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**THE EFFECTS OF MUTAGENESIS ON THE REACTIVE CENTRE LOOP OF TWO
THROMBIN-INHIBITORY SERPINS, ANTITHROMBIN AND HEPARIN
COFACTOR II**

By

MICHAEL ANDREW CUNNINGHAM, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

DOCTOR OF PHILOSOPHY

SCIENCE

McMaster University

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REACTIVE CENTRE LOOP MUTAGENESIS OF AT AND HCII

**Doctor of Philosophy (2000)
(Medical Sciences)**

**McMaster University
Hamilton, Ontario**

TITLE: The Effects of Mutagenesis on the Reactive Centre Loop of Two Thrombin-Inhibitory Serpins, Antithrombin and Heparin Cofactor II

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NUMBER OF PAGES: xx, 218

ABSTRACT

Antithrombin (AT) and heparin cofactor II (HCII) are the predominant inhibitors of thrombin in plasma. They belong to the serine protease inhibitor, or serpin, family of proteins and they inhibit their target proteases through a mechanism that is unique to this family of molecules. AT and HCII provide an ideal substrate on the reactive centre loop and subsequently form 1:1 stoichiometric inhibitory complexes with their target proteases through a mechanism that results in major conformational changes in these serpins. While thrombin acts as a target protease with these serpins, neutrophil elastase (NE), another serine protease, reacts with AT and HCII within the reactive centre loop. Unlike thrombin, however, the interaction of NE with AT and HCII results in cleavage and inactivation of these serpins, without forming inhibitory complexes with NE. The aim of this thesis was to analyze the effects of mutagenesis of the NE cleavage sites within AT and HCII to determine the limits of amino acid substitutions that would permit AT and HCII to retain function but be less susceptible to inactivation by NE.

Analyses of proteins expressed in a cell-free expression system and then in COS-1 mammalian cell culture were used to study the effects of mutagenesis at P4 and then at P4 and P5 in rabbit and human AT. While charged and polar amino acid substitutions severely reduced the function of AT, substitution of the bulkiest residue, tryptophan, at P4 and then at P5 and P5 had minimal effects on the thrombin-inhibitory activity of AT. However, the susceptibility to NE cleavage did not appear to be affected by any substitutions that were made in AT. Analysis of amino acid substitutions in bacterially-

derived HCII P6 variants demonstrated a similar flexibility in amino acid composition at the site of NE cleavage, with respect to the ability to inhibit thrombin, although substitution of polar and bulky residues within the reactive centre loop of this serpin increased its resistance to NE inactivation.

These results demonstrate that the amino acid composition of the reactive centre loop of AT and HCII is flexible to allow the maintenance of thrombin-inhibitory activity, although some limitations do exist. The effects of residue substitutions within the reactive centre loop on the susceptibility to NE cleavage are less clear, but the presence of bulky amino acids at the primary NE cleavage site, at least in HCII, appears to reduce the proteolytic activity of this protease. The production of an NE-resistant thrombin-inhibitory serpin provides the basis for the possible development of “hardened serpins” which might be of therapeutic importance in the future.

ACKNOWLEDGEMENTS

I would like to thank Dr. W.P. Sheffield for the opportunity to complete this thesis. Bill, I cannot thank you enough for all of your support, guidance and generosity, especially through some of the more difficult portions of my tenure at McMaster. If I turn out to be half the investigator that you are, I will consider myself a success! To my supervisory committee members, Drs. Mark Hatton and Rick Austin, thank you for all of your advice and constructive criticism of my project. A special thanks to Dr. Morris Blajchman for his thoughtful discussions and suggestions at regular Friday morning lab meetings. I would also like to acknowledge and thank the Heart and Stroke Foundation of Canada for their generous financial support.

Many thanks to all past and present lab members who helped me with my project, both scientifically and socially. I cannot thank Varsha Bhakta enough for all of her help and encouragement through many temper tantrums and frustrating experiments. Thanks to Myron Kulczyk for help with conducting ELISAs, teaching me “Myronese”, and for all the necessary social distractions that helped me complete this project. Dr. Michael Wells (that still sounds funny!), thanks for being such a great friend and helping me, especially in the early days, with my project. I’ll never forget how you and Myron taught me how to properly immunize chickens..... To all friends and acquaintances at MAC who have come and gone, I’ll never forget my time here, so thanks for the memories.

Last, but surely not least, thank you to my family. You have all been an unwavering source of support and encouragement, and I could never have finished this project without you all there with me!

