) is serpin-specific for thrombin (*19*) with an atypical Leu-Ser reactive center bond (*20*). In this study, we investigated the consequences of substituting all or part of the RCL of HCII for the corresponding residues of α_1-PI, reasoning that it might be possible to transfer thrombin specificity and low anti-APC reactivity to α_1-PI by this approach. We report that it was possible to enhance α_1-PI reactivity to within less than 2-fold of the progressive rate of the inhibition of thrombin by HCII by full-loop exchange but that maximal thrombin inhibition was dependent on a P1 Arg residue even in the context of HCII loop exchange. The reductions in anti-APC activity gained through this approach came at the cost of increases in the stoichiometry of inhibition (SI).

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology at McMaster University, Hamilton, ON, Canada. Human hepatoma HepG2 cells were from the ATCC (Manassas, VA). Purified human α_1-PI, neutrophil elastase, and PPACK were from Calbiochem (La Jolla, CA). Purified human APC and arabinose were from Enzyme Research Laboratories (South Bend, IN). Restriction enzymes and protein and DNA molecular mass standards were from MBI Fermentas (Burlington, ON). Ni-NTA agarose and plasmid DNA isolation kits were from Qiagen (Chatsworth, CA). *Escherichia coli* DH5α-competent cells and the pBAD(B) expression plasmid were from Invitrogen (Carlsbad, CA). Plasmid pGEM5zf(+) was from Fisher Scientific (Unionville, ON). DEAE-Sephacel Fast Flow resin was purchased from Amersham Biosciences (Baie d'Urfe, PQ). The S-2238 and S-2366 chromogenic substrates were from DiaPharma Group (West Chester, OH). Complete protease inhibitor tablets were from Roche Diagnostics Canada (Laval, QFC). IgG-peroxidase conjugated and nonconjugated sheep anti-human α_1-PI IgG were from Affinity Biologicals (Ancaster, ON). Carrier free ^{125}Iodine was purchased from Perkin−Elmer (Boston, MA), while the iodination reagent Iodogen was bought from Pierce (Rockford, IL). All other chemicals not listed above were of the highest quality available.

Construction of Wild-Type (WT) pBAD-$H_6\alpha_1$-PI Expression Vector. A human α_1-PI cDNA was cloned by RT-PCR using total RNA isolated from human hepatoma HepG2 cells and primers specific to the published α_1-PI sequence (*21*) and inserted into the *Eco*R V site of the plasmid pGEM5zf(+) (Fisher). Several plasmids were subjected to DNA sequencing, and one found to be free of unwanted PCR-derived mutations was selected for continuing experimentation. Codons specifying mature α_1-PI were amplified using PCR under standard conditions, using oligonucleotide primers 16504 (5′-GAACCATGGG GCATCATCAT CATCATGAGG ATCCCCAGGG AGAT-3′) and 16503 (5′-CCGGAATTCT TATTTTTGGG TGGGATTCAC-3′). The resulting PCR product was restricted with *Nco* I and *Bam*H I and inserted between the corresponding sites of pBAD(B) (Invitrogen) to form pBAD-H_6API, encoding mature human α_1-PI with an N-terminal Met-Gly-hexahistidine octapeptide extension. DNA sequence analysis was again performed to ensure the absence of secondary mutations.

Construction of pBAD-H_6API (M358R) and pBAD-HAPI (M358L) Expression Vectors. Single-codon mutations at residue 358 (P1) of α_1-PI were constructed using a megaprimer protocol (*22*), employing oligonucleotides designed to alter codons for the P1 Met (ATG) into Arg (AGG) and Leu (TTG), respectively. As with the WT expression plasmid, following transformation of *E. coli* TOP10 cells to ampicillin resistance, the sequence of each α_1-PI mutant was confirmed by automated DNA sequence analysis.

Construction of pBAD-H_6API-eRCL Expression Vector. To perform more extensive RCL mutagenesis, a series of PCR and subcloning steps analogous to those described above were carried out that deleted codons 342−361 inclusive, forming plasmid pBAD-H_6API-eRCL (for empty RCL). This plasmid differs from pBAD-H_6API in that it contains unique *Cla* I (Ile340/Asp341) and *Sau* I (Pro362/Glu363) restriction sites introduced by silent mutations, separated by a termination codon. Restriction endonuclease digestion of pBAD-H_6API-eRCL with *Cla* I and *Sau* I and insertion of annealed oligonucleotides with *Cla* I and *Sau* I compatible ends permitted the substitution of any codons for the natural RCL. For instance, the entire α_1-PI RCL was exchanged for that of HCII using annealed oligonucleotides 19054 (5′-CGATGAGGAA GGTACCCAAG CCACCACTGT GACCACGGTG GGGTTCATGC CGCTGTCCAC CCAACC-3′) and 19055 (5′-TCAGGTTGGG TGGACAGCGG CATGAACCCC ACCGTGGTCA CAGTGGTGGC TTGGGTACCT TCCTCAT-3′). An analogous procedure differing only in the sequences of the annealed oligonucleotides was used to create the ten expression plasmids whose altered RCLs are shown in Table 1. In all cases, the ligated expression plasmids were used to transform *E. coli* TOP10, and DNA