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LIST OF SYMBOLS AND ABBREVIATIONS

α	alpha
α -AT	alpha-isoform of antithrombin
α -MEM	alpha-minimal essential medium medium
β	beta
β -AT	β -isoform of antithrombin
μ Ci	microcurie(s)
μ g	microgram
μ l	microlitre(s)
μ M	micromolar
$^{\circ}$ C	degrees Celsius
A	amp(s)
Å	angstrom
Ala	alanine
APS	ammonium persulfate
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AT	antithrombin
AT-I	antithrombin-I
AT-II	antithrombin-II

AT-III	antithrombin-III
AT-IV	antithrombin-IV
ATP	adenosine 5'-triphosphate
AT-V	antithrombin-V
AT-VI	antithrombin-VI
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	baby hamster kidney cell line
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid(s)
CHO	chinese hamster ovary
COS	transformed African green monkey kidney cells
C-terminal	carboxy-terminal
Cys	cysteine
D	dextrorotatory
Da	dalton(s)
ddH₂O	distilled, deionized water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
g	gram(s)
G418	geneticin
Glu	glutamic acid
Gly	glycine
GST	glutathione-S-transferase
h	hour(s)
H ₆ -HCII	amino-terminal hexahistidinyI-tagged heparin cofactor II
H ₆ -Δ66HCII	amino-terminal hexahistidinyI-tagged heparin cofactor II lacking residues 1 to 66
HCII	heparin cofactor II
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid
His	histidine
ICU	intensive care unit
IgG	immunoglobulin G
Ile	isoleucine
IPTG	β-D-idopropyl-thiogalactopyranoside
k ₂	second order rate constant

KAc	potassium acetate
kb	kilobase(s)
kDa	kiloDalton(s)
k_{obs}	pseudo-first-order rate constant
L	levorotatory
l	litre(s)
LB	Luria-Bertani broth
Leu	leucine
LRP	low density lipoprotein receptor-related protein
Lys	lysine
M	molar
Met	methionine
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mmol	millimole(s)
mRNA	messenger ribonucleic acid(s)
NaOAc	sodium acetate
NBT	nitro blue tetrazolium
NE	neutrophil elastase
ng	nanogram(s)

nm	nanometre(s)
nM	nanomolar
NPGB	N-paranitroguanidinobenzoate
N-terminal	amino-terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Phe	phenylalanine
PMSF	phenylmethylsulfonyl fluoride
PPACK	D-phenyl-L-propyl-L-arginyl-chloromethylketone
PPE	procine pancreatic elastase
Pro	proline
PVDF	polyvinylidene difluoride
RPM	revolutions per minute
S-2238	H-D-phenylalanyl-L-pepecolyl-L-arginine-p-nitroaniline dihydrochloride
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SECR	serpine enzyme complex receptor
Ser	serine

SI	stoichiometry of inhibition
TAT	thrombin-antithrombin complex
TBS	tris-buffered saline
TBST	tris-buffered saline with tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine hydrochloride
TFPI	Tissue Factor Pathway Inhibitor
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
U	units
UV	ultraviolet
V	volts
v/v	volume:volume ratio
Val	valine
w/v	weight:volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

1. INTRODUCTION

Blood coagulation occurs as the result of a cascade of enzymatic reactions involving several serine proteases, which ultimately results in the activation of thrombin, the pivotal serine protease involved in the formation of a fibrin clot (Roberts and Tabares, 1995). The serine protease family of proteins, which hydrolyze peptide bonds using an active site serine residue, consists of many proteins which have roles in several physiological functions including blood coagulation, fibrinolysis, digestion and activation of the complement system. During coagulation, there are successive conversions of inactive zymogens into active serine proteases, of which α -thrombin is the ultimate product. While α -thrombin has other physiological roles in smooth muscle cell proliferation, angiogenesis, mitogenesis, and chemotaxis of lymphocytes, the central role of thrombin is in coagulation, where it proteolytically cleaves soluble fibrinogen to form insoluble fibrin and ultimately a hemostatic fibrin clot (Friezner-Degen, 1995).

Thrombin activity is also associated with several pathological states including atherosclerosis, cancer, viral infectivity, and thrombosis (Friezner-Degen, 1995). It is logical, therefore, that thrombin be a strictly regulated molecule, and several proteins exist which control its activity. Physiological inhibition of thrombin, and other serine proteases, is achieved predominantly by the serine protease inhibitor, or *serpin*, family of proteins (Carrell *et al.*, 1987). Serpins are a unique family of proteins in that they inactivate their target proteases by forming a covalent, denaturation-resistant inhibitory complex (Carrell *et al.*, 1987). In plasma, the primary physiological inhibitors of

thrombin are the serpins antithrombin (AT) and heparin cofactor II (HCII) (Rosenberg and Damus, 1973; Tollefsen *et al.*, 1982).

These thrombin inhibitory serpins inactivate their target proteases by acting as suicide inhibitors; they bind to their target proteases by providing an ideal substrate, the reactive centre loop, on their surface as “bait”. Once bound, target proteases become trapped in a stable complex (Gettins *et al.*, 1996). Serpin-protease complexes are then cleared from the circulation more rapidly than either of their constituent proteins (Pizzo *et al.*, 1988).

While thrombin forms a stable serpin-protease complex, non-target proteases cleave the serpin reactive centre loop without becoming trapped. In the case of AT (Carrell and Owen, 1985) and HCII (Pratt *et al.*, 1990), neutrophil elastase (NE) acts as a non-target protease, cleaving both serpins at sites proximal to the reactive centre. The importance of the reactive centre loop in both kinds of serpin-protease interactions is illustrated by variant serpins with reactive centre loop mutations that see thrombin as a non-target protease. Because much of the reactive centre loop is not conserved amongst serpins (Stein and Carrell, 1995), particularly the sites of elastase cleavage, a mutagenesis study was conducted to provide information describing to what extent the serpin reactive centre loop determines target or non-target status, specifically with respect to the interactions of AT and HCII with thrombin and neutrophil elastase.

The following pages provide an overview of AT, HCII and the serpins, and describe in detail their interactions with target (thrombin) and non-target (NE) proteases.

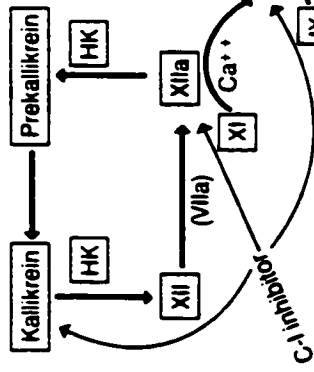
In addition, the results of a mutational analysis of the reactive centre loop of these serpins on the interactions with thrombin and NE are reported.

1.1 Overview of Coagulation

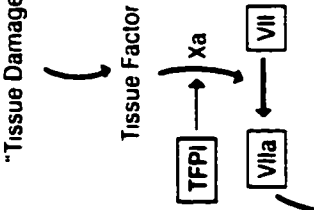
Blood coagulation occurs through the activation of several clotting factors within the coagulation cascade (Figure 1). Coagulation can be initiated through two pathways, designated the intrinsic pathway and the extrinsic pathway, which rely on the successive activation of zymogens eventually resulting in the formation of fibrin (reviewed in Roberts and Tabares, 1995). Cellular components of the blood, such as platelets, contribute to effective hemostasis by providing a phospholipid surface for the activation of some zymogens, in the coagulation cascade, and in addition, interact with other platelets and fibrin products to fortify the hemostatic plug and stop the flow of blood at the site of injury. The intrinsic pathway initiates coagulation by a series of interactions between substances that are located within the circulation. These substances include factors XII, XI, IX, VIII, and X, phospholipids, and calcium. This pathway is activated when blood makes contact with a foreign surface within the blood vessel. Activation of this pathway converts factor XII to XIIa, which in turn, activates factor XI to XIa. Factor XIa then activates factor IXa from IX, which leads to the formation of a structure of several proteins called the “intrinsic tenase complex” which consists of factors IXa and VIIIa. In the presence of calcium and a phospholipid surface, this intrinsic tenase complex activates factor Xa from the proenzyme factor X.

Coagulation can also be initiated through the “extrinsic pathway”, which consists of tissue factor, factor VII, and calcium (Davie *et al.*, 1991). This pathway is activated

Contact Factor Pathway
(Intrinsic Pathway)

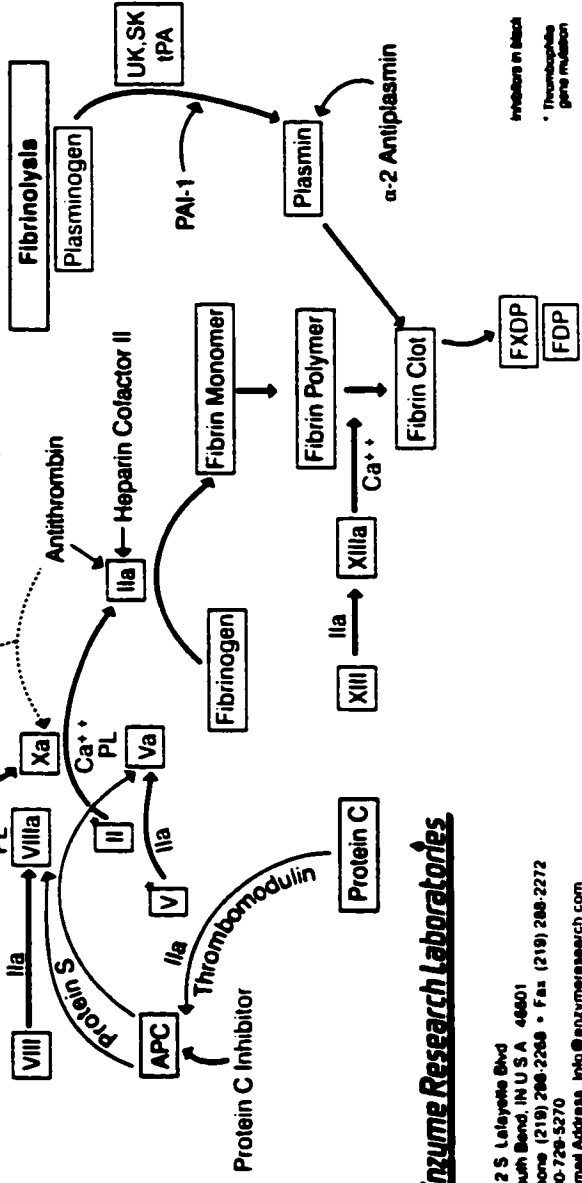


Tissue Factor Pathway
(Extrinsic Pathway)