Table 1: RCL Sequences of Natural and Engineered Serpins

name	RCL sequence from P20−P9′	SDS-stable complex with nonradioactive thrombin detected
α_1-PI	TIDEKGTEAAGAMFLEAIPMSIPPE	−[a]
HCII	TVNEEGTQATTVTTVGFMPLSTQVR	+[b]
α_1-PI (M358R)	TIDEKGTEAAGAMFLEAIP**R**SIPPE	+++
α_1-PI (P16−P3′)	TIDE**EGTQATTVTTVGFMPLSTQ**PE	+
α_1-PI (P16−P1)	TIDE**EGTQATTVTTVGFMPL**SIPPE	−
α_1-PI (P10−P3′)	TIDEKGTEAA**TVTTVGFMPLSTQ**PE	+
α_1-PI (P5−P3′)	TIDEKGTEAAGAMFL**GFMPLSTQ**PE	+
α_1-PI (P1−P3′)	TIDEKGTEAAGAMFLEAIP**LSTQ**PE	−
α_1-PI (P1′−P3′)	TIDEKGTEAAGAMFLEAIPM**STQ**PE	++
α_1-PI (I360T)	TIDEKGTEAAGAMFLEAIPMS**T**PPE	−
α_1-PI (P361Q)	TIDEKGTEAAGAMFLEAIPMS**Q**PE	−
α_1-PI (M358L)	TIDEKGTEAAGAMFLEAIP**L**SIPPE	−
α_1-PI (P16−P3′/M358R)	TIDE**EGTQATTVTTVGFMPRSTQ**PE	+++
LS (P7−P3′ antithrombin)[c]	TIDEKGTEAAGAM**AVVIAGRSLN**PE	+++

[a] Indicates no complex detected. [b] Indicates complex detected; additional plus signs indicate greater extent of reactions. [c] From ref *16*.

sequencing was used to validate the constructs prior to their use to express the chimeric recombinant α_1-PI proteins.

Expression and Purification of Recombinant α_1-PI Variants. Competent *E. coli* TOP10 cells transformed with either WT α_1-PI or mutant α_1-PI plasmids were used to inoculate a culture of Luria Broth (LB) medium supplemented with 100 μg/mL ampicillin and grown overnight in a shaking incubator at 37 °C. The culture was then diluted 1:100 in fresh LB medium containing ampicillin and grown to an OD_{600} of approximately 0.5 before induction with 0.002% arabinose. After induction for 4−5 h, cells were harvested by centrifugation and resuspended in lysis buffer (50 mM sodium phosphate at pH 8.0, 300 mM NaCl, and 10 mM imidazole) supplemented with protease inhibitors (Roche). After lysis of the cells by sonication, Triton X-100 was added to the lysate at a final concentration of 1% and mixed for 20 min on a rocking platform. Cell debris was removed by centrifugation, and the supernatant was collected for purification on an Ni-NTA-agarose column. After sample application, the column was washed with 50 mM sodium phosphate at pH 7.4, 300 mM NaCl, 20 mM imidazole, and 0.02% sodium azide. Tightly associated nickel-binding proteins were then eluted using the same buffer supplemented with 100 mM imidazole. The elution fractions were then pooled and dialyzed overnight against 20 mM sodium phosphate at pH 6.8 prior to ion-exchange chromatography using a DEAE-Sephacel column developed with a linear NaCl gradient from 0 to 150 mM NaCl in 20 mM sodium phosphate at pH 6.8. Fractions containing the proteins of interest, as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), were pooled and concentrated using a centrifugal concentrator unit (Fisher Scientific). Final protein concentrations were determined by ELISA using affinity-purified sheep anti-human α_1-PI HCII antibodies (Affinity Biologicals).

Gel-based Analysis of Reactions of Recombinant α_1-PI Variants with Iodinated Proteases. Purified human α-thrombin, APC, and factor XIa were separately radiolabeled with ^{125}I using the Iodogen method, as described (*23*), and separated from unincorporated ^{125}I by exhaustive dialysis. Purified α_1-PI variants (150 nM) were combined with either 15 nM ^{125}I-radiolabeled human α-thrombin, 30 nM ^{125}I-radiolabeled human APC, or 20 nM ^{125}I-radiolabeled human factor XIa, all in 20 mM sodium phosphate at pH 7.4, 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG 8000 (PPNE buffer). Reactions were allowed to progress for times ranging from 0 to 15 min at 37 °C and stopped by the addition of PPACK (Calbiochem) to a 50 μM final concentration. Reactions were then analyzed by SDS−PAGE under reducing (thrombin and factor XIa) or nonreducing conditions (APC), employing autoradiography of the dried gel.

Gel-based Analysis of Reactions of Recombinant α_1-PI Variants with Nonradioactive Proteases. Recombinant α_1-PI variants (150 nM) were incubated with 15 nM human α-thrombin in PPNE buffer as described above, except that, following SDS−PAGE, the gels were electroblotted onto nitrocellulose membranes and analyzed by enhanced chemiluminescent (ECL) immunoblotting (Amersham Biosciences), using an affinity-purified, peroxidase-conjugated sheep anti-human α_1-PI IgG.

Rates of Thrombin and APC Inhibition by α_1-PI. The second-order rate constants (k_2) for thrombin inhibition by α_1-PI were determined under pseudo-first-order conditions using a discontinuous assay, as previously described (*24*, *25*). In the first stage, recombinant α_1-PI or HCII or variants (140 nM) was incubated with either 7 nM thrombin or APC in PPNE buffer for various times at room temperature. The second stage was initiated with the addition of 200 μM S-2238 (for thrombin) or 400 μM S-2366 (for APC) chromogenic substrates. Residual protease activity was calculated by measuring absorbance at 405 nm for 5 min using an EL808 plate reader (BioTek Instruments, Winooski, VT). Because of the use of pseudo-first-order conditions, the pseudo-first-order rate constant (k_{obs}) could be determined using the equation, $k_{obs}t = \ln([P]_0/[P]_t)$, where $[P]_0$ is the initial protease activity and $[P]_t$ is the protease activity at time t. The pseudo-first-order rate constant was determined by plotting $\ln([P]_0/[P]_t)$ versus time. Subsequently, the k_2 was calculated by dividing k_{obs} with the initial concentration of α_1-PI.