Protein Concentrations

Component	Molecular Weight	Plasma Concentration µg/ml	Plasma Concentration µM
Fibrinogen (I)	330,000	3000	9.09
Prothrombin (II)	72,000	100	1.369
Factor V	330,000	10	0.03
Factor VII	50,000	0.5	0.01
Factor VIII	330,000	0.1	0.0003
Factor IX	58,000	5	0.08628
Factor X	58,000	6	0.13605
Factor XI	160,000	5	0.031
Factor XII	80,000	30	0.375
Factor XIII	370,000	10	0.03125
Protein C	62,000	4	0.0645
Protein S	62,000	2.2	0.0355
Prothrombin	72,000	2	0.0272
Prekallikrein	110,000	70	0.8363
HK	450,000	300	0.6667
Fibrinogen	330,000	290	5
Antithrombin III	58,000	216	2.4
Plasminogen	90,000	0.1	0.001887
Urokinase	53,000	90	1.3636
Heparin Cofactor II	66,000	60	0.924
Alpha ₂ Antiplasmin	63,000	57	0.7152
Protein C Inhibitor	57,000	4	0.0752
Alpha ₂ Macroglobulin	725,000	2100	2.8866



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Inhibitors in blood
• Thrombolytic
• plasmin

by the presence of tissue factor, a protein that is normally expressed in the adventitia of blood vessels and in extravascular tissue, and is exposed to flowing blood only after a vessel wall injury. Under normal conditions, tissue factor does not make contact with flowing blood because it is present under the endothelial cell layer, which lines blood vessels. When the extrinsic pathway is activated by a vascular injury, tissue factor forms a complex with factor VII/VIIa and calcium, to form the “extrinsic tenase complex” (Nemerson and Repke, 1985; Rao and Rapaport, 1988; Sakai *et al.*, 1989). In this complex, factor VII is converted to VIIa. Tissue factor/VIIa converts factor X to Xa and factor IX to IXa.

Following the initiation of coagulation by either the intrinsic or extrinsic pathways that form factor Xa, the propagation of coagulation occurs through a common pathway. This pathway relies on the presence of several factors, including factor Xa, factor Va, phospholipids, calcium, prothrombin, thrombin, fibrinogen, and fibrin. After factor Xa is activated by either the intrinsic or extrinsic pathways, it proceeds to activate thrombin from prothrombin, which relies on a phospholipid surface and activated factor Va to catalyze the reaction (Davie *et al.*, 1991). Typically, this reaction occurs on the surface of activated platelets. Thrombin then converts fibrinogen to fibrin, which is a principal protein associated with a clot. Fibrin is stabilized through the cross-linking activity of factor XIIIa, which is produced from the zymogen factor XIII by thrombin.

The generation of small amounts of factor Xa through the activation of the intrinsic or extrinsic pathway can lead to some thrombin generation, particularly in the presence of factor Va. However, this pathway is insufficient to allow continued

coagulation, mainly because of efficient inhibition of the tissue factor/VIIa complex by tissue factor pathway inhibitor (TFPI), which can occur via a ternary tissue factor/VIIa/Xa/TFPI complex (Broze and Meletich, 1987; Rao and Rapaport, 1987). Coagulation is efficiently promoted by the action of factor IXa and the feedback action of thrombin to activate cofactors VIII and V.

Thrombin is the integral serine protease involved in coagulation. In addition to catalyzing the formation of fibrin from fibrinogen, it also amplifies the coagulation reaction by its interaction with several factors in the cascade. For instance, thrombin activates factors VIIIa and IXa, from factors VIII and IX, respectively, which accelerates the formation of factor Xa in the tenase complex reactions described above (Davie *et al.*, 1991). Thrombin can also cleave prothrombin to increase its own levels in the forming clot. Amplification of the coagulation cascade also occurs via factor Xa activating factor VIIa from factor VII, which results in increasing factor Xa levels through increased activity of the extrinsic tenase complex (Davie *et al.*, 1991). The result of these amplification reactions is to ultimately enhance thrombin activation and accelerate the formation of the hemostatic clot to repair the site of vascular injury as quickly as possible.

Despite the efficiency of the coagulation cascade in repairing vascular injuries through the formation of hemostatic clots, this process must be tightly regulated to prevent uncontrolled coagulation and aberrant thrombosis. To this end, several regulators of coagulation are present in the blood to control hemostasis. These inhibitors include tissue factor pathway inhibitor, which, as mentioned above, down-regulates the rate of

coagulation by inhibiting the activity of the extrinsic tenase complex, by binding to factor Xa and tissue factor-factor VIIa (Broze and Meletich, 1987; Rao and Rapaport, 1987). Protein C is another inhibitor of coagulation. It is associated with thrombomodulin, an integral membrane protein located on endothelial cells. When thrombin interacts with thrombomodulin, it converts protein C to activated protein C, which, in conjunction a cofactor called protein S, inactivates factors VIIIa and Va to reduce the formation of factor Xa and thrombin (Stenflo, 1976; Kisiel, 1979; Vehar and Davie, 1980). The two principal inhibitors of thrombin in plasma, however, are antithrombin and heparin cofactor II (Rosenburg and Damus, 1973; Tollefsen *et al.*, 1982). These two molecules are members of the serine protease inhibitors family of proteins which inhibit thrombin, and in the case of antithrombin, also inhibit several other serine proteases of the coagulation cascade. These two molecules and their roles in hemostasis will be described in detail in subsequent sections.

1.2 Historical Background

An anticoagulant component in plasma that inhibited thrombin was first discovered by Morawitz (1905). Later, the term “heparin cofactor” was established to describe an unidentified plasma component that was necessary to observe the anticoagulant effects of heparin (Brinkhous, *et al.*, 1939). Seegers *et al.*, (1954) subsequently devised a numerical classification system for several different antithrombin activities that were found in plasma. Antithrombin-I described the ability of a fibrin clot to absorb thrombin, while antithrombin-II described the observed heparin cofactor activity. Antithrombin-III described the plasma component that was responsible for

conferring the progressive inhibition of thrombin in plasma. Antithrombin-IV represented the thrombin-inhibitory activity that was associated with prothrombin activation, while antithrombin-V referred to the anti-thrombin activity of a pathological globulin that was present in the serum of an individual with hypergammaglobulinemia. Finally, AT-VI was designated as the anti-thrombin properties of fibrin degradation products. Further information on the nature of AT-II and AT-III was gained by the study of a family with recurrent venous thromboembolism who demonstrated the partial loss of both progressive antithrombin (AT-II) and heparin cofactor (AT-III) activities (Egeberg, 1965). These observations suggested that AT-II and AT-III were possibly the same. The subsequent discovery that an α_2 -globulin had both progressive and heparin cofactor activities further supported this hypothesis (Abilgaard, 1967; Abilgaard, 1969). The eventual isolation of large quantities of this protein provided convincing evidence that one factor possessed both progressive and heparin cofactor activities (Rosenberg and Damus, 1973). This protein was initially named AT-III, but more recently has come to be known merely as AT (Lane *et al.*, 1993). The other AT terms are no longer used.

More recently, an additional antithrombin protein was discovered in plasma that had similar antithrombin activity to AT. Tollefsen and Blank (1981) demonstrated that incubation of human plasma with radiolabeled thrombin resulted in the formation of two distinct high molecular weight species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, one of which did not correspond to the thrombin-antithrombin (TAT) complex, and which did not correspond to a complex between thrombin and any of the other protease inhibitors known at the time. Subsequently, the

same investigators isolated a previously unrecognized heparin-dependent inhibitor of thrombin from plasma and designated it heparin cofactor II (Tollefsen, *et al.*, 1982). In addition, they showed that HCII activity was accelerated by dermatan sulfate, another glycosaminoglycan (Tollefsen *et al.*, 1983). This characteristic of HCII is unique to this serpin, since AT activity is unaffected by the presence of dermatan sulfate.

1.3 The Serpin Superfamily of Proteins

The serine protease inhibitor, or serpin, superfamily of proteins was first proposed and described by Hunt and Dayhoff (1980) after they recognized that several proteins including ovalbumin, α_1 -proteinase inhibitor, and antithrombin had similarities in their primary protein structures. It was not until 1985 however, that Carrell and Travis (1985) devised the name, *serpins*, for this newly described family of proteins, an acronym which described several proteins of this family, most of which functioned as serine protease inhibitors. To date, over 100 different proteins have been included in the serpin superfamily of proteins, including species variants and other gene products that have had their primary protein structures deduced from their respective cDNA sequences (Gettins *et al.*, 1996). As more proteins are added to the serpin family of proteins, it has become evident that the term *serpins* may be a misleading name for this group of proteins. Some of the *serpins* have no known inhibitory activity (*ie.* ovalbumin, angiotensinogen (Doolittle, 1983), corticosteroid-binding protein (Hammond *et al.*, 1987), thyroxine-binding globulin (Flink *et al.*, 1986), and maspin (Zou *et al.*, 1994)), while others specifically inhibit cysteine proteases (*ie.* cowpox viral protein CrmA (Komiyama *et al.*,

1994), and squamous cell carcinoma antigen (Takeda *et al.*, 1995)). Most serpins display 25-30% amino acid identity.

More recently, a sub-family of serpins, named ov-serpins, has been proposed, that groups proteins with similar protein and gene characteristics to ovalbumin (Remold-O'Donnell, 1993). This classification identifies serpins with 40-60% protein sequence homology to ovalbumin, a higher protein homology than that observed with serpins as a whole, as well as those with no cleavable hydrophobic signal sequence. In addition, this sub-family of serpins has common intron/exon characteristics at the gene level.