Stoichiometries of Inhibition. The number of molecules of recombinant α_1-PI required to inhibit 1 molecule of thrombin or APC was calculated as previously described (*24*, *25*). A constant amount of thrombin (5 nM) was incubated with varying amounts of recombinant α_1-PI in PPNE buffer supplemented with 2 mg/mL BSA for 2 h at room temperature. The reaction was diluted into 200 μM S-2238 in PPNE, and residual thrombin activity was measured as described

above. A plot of thrombin activity versus the ratio of α_1-PI/thrombin was used to determine the ratio of α_1-PI/thrombin at zero thrombin activity using extrapolation by linear regression, as was done for APC using a plot of APC activity versus the ratio of α_1-PI/APC.

General Procedure. Standard methods of DNA and *E. coli* manipulation and routine protocols for SDS−PAGE, immunoblotting, and autoradiography were employed. Recombinant hexahistidine-tagged HCII was expressed and purified as described (*24*) and used in the absence of heparin in kinetic assays exactly as described for α_1-PI and its recombinant derivatives above. Autoradiographic images were quantified using an LKB Bromma Ultroscan XL enhanced laser densitometer.

RESULTS

Expression and Characterization of Recombinant α_1-PI *Variants.* In this study, expression plasmids encoding N-terminally hexahistidine-tagged WT, M358R, M358L, or RCL exchanged versions of α_1-PI were created and expressed. The exact sequences of the RCL in the chimeric proteins are shown in Table 1, aligned with the corresponding residues of α_1-PI and HCII. For simplicity, the single-residue mutants are referred to in this paper in standard notation (e.g., M358R), while the loop-exchanged mutants are identified by the portions of the RCL exchanged for the corresponding peptide from HCII (e.g., P16−P3′ refers to the chimera in which the P16−P3′ residues of α_1-PI were exchanged for those residues from HCII). All proteins reacted with both anti-hexahistidine- and anti-α_1-PI-specific antibodies and could be purified to homogeneity using nickel chelate and ion-exchange chromatography, with the exception of the P5−P3′ and P1′−P3′ variants. The latter contained minor proteolytic breakdown products of α_1-PI lacking, on the basis of their antibody and thrombin reactivity, C-terminal residues of the inhibitor (results not shown). The sole or major polypeptide species in the recombinant α_1-PI preparations migrated on SDS−PAGE in good correspondence with the calculated molecular weight of N-terminally hexahistidine-tagged α_1-PI (45 335 Da). The yield of purified WT and most variants was approximately 0.3 mg/L of bacterial culture, with the exception of P5−P3′, where the yield was reduced approximately 10-fold. The concentration of purified recombinant α_1-PI was determined by ELISA, using plasma-derived α_1-PI as the standard; the results agreed well with total protein assays calibrated with either plasma-derived α_1-PI or BSA.

Inhibition of Radiolabeled Thrombin by α_1-PI *Variants.* Semiquantitative gel-based reactions were initially undertaken to identify which of the 12 α_1-PI-related recombinant proteins were active in forming denaturation-stable complexes with thrombin. Under the conditions employed in experiments shown in Figure 1, no reaction was detected for WT α_1-PI, M358L, the most complete RCL exchange mutant (P16−P3′), less extensive exchange mutants P16−P1 or P1−P3′, or point mutants I360T or P361Q. In contrast, SDS-stable complexes formed with similar speed and intensity in the case of the M358R variant and a complete loop exchange variant in which the P1 residue was additionally altered to Arg (P16−P3′/M358R). When the SDS-stable α_1-PI-thrombin complex band intensity on appropriately exposed autoradiograms was determined by laser densitometry and normalized to the intensity seen with the M358R variant, the relative complex levels were 1.0, 1.1, 0.2, 0.07, and 0.02 for M358R, P16−P3′/M358R, P1′−P3′, P10−P3′, and P5−P3′, respectively.

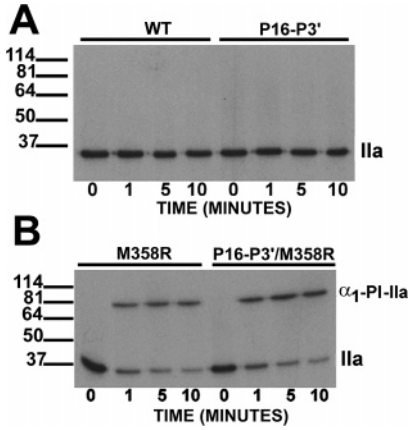

FIGURE 1: Electrophoretic detection of complex formation between ^{125}I-human α-thrombin and recombinant α_1-PI proteins. An autoradiogram of a dried 10% SDS−polyacrylamide gel containing the products of timed reactions between WT or variant α_1-PI proteins and α-thrombin is shown. The reactants, consisting of 150 nM α_1-PI and 15 nM ^{125}I-human α-thrombin, were incubated at 37 °C for 1, 5, or 10 min prior to reaction termination and electrophoresis under reducing conditions. Samples of 0 min came from a mock reaction lacking α_1-PI. The positions of prestained molecular weight markers and their sizes in kilodaltons are shown on the left.

The possibility of global misfolding of nonreactive variants was rendered unlikely by the finding that several of these proteins were capable of forming serpin−enzyme complexes, either with other proteases or with thrombin under more favorable reaction conditions. For example, the WT and M358L forms of α_1-PI were completely converted to either cleaved or complexed forms by NE, in reactions performed at 1:1 molar ratios of serpin/enzyme for 10 min. Under identical conditions, the M358R and P16−P3′ variants were only partially reactive. Some complex formation with thrombin could also be detected for all of these forms of α_1-PI reacted at equimolar, micromolar concentrations, for 10 min at 37 °C (data not shown).