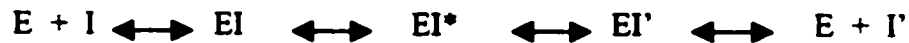
Serpins are an ancient family of proteins, having evolved over 500 million years from a gene encoding a single protein (Dayhoff and Hunt, 1980; Bao *et al.*, 1987). These molecules are found in several organisms including vertebrates, plants, insects, and viruses, but interestingly, not from bacteria. The best-characterized serpins are those found in human plasma, and appear to be involved predominantly in the regulation of coagulation, fibrinolysis, the immune system or inflammation. However, other roles of serpins are possible given their identification in mammary tumours (Zou *et al.*, 1994), astrocytes (Abraham *et al.*, 1993; Pasternack *et al.*, 1989), keratinocytes (Jensen *et al.*, 1995), and in a retinal epithelial cell line (Steele *et al.*, 1993). All serpins contain a core of approximately 360 amino acids with variable amino- or carboxy-terminal extensions that have evolved over time. For example, several serpins, including antithrombin, heparin cofactor II, plasminogen activator inhibitor-1 and protein C inhibitor all have an N-terminal heparin-binding domain that accelerates their inhibitory activities. In addition, there are several post-translational modifications on some serpins that

distinguish them from other serpins. The degree of glycosylation varies between serpins and other modifications, such as O-linked sulfation in HCII, are unique to some serpins. The primary protein sequence homology between serpins containing the core domain of 360 residues is, on average, only 25-30%. However, the tertiary structure that results from the physiological folding of this core domain is responsible for conferring a much more homologous tertiary structure that is conserved amongst the serpins (Gettins *et al.*, 1996). Thus, serpins are thought to share a common tertiary structure, composed of nine α -helices and three β -sheets.

The interest in studying the mechanism of action of protease inhibition by serpins originated from the observation that serpins inhibit their target proteases by a different mechanism than the “standard-mechanism” inhibitors of serine proteases, which have been extensively studied. Standard mechanism inhibitors are relatively small proteins that are usually less than 100 residues in primary sequence (Laskowski and Kato, 1980). These protease inhibitors have been crystallized and their mechanism of action has been well-characterized (Bode and Huber, 1992). These inhibitors bind to the active site of their target proteases by presenting an exposed loop, known as the reactive centre loop, in a canonical conformation that allows the reactive centre loop to react with the protease in a substrate-like manner (Laskowski and Kato, 1980). Standard mechanism inhibitors maintain a canonical formation of the reactive centre loop by intramolecular bonds between the loop and the body of the molecule. Stabilization of the reactive centre loop by intramolecular interactions is very effective, as shown by the minor change in conformation of the inhibitor after binding to its target protease. The protease specificity

of standard mechanism inhibitors is predominantly determined by the amino acid sequence of the reactive centre loop. An ideal reactive centre loop is complementary to the active site binding pocket in the protease.

The reaction of a standard mechanism inhibitor with its target protease occurs (Stone *et al.*, 1997), as described in the following reaction:



The protease (E) interacts with the inhibitor (I) to form initially a Michaelis type complex (EI) stabilized solely by non-covalent interactions. Subsequently, a series of covalent interactions occur (designated EI*), which include the formation of tetrahedral and acyl intermediates. The formation of the covalent intermediate molecules has been shown to involve a covalent bond between the inhibitor and the active site serine, present in all serine proteases. Normally standard mechanism inhibitors are not cleaved by their target proteases unless the interaction between the two molecules occurs over long periods of time. If the reaction between enzyme and inhibitor is permitted to go to completion, the inhibitor is cleaved and another non-covalent complex (EI') forms between the enzyme and the cleaved inhibitor. Dissociation of the complex then occurs, which yields an inactive inhibitor (I') and an active protease (E). As shown in the scheme above, all steps in the reaction are reversible. This reversibility demonstrates the intrinsic conformational stability of the canonical conformation of the standard mechanism inhibitor even after cleavage by the protease. While kinetic evidence exists for all stages of the reaction shown above, the crystal structures that have been obtained have been found to be solely in the Michaelis (EI) form (Bode and Huber, 1992). In

addition, chemically modified serine proteases, in which the active site serine has been inactivated and which are catalytically inactive, still form relatively tight complexes with their standard mechanism inhibitors (Laskowski and Kato, 1980). These two observations demonstrate that the active site serine of the serine protease actually contributes little towards the stability of the protease-inhibitor complex.

While the mechanism of action of standard mechanism inhibitors has been well characterized, less is known about the mechanism by which serpins inhibit their target proteases. Unlike standard inhibitors, no crystal structure exists for the serpin-enzyme complex. This deficiency has made studying these molecules more difficult and dependent on circumstantial evidence. Several differences between standard inhibitors and serpins exist, which have been demonstrated experimentally (Stone *et al.*, 1997). Unlike standard inhibitors, the reactive centre loop of serpins is flexible and can maintain several possible conformations. In addition, the reactive centre loop of serpins changes its conformation significantly after interaction with its target protease, whereas the reactive centre loop of standard inhibitors is more rigid in its conformation. Also, the serpin-enzyme reaction, unlike that observed between standard inhibitor and its protease, is irreversible. Finally, experimental evidence has shown that the active site serine in the protease is essential in the interaction between serpin and protease that ultimately results in the inhibitory serpin-enzyme complex (Stone *et al.*, 1997).