Nonradioactive Thrombin Complexing Assays. Before proceeding to kinetic analysis of the reactions of the mutant proteins with thrombin, the state of the variants following reaction with thrombin under pseudo-first-order conditions was assessed using nonradioactive methods. SDS gel analysis of aliquots of the reaction of thrombin with various forms of WT and the M358L, P1−P3′, and P16−P1 variants neither formed complexes with thrombin nor were cleaved by the protease (data not shown). In contrast, as shown in Figure 2, M358R acted both as an inhibitor and a substrate of thrombin, as did P16−P3′/M358R; cleavage of the latter was more extensive than that of the former (compare "α_1-PI (cl)" bands in A and B). The P1′−P3′ and P16−P3′ variants formed complexes with thrombin less effectively than either P1 Arg-containing variant, with the former being more reactive; no evidence of variant α_1-PI cleavage was seen in either case, although a breakdown product complicated this assessment in the case of the P1′−P3′ variant (Figure 2). The detection of limited complex formation in the nonradioactive experiments with P16−P3′ and not in the radioactive ones may have derived from a loss of specific

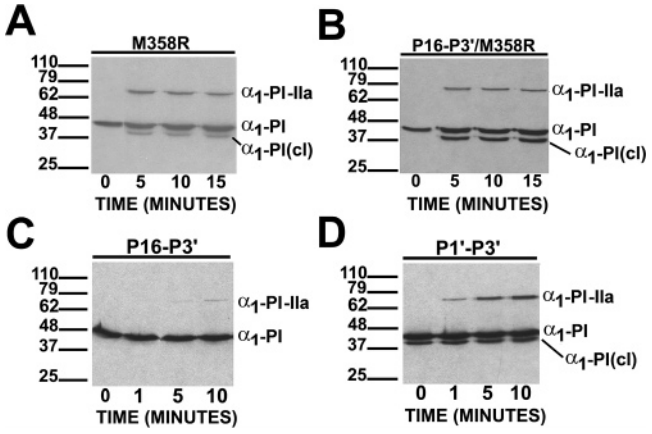

FIGURE 2: Electrophoretic detection of the effects of incubation with thrombin on recombinant α_1-PI proteins. Immunoblots probed with an affinity-purified sheep anti-human α_1-PI horseradish peroxidase-conjugated antibody and visualized using enhanced chemiluminescent substrate cleavage are shown. The reactants, consisting of 150 nM α_1-PI and 15 nM α-thrombin, were incubated at 37 °C for 1, 5, and 10 min prior to reaction termination, electrophoresis, and immunoblotting. Samples of 0 min came from a mock reaction lacking α-thrombin. The positions of prestained molecular weight markers and their sizes in kilodaltons are shown on the left.

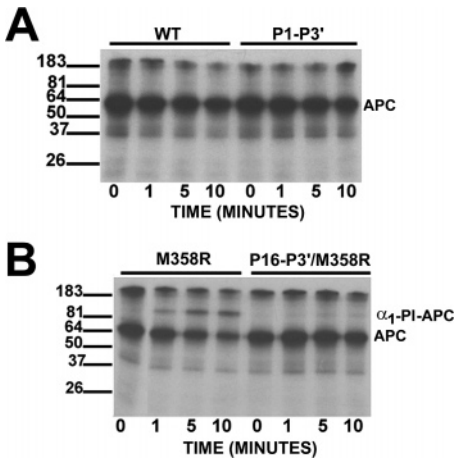

FIGURE 3: Electrophoretic detection of complex formation between ^{125}I-human APC and recombinant α_1-PI proteins. An autoradiogram of a dried 10% SDS−polyacrylamide gel containing the products of timed reactions between WT or variant α_1-PI proteins and α-thrombin is shown. The reactants, consisting of 150 nM α_1-PI and 30 nM ^{125}I-human APC, were incubated at 37 °C for 1, 5, or 10 min prior to reaction termination and electrophoresis. Samples of 0 min came from a mock reaction lacking α_1-PI. The positions of prestained molecular weight markers and their sizes in kilodaltons are shown on the left.

activity of the protease because of iodination; the results were otherwise consistent between the two approaches.

Reaction of Variants with APC and Factor XIa. The reaction of the WT α_1-PI and the three variants most active in thrombin complexing with activated protein C and factor XIa was next examined. As shown in Figure 3, iodinated activated protein C migrated as a single major band on nonreduced SDS gels, which were selected over reduced gels to maximize resolution of serpin−APC complexes. There were also some reducing agent-sensitive high molecular weight aggregates detected in the APC preparation. Incubation of radiolabeled APC with the M358R variant but not the WT led to the appearance of α_1-PI−APC complexes of mobility intermediate between unreacted APC and the

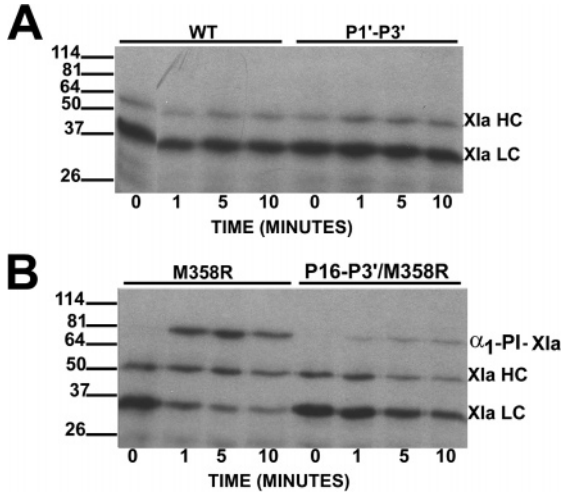

FIGURE 4: Electrophoretic detection of complex formation between ^{125}I-human factor XIa and recombinant α_1-PI proteins. An autoradiogram of a dried 10% SDS−polyacrylamide gel containing the products of timed reactions between WT or variant α_1-PI proteins and ^{125}I-human factor XIa. The reactants, consisting of 150 nM α_1-PI and 20 nM ^{125}I-human factor XIa, were incubated at 37 °C for 1, 5, or 10 min prior to reaction termination and electrophoresis under reducing conditions. Samples of 0 min came from a mock reaction lacking α_1-PI. The positions of prestained molecular weight markers and their sizes in kilodaltons are shown on the left.

aggregates, which increased over time; in contrast, little or no such complexes formed in the equivalent reactions that contained P16−P3′/M358R. Analogous results were found in parallel reactions with iodinated factor XIa, which was resolved into 52-kDa heavy and 28-kDa light chains on reducing SDS−PAGE (Figure 4); the latter contained the active site, as shown by its conversion into a serpin−enzyme complex by M358R and, to a lesser extent, P16−P3′/M358R. As in the case of reactions with labeled APC, the WT and P1′−P3′ variants were unreactive under the conditions employed (Figure 4).

Kinetic Characterization of α_1-PI Variants. Quantitative kinetic analysis of the thrombin and APC-inhibitory activities of the most active α_1-PI variants was next performed to obtain measurements of the second-order rate constants and stoichiometries of inhibition. Those variants capable of forming SDS-stable complexes with either thrombin or APC were analyzed, with the exception of the P5−P3′ variant, which was the sole altered α_1-PI protein whose expression and stability in *E. coli* was reduced compared to that of the WT (data not shown). As shown in Table 2, the M358R variant exhibited a mean second-order rate constant of approximately $1.00 \pm 0.05 \times 10^7$ M^{-1} min^{-1}, a value intermediate between the 6.0×10^6 M^{-1} min^{-1} and 2.9×10^7 M^{-1} min^{-1} values previously reported for bacterially expressed M358R (*17*, *26*). The mean SI was 3.2 in our hands but was either not determined or 1.1 in previous studies (*17*, *26*). The P16−P3′/M358R variant had a rate constant 3-fold less than that of the M358R variant, with a 3−4-fold increase in SI, while the rate constant reduction for the P1′−P3′ variant was 80-fold with a 4−5-fold increase in SI. The P16−P3′ complete loop exchange and the less extensive P10−P3′ mutations both resulted in a greater than 700-fold rate constant reduction.

We also examined the kinetic consequences of the P16−P3′/M358R mutation on the interaction of APC with variant

Table 2: Inhibition Rate Constants (k_2) and SI for Serpins or Serpin Variants with Thrombin and Activated Protein C

serpin or serpin variant	k_2 versus IIa $\times 10^3$ (M^{-1} min^{-1})	k_2 versus APC $\times 10^3$ (M^{-1} min^{-1})	SI versus IIa
$H_6\alpha_1$-PI (M358R)	10 100 ± 480[a,b]	1300 ± 80[b]	3.2 ± 0.3[c]
$H_6\alpha_1$-PI (P16−P3′/M358R)	3100 ± 100[b]	19 ± 2[b]	11.4 ± 0.5[c]
$H_6\alpha_1$-PI (P1′-P3′)	130 ± 13[d]	ND[e]	13.7 ± 1.3[c]
$H_6\alpha_1$-PI (I360T)	2.4 ± 0.3[d]	ND	ND
$H_6\alpha_1$-PI (P361Q)	2.3 ± 0.2[d]	ND	ND
H_6HCII (WT)	27 ± 5[d]	ND	4.0[f]
$H_6\alpha_1$-PI (P16−P3′)	15 ± 2[d]	ND	ND
$H_6\alpha_1$-PI (P10−P3′)	13 ± 1[d]	ND	ND
$H_6\alpha_1$-PI	3.0 ± 0.2[d]	ND	ND
α_1-PI	2.9[g]	ND	ND
LS[h]	2800[h]	0.34[h]	ND

[a] Values are expressed as the mean ± standard error of the mean. [b] $n = 5$. [c] $n = 4$. [d] $n = 3$. [e] ND = not determined. [f] Mean of three determinations. [g] Value from ref *29*. [h] α_1-PI with an AVVIAGRSLN P7−P3′ substitution, from ref *16*.

proteinase inhibitors. In our hands, the M358R variant inhibited APC with a rate constant of $1.30 \pm 0.08 \times 10^6$ M^{-1} min^{-1}, about 3-fold lower than the previously reported value (*17*). The positioning of the M358R variant within the exchanged HCII RCL in P16−P3′/M358R reduced the rate of APC inhibition 65-fold (Table 2).

DISCUSSION

The major findings of this study were that α_1-PI gained most of the progressive antithrombin activity of HCII when the HCII RCL was substituted for its own, that exceeding this level of activity required a P1 Arg in either the substituted or natural loop, that α_1-PI-gained enhanced specificity when the P1 Arg and HCII RCL substitutions were combined, and that enhanced specificity came at the cost of increased substrate behavior. Thus, the inhibition characteristics of serpins can be changed by mutagenesis, but the prediction of reactivity and specificity remains difficult, likely because of the complexity of the serpin mechanism and the selection of optimal serpin−protease pairs through evolution.

In this study, WT recombinant α_1-PI and the widely investigated α_1-PI (M358R) "Pittsburgh" variant were produced in *E. coli* as N-terminally hexahistidine-tagged soluble proteins, using an arabinose-inducible expression system. We have previously used this system to produce N-terminally hexahistidine-tagged HCII (*27*) and extended this approach to α_1-PI for consistency and to compare chimeric proteins comprised of portions of both serpins. Our approach therefore differed in two respects from previous studies in which α_1-PI and α_1-PI (M358R) had been expressed in bacterial systems: in the presence of the His tag, introduced to facilitate purification, and in the fact that they had not been renatured from insoluble inclusion bodies (*28*). While the yield of recombinant serpin was much greater in such systems than in this study, the His tag allowed for simple purification and the soluble nature of the expression products eliminated the need for renaturation, arguably more faithfully mimicking the natural synthesis of these proteins.