1.3.1 Mechanism of Protease Inhibition by Serpins

The reaction of serpins with their target proteases occurs in a reaction that can have two outcomes, and is described as a branched reaction (Rubin *et al.*, 1990; Patston

et al., 1991). Like standard mechanism inhibitors, serpins form an initial non-covalent, reversible, low affinity complex with their proteases called the Michaelis complex. In the subsequent reaction of serpin and protease, a covalent bond forms between the two molecules. At this point in the interaction, the reaction can proceed in one of two possible directions. This branchpoint complex is believed to consist of a tetrahedral intermediate between the serpin and the protease (O'Malley *et al.*, 1997). Progression of the reaction can proceed from this point to the formation of a covalent serpin-enzyme complex or to the formation of a cleaved serpin and intact protease.

The reactive centre loop of serpins is an essential structural feature for serpin-protease interactions. The first serpin structure to be determined was that of cleaved α_1 -proteinase inhibitor (Loebermann *et al.*, 1984). The serpin had been cleaved at the reactive site and the reactive centre loop was observed to be incorporated as the central strand of the A β -sheet within the body of the molecule. The crystal of the latent form of plasminogen activator inhibitor 1 (PAI-1), that contains an intact reactive centre loop has also demonstrated that the loop is inserted into the A β -sheet (Mottonen *et al.*, 1992). These two serpins, however, are inactive and cannot inhibit their target proteases. Determination of the structure of the reactive centre loop in a native, active serpin has been more difficult to achieve. However, the data obtained to date consistently show that the reactive centre loop of active serpins is not incorporated into the body of the molecule, but is in an exposed conformation. The conformation of the exposed reactive centre loop does not appear to be consistent amongst the inhibitory serpins that have been crystallized to date. While the reactive centre loop of antithrombin appears to maintain

β -strand characteristics (Carrell *et al.*, 1994), the reactive centre loop of α_1 -antichymotrypsin maintains a distorted α -helical conformation (Wei *et al.*, 1994). Most recently, the reactive centre loop of α_1 -proteinase inhibitor has been observed to maintain a canonical conformation similar to that of other non-serpin inhibitors (Elliot *et al.*, 1996).

From all of these structures, it is evident that the reactive centre loop is not stabilized in a manner similar to that of non-serpin inhibitors, which employ intramolecular stabilization. Rather, there is significant plasticity with respect to the conformation of the reactive centre loop that the serpin can adopt. In addition, with variable reactive centre loop conformations, the tight binding of serpins and proteases appears not to occur as a result of a preformed canonical reactive centre loop interacting with the active site of the protease. Instead, it is possible that inhibitory serpins and their target proteases interact through an “induced fit” model, which relies on minor modifications of conformation between the two molecules for efficient interaction. The data obtained from serpin protease binding indicate that, although the precise mechanism is unclear, these molecules interact in a manner that is distinct from the tight-binding observed between a protease and the canonical reactive centre loop of standard mechanism inhibitors.

The critical step in the ability of a serpin to inhibit its target protease is the insertion of the reactive centre loop into the A β -sheet after the serpin-protease interaction. Crystal structure and biophysical analyses of latent and cleaved serpins indicate that insertion of the reactive centre loop into the A β -sheet causes the serpin to

maintain a more thermodynamically favourable conformation, indicating that when the reactive centre loop is in an exposed conformation, the serpin maintains a stressed, or metastable conformation. Several studies support the notion that the reactive centre loop of inhibitory serpins must be presented in this thermodynamically unfavourable state and then insert into the β -sheet, in order to be able to inhibit its target protease. Plakalbumin, a cleaved form of the non-inhibitory serpin ovalbumin, does not demonstrate insertion of the reactive centre loop into the β -sheet (Wright *et al.*, 1990), which supports the hypothesis that insertion of the reactive centre loop is a property unique to inhibitory serpins and is required for their inhibitory abilities. In addition, mutants of the hinge region of inhibitory serpins, corresponding to residues P15 to P10 in the reactive centre loop, convert serpins from being inhibitors of their target proteases to substrates (Holmes *et al.*, 1987; Hopkins *et al.*, 1993; Hood *et al.*, 1994; Hopkins *et al.*, 1995). As shown in the crystal structure of AT, the hinge region corresponds to the N-terminal region of the reactive centre loop that partially inserts into the underlying A β -sheet and allows the reactive centre loop to maintain its metastable conformation in the active protein (Carrell *et al.*, 1994). The hinge region of inhibitory serpins is highly conserved, especially at P12 where an alanine residue exists, without exception, in all inhibitory serpins. The substitution in Antithrombin-Hamilton, a naturally occurring AT variant where threonine has been substituted for alanine at P12, causes the thrombin-inhibitory serpin to become a substrate for thrombin (Austin *et al.*, 1991b). In addition, several non-inhibitory serpins do not contain alanine at this position.