Despite the differences in their mode of production, our purified preparations exhibited similar properties compared to renatured expression products with respect to their inhibition of target proteases. Because the purpose of this study was to investigate the potential antithrombin activity of engineered α_1-PI variants, the base protein for comparative purposes was the M358R variant. The tagged M358R variant compared favorably with previous studies in which untagged α_1-PI (M358R) had been expressed in bacteria, with a second-order rate constant of thrombin inhibition of 1.0×10^7 M^{-1} min^{-1}, a value intermediate between the 6.0×10^6 M^{-1} min^{-1} and 2.9×10^7 M^{-1} min^{-1} reported values (*17*, *26*). Our M358R variant exhibited an elevated SI of 3.2 compared to the sole previously published value of 1.1 (*26*), but electrophoretic analysis at equimolar protease−inhibitor ratios revealed that the variant reacted completely, forming either serpin−enzyme complexes or cleaved forms. This finding ruled out trivial explanations for the elevated SI such as a subpopulation of unreactive material in our preparations and suggested the appropriateness of extending this approach to include novel mutations. Importantly, His-tagged WT α_1-PI exhibited the low but detectable second-order rate constant for thrombin inhibition of 3.0×10^3 M^{-1} min^{-1}, one indistinguishable from that previously reported for plasma-derived α_1-PI (*29*), again suggesting the validity of our approach.

The measurement of the second-order rate constant has been widely employed as a means to quantify the fitness of natural and recombinant serpins as protease inhibitors and must be performed either under pseudo-first-order conditions or solved by iterative curve-fitting if performed under second-order conditions. We and others have employed the technically simpler former approach but with the understanding that the second-order rate constant can be misleading in mutagenesis studies unless the SI is also determined. This inaccuracy can arise because of the excess serpin employed, which can mask substrate behavior. From one perspective, an elevated SI leads to underestimation of the rate constant, because of recycling of the protease (*1*); on the other hand, it indicates a propensity to generate cleaved inhibitor and allow escape of target protease molecules, neither of which are desirable in seeking to apply serpins as therapeutic agents (*16*). From either perspective, SI determination is necessary to complement second-order rate constant determination to obtain an accurate picture of the characteristics of variant serpins.

An increase in the rate of thrombin inhibition by α_1-PI of almost 4 orders of magnitude is conferred on the inhibitor by the M358R mutation (*17*). To focus on variants of α_1-PI containing HCII RCL substitutions that increased the efficacy of α_1-PI as a thrombin inhibitor above its natural levels, we selected initial experimental conditions under which the ability of WT α_1-PI to form SDS-stable complexes with

thrombin was undetectable. This strategy allowed us to select those variants forming detectable complexes for further kinetic analysis. Thus, the substitution of the RCL of HCII for that of α_1-PI, the P16−P3′ substitution, increased the ability of α_1-PI to inhibit thrombin by approximately 5-fold, in part supporting our initial hypothesis that substitution of the RCL of thrombin-specific HCII would increase the ability of α_1-PI to inhibit thrombin. The HCII RCL substitution brought the second-order rate constant of thrombin inhibition of the resulting chimeric inhibitor to within less than 2-fold of that of bacterially expressed, hexahistidine-tagged HCII (*24*) tested under identical conditions in this study. This finding showed that the Leu-Ser reactive center, atypical for a thrombin inhibitor, could function more effectively than Met-Ser in the context of the transferred RCL but not in that of α_1-PI, as seen in the M358L single residue substitution. The enhancement was dependent on the transfer of portions of both the proximal and distal loop, as shown by its absence in the case of both the P16−P1 variant and the P1−P3′ variants; thrombin inhibition by an α_1-PI with a P1 Leu could only be accelerated over WT rates by the substitution of at least the P5−P3′ residues of HCII. However, in the presence of the P1 Met, the substitution of the TQ P2′−P3′ dipeptide for the natural IP residues lead to an over 40-fold enhancement of the rate of thrombin inhibition. Both alterations were required for this effect, because in isolation, neither point mutation increased the rate of thrombin inhibition above WT levels (Table 2). These results are consistent with the greater portion of the RCL shown to contact active-site-mutated thrombin in its crystallized encounter complex with HCII than that of the corresponding trypsin-α_1-PI (M358R) complex, where P4−P5′ residues, rather than P2−P2′ residues alone, formed close contacts with the protease (*7*, *29*). While not attempted in this study, combining the TQ P2′−P3′ dipeptide and M358R substitutions in (M358R) might therefore further enhance the antithrombin activity of the resulting chimeric α_1-PI.

For maximal thrombin-inhibitory activity, an Arg-Ser reactive center appeared to be essential. Substitution of Arg for Leu in P16−P3′ increased the rate of inhibition of thrombin by the chimeric inhibitor by over 200-fold, to within 3-fold of that observed for the M358R variant, demonstrating the dominant role of the P1 Arg in thrombin inhibition, even when presented in the context of a different reactive center loop, that of HCII, in the α_1-PI scaffold. We investigated the reactivity of this P16−P3′/M358R combination mutant with two other coagulation proteases, factor XIa and APC, because of the previously reported effective inhibition of these proteases by the M358R variant (*14*, *15*). The combination mutant reacted with both proteases but to a diminished and greatly diminished extent, respectively. Quantitation of the anti-APC inhibitory activity revealed a 70-fold reduction in the effectiveness of the combination mutant as an APC inhibitor compared to M358R.

It has been previously argued by Hopkins et al. and others that to transform α_1-PI into an effective antithrombotic agent, its thrombin activity should be maximized and its anti-APC activity minimized (*17*). More recently, this concept has been strengthened by data suggesting that α_1-PI M358R administration worsened the outcome in a baboon model of sepsis (*16*) and by a clinical trial showing the efficacy of recombinant APC in decreasing mortality in septic patients (*30*). The P16−P3′/M358R variant demonstrated similar antithrombin activity to the most selective of the α_1-PI variants initially described by Hopkins et al. (*17*) but retained greater anti-APC activity than that "LS" protein, α_1-PI with a P7−P3′ substitution of antithrombin residues, including P1 Arg. This residual activity may derive from the HCII P2 Pro residue, also found in M358R but lacking in antithrombin and LS, as previously suggested (*17*); however, in a follow-up study of additional substitutions based on the same antithrombin to α_1-PI concept, other cooperative effects were noted between different substitutions (*18*).

Alterations to the RCL of α_1-PI also affected the initial partitioning between substrate and inhibitor pathways. It is well-established that the serpin mechanism branches at the point at which cleaved inhibitor and intact protease can be released or at which denaturation-stable complexes of cleaved inhibitor and inhibited protease are formed (*1*). The distribution between these outcomes can be altered by varying the ionic strength or by mutation, as previously demonstrated for α_1-PI (G349P), the P10 residue (*31*), and in a loop exchange of the P6−P2 residues of α_1-PI for those of ovalbumin (*32*). The extent of substrate behavior of the most thrombin-inhibitory chimeras produced in this study was assessed by measuring the SI, which corresponds to the number of serpin molecules required to inhibit 1 protease molecule (*25*). The SI was increased over that of M358R by 3−4-fold for both P16−P3′/M358R and for P1′−P3′. In our hands, both M358R and recombinant HCII made in the same system with the same hexahistidine tag exhibited an SI of 3−4. For HCII, it is well-established that the presence of a P1 Arg in its RCL leads to both an elevated stoichiometry and complex instability in the presence of heparin (*33*, *34*). Our results extend this finding to substrate behavior in the context of the HCII/P1 Arg RCL in another serpin.

The altered RCL of P16−P3′/M358R appeared to demonstrate a reduced compatibility, compared to M358R or WT α_1-PI, with the body of the serpin, resulting in an increased stoichiometry. While the reasons for this are not clear, there are a number of possibilities. The HCII or modified HCII loop may be sufficiently well-conformed to be recognized and cleaved by thrombin but cause a subtle misalignment such that the subsequent insertion of the cleaved RCL is impaired in some modified serpin−protease encounters. Alternatively, it is not the initial angle of insertion of the cleaved RCL that results in a failure to form a stable complex but conformational incompatibility between the loop that is attempting to insert and either its presumptive partner strands in β-sheet A or another neighboring structural element. An indirect effect is likely in the case of the P1′−P3′ variant, because the altered residues are separated from the inserting RCL by reactive center cleavage and cannot therefore play a direct role in failure of the loop to insert into the RCL in most serpin−protease encounters. A precedent for this increased stoichiometry in mutations of the M358R α_1-PI distal hinge (P1′−P10′) was reported by Bottomley et al. (*26*) who found 3.5−177-fold increases in SI for M358R variants with Ala substitutions at positions P6′−P8′, inclusive.

While the backbones of the RCLs of α_1-PI (M358R) and HCII in their respective encounter complexes with S195A−trypsin and S195A−thrombin are superimposable and the Arg and Leu side chains point in the same direction (*7*, *35*),

our findings suggest that these loops are not functionally interchangeable. Unlike the corresponding residues of canonical inhibitors, serpin RCLs must not only interact with the protease but they must also be capable of strand insertion more rapid than completion of the proteolysis reaction. Thus, the side chains of the serpin RCL residues and not just the polypeptide backbone are important in serpin function. An additional possible contributor to the optimal structure of serpin RCLs could be loop−body interactions. In this regard, the crystal structure of native α_1-PI revealed contacts between P5 Glu and several residues in the body of α_1-PI, such as R196, M226, R223, K243, and R281, and the RCL remains superimposable between free and encounter complex forms of crystallized α_1-PI, arguing for their maintenance (*36*). Such interactions were not possible in the P16−P3′/M358R variant created in this study, and this absence may have had an effect on subsequent loop movements critically related to the SI.

Replacing the RCL with that of HCII increased the antithrombin activity of the resulting chimera to within less than 2-fold that of HCII and also appeared to transfer its specificity, because no reactions with factor XIa or APC were detected. Combining the P16−P3′ substitution with the M358R mutation enhanced thrombin reactivity and partially increased specificity in comparison to M358R. However, these alterations also increased the tendency of the engineered α_1-PI proteins to act as substrates, a change not previously investigated in earlier and extensive antithrombin loop-exchange mutagenesis of α_1-PI. While from a kinetic perspective, it has been persuasively argued that an increased SI results in an underestimate of the second-order rate constant of inhibition (*1*), in a pharmacological context, increased substrate behavior and potential recycling of thrombin are not desirable outcomes (*16*). Our results highlight the necessity in attempting to engineer serpins of ensuring not just effective initial binding of the protease and cleavage of the inhibitor but also efficient and durable inhibitory complex formation and suggest the possible utility of continuing to apply a new paradigm, that of using the HCII RCL as an iterative template for the design of antithrombotic variants of α_1-PI.

ACKNOWLEDGMENT

This work was supported by Grant-In-Aid T-4903 from the Heart and Stroke Foundation of Ontario. M.L.F. held an Ontario Graduate Scholarship (OGS) award from the Government of Ontario. We thank Sharon Gataiance (McMaster University) for her assistance in protease iodination, Jason Sutherland (McMaster University) both for performing experiments with recombinant HCII and for a careful review of the manuscript, and Vikram Ravindran (McMaster University) for performing initial experiments with some mutant proteins.

REFERENCES

1. Gettins, P. G. (2002) Serpin structure, mechanism, and function, *Chem. Rev. 102*, 4751−4804.
2. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-O'Donnell, E., Salvesen, G. S., Travis, J., and Whisstock, J. C. (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature, *J. Biol. Chem. 276*, 33293−33296.
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. Im, H., Woo, M. S., Hwang, K. Y., and Yu, M. H. (2002) Interactions causing the kinetic trap in serpin protein folding, *J. Biol. Chem. 277*, 46347−46354.
5. Futamura, A., Stratikos, E., Olson, S. T., and Gettins, P. G. (1998) Change in environment of the P1 side chain upon progression from the Michaelis complex to the covalent serpin-proteinase complex, *Biochemistry 37*, 13110−13119.
6. 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 β-sheet A, *Proc. Natl. Acad. Sci. U.S.A. 96*, 4808−4813.
7. Huntington, J. A., Read, J. R., and Carrell, R. W. (2000) Structure of a serpin-protease complex shows inhibition by deformation, *Nature 407*, 923−926.
8. Laurell, C. B. (1972) A screening test for 1-antitrypsin deficiency, *Scand. J. Clin. Lab. Invest. 29*, 247−248.
9. Morgan, K., and Kalsheker, N. A. (1997) Regulation of the serine proteinase inhibitor (SERPIN) gene α 1-antitrypsin: A paradigm for other SERPINs, *Int. J. Biochem. Cell Biol. 29*, 1501−1511.
10. Loebermann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) Human α 1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model, and preliminary analysis of the implications for function, *J. Mol. Biol. 177*, 531−557.
11. Laurell, C. B., and Eriksson, S. (1965) The serum α-1-antitrypsin in families with hypo-α-1-antitrypsinemia, *Clin. Chim. Acta 11*, 395−398.
12. Pannell, R., Johnson, D., and Travis, J. (1974) Isolation and properties of human plasma α-1-proteinase inhibitor, *Biochemistry 13*, 5439−5445.
13. Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) Mutation of antitrypsin to antithrombin. α-1-Antitrypsin Pittsburgh (358 Met leads to Arg), a fatal bleeding disorder, *N. Engl. J. Med. 309*, 694−698.
14. Scott, C. F., Carrell, R. W., Glaser, C. B., Kueppers, F., Lewis, J. H., and Colman, R. W. (1986) α-1-Antitrypsin-Pittsburgh. A potent inhibitor of human plasma factor XIa, kallikrein, and factor XIIf, *J. Clin. Invest. 77*, 631−634.
15. Heeb, M. J., Bischoff, R., Courtney, M., and Griffin, J. H. (1990) Inhibition of activated protein C by recombinant α 1-antitrypsin variants with substitution of arginine or leucine for methionine358, *J Biol. Chem. 265*, 2365−2369.
16. 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.
17. 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.
18. 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.
19. Parker, K. A., and Tollefsen, D. M. (1985) The protease specificity of heparin cofactor II. Inhibition of thrombin generated during coagulation, *J. Biol. Chem. 260*, 3501−3505.
20. Ragg, H. (1986) A new member of the plasma protease inhibitor gene family, *Nucleic Acids Res. 14*, 1073−1088.
21. Long, G. L., Chandra, T., Woo, S. L., Davie, E. W., and Kurachi, K. (1984) Complete sequence of the cDNA for human α 1-antitrypsin and the gene for the S variant, *Biochemistry 23*, 4828−4837.
22. Chen, B., and Przybyla, A. E. (1994) An efficient site-directed mutagenesis method based on PCR, *BioTechniques 17*, 657−659.
23. Fraker, P. J., and Speck, J. C., Jr. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril, *Biochem. Biophys. Res. Commun. 80*, 849−857.
24. 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.

25. Olson, S. T., Bjork, I., and Shore, J. D. (1993) Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin, *Methods Enzymol. 222*, 525−559.
26. Bottomley, S. P., Lawrenson, I. D., Tew, D., Dai, W., Whisstock, J. C., and Pike, R. N. (2001) The role of strand 1 of the C β-sheet in the structure and function of α(1)-antitrypsin, *Protein Sci. 10*, 2518−2524.
27. 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.
28. Hopkins, P. C., Carrell, R. W., and Stone, S. R. (1993) Effects of mutations in the hinge region of serpins, *Biochemistry 32*, 7650−7657.
29. Travis, J., Matheson, N. R., George, P. M., and Carrell, R. W. (1986) Kinetic studies on the interaction of α 1-proteinase inhibitor (Pittsburgh) with trypsin-like serine proteinases, *Biol. Chem. 367*, 853−859.
30. 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.
31. Hopkins, P. C., Carrell, R. W., and Stone, S. R. (1993) Effects of mutations in the hinge region of serpins, *Biochemistry 32*, 7650−7657.
32. Chaillan-Huntington, C. E., Gettins, P. G., Huntington, J. A., and Patston, P. A. (1997) The P6−P2 region of serpins is critical for proteinase inhibition and complex stability, *Biochemistry 36*, 9562−9570.
33. Han, J. H., van Deerlin, V. M., and Tollefsen, D. M. (1997) Heparin facilitates dissociation of complexes between thrombin and a reactive site mutant (L444R) of heparin cofactor II, *J. Biol. Chem. 272*, 8243−8249.
34. Ciaccia, A. V., Willemze, A. J., and Church, F. C. (1997) Heparin promotes proteolytic inactivation by thrombin of a reactive site mutant (L444R) of recombinant heparin cofactor II, *J. Biol. Chem. 272*, 888−893.
35. 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.
36. Elliott, P. R., Lomas, D. A., Carrell, R. W., and Abrahams, J. P. (1996) Inhibitory conformation of the reactive loop of α 1-antitrypsin, *Nat. Struct. Biol. 3*, 676−681.

BI048833F