Peptide studies have also demonstrated the necessity of reactive centre loop insertion for serpin function. Peptides corresponding to reactive centre loop residues P14 to P1 in AT (Björk *et al.*, 1992; Björk *et al.*, 1992b) and α_1 -proteinase inhibitor (Schulze *et al.*, 1990) have been shown to compete with the reactive centre loop of inhibitory serpins for insertion into the A β -sheet. Competitive insertion of the reactive centre loop peptides resulted in the conversion of the analyzed serpins from being inhibitors to becoming substrates of their target proteases, and further demonstrates that loop insertion is a requirement for the inhibitory ability of serpins.

In contrast to standard mechanism inhibitors, the interaction of the protease active site serine with the reactive centre loop of serpins is crucial in forming an inhibitory serpin-enzyme complex. Binding kinetics of serpin-enzyme interactions have demonstrated tight binding between the two proteins as determined by dissociation constants of less than 1 nM. Conversely, when the serpins AT, α_1 -proteinase inhibitor and PAI-1 were incubated with anhydrotrypsin, a chemically-modified trypsin molecule with an inactive serine in the active site, the dissociation constants were increased 10000-fold (Olson *et al.*, 1995). Some studies have shown that the interaction of the reactive centre loop of serpins with the active site serine of the protease contributes up to 50% of the binding energy of the serpin-enzyme complex. However, the contribution of the serine residue varies for different serpin-protease pairs (Stone and Le Bonniec, 1996). This suggests that the complementarity of the entire reactive centre loop to the binding pocket of the serine protease may also make a significant contribution to the binding energy of the serpin-enzyme complex.

Subsequent to the interaction of the reactive centre loop with the active site serine in a Michaelis complex, the serpin is cleaved at the active site to yield a serpin which is covalently bound to the protease via the serine residue in the protease and the P1 residue in the serpin (Engh *et al.*, 1995). Unlike standard mechanism inhibitors, the formation of Michaelis complexes between serpins and their target proteases is insufficient to inhibit the enzyme (Stone *et al.*, 1997). The generation of the covalent association between serpin and protease supports this hypothesis, and is believed to occur through the formation of a tetrahedral and then an acyl intermediate (Engh *et al.*, 1995). NMR studies of an elastase- α 1-proteinase inhibitor complex suggest that the complex exists as a tetrahedral intermediate (Matheson *et al.*, 1991). Other studies have shown that the α -amino groups of the P1' residue are available for chemical modification, suggesting that cleavage of the reactive site P1-P1' bond occurs through the formation of an acyl intermediate (Lawrence *et al.*, 1995; Wilczynska *et al.*, 1995). In both of these reaction intermediates, an active site serine covalent bond is involved in the process, which demonstrates that a Michaelis complex does not account for the tight-binding characteristics between serpin and protease. Furthermore, evidence shows that the cleaved bond of the reactive centre loop of serpins are separated by 70 Å when the reactive centre loop is inserted into the A β -sheet, which indicates that resynthesis of the cleaved bond in the serpin is impossible (Loebermann *et al.*, 1984; Stratikos and Gettins, 1999). Thus, the reaction of serpin and protease appears irreversible. The irreversibility of the serpin-protease interaction is another difference between non-serpin and serpin inhibitory mechanisms.

The rate of insertion of the reactive centre loop of a serpin complexed with its protease is believed to dictate whether or not the serpin follows the “inhibitor” or “substrate” branch of the serpin-enzyme reaction. It has been suggested that the insertion of the reactive centre loop protects the acyl-enzyme bond from hydrolysis by nucleophilic attack of water, but the rate of the insertion is important in determining if hydrolysis occurs (Wright and Scarsdale, 1995). If loop insertion proceeds slowly, then deacylation is believed to occur, resulting in cleavage of the serpin with a resultant substrate reaction. Appropriate, rapid insertion of the reactive centre loop would ensure that the serpin-enzyme complex is not dissociated before the formation of a thermodynamically favourable complex.

However, very recent data suggest that insertion of the reactive centre loop occurs before acylation (Mellet and Bieth, 2000). Fluorescently labeled elastase and α_1 -proteinase inhibitor were incubated, and the kinetics of Michaelis complex decay in addition to rates of elastase inhibition were determined over a series of pH ranges. The investigators determined that both reactions occurred at similar rates and that the rates were independent of pH. Since acylation is pH dependent, they suggested that insertion of the reactive centre loop into an inhibitory conformation occurred before acylation and further postulated that acylation was responsible for stabilizing the inhibitory complex in an irreversible conformation.

1.3.2 Tertiary Structure of the Serpin-Enzyme Complex

As previously mentioned, no crystal structures have been elucidated to determine the structure of the serpin-enzyme complex. However, molecular modeling, using crystal

structure data from various serpin and protease structures, has been used to predict the structures for elastase- α_1 -proteinase inhibitor and TAT complexes (Wright and Scarsdale, 1995; Schreuder *et al.*, 1994). The predicted elastase- α_1 -proteinase inhibitor complex structure suggests that elastase is flipped to the opposite pole of α_1 -proteinase inhibitor, which supports the hypothesis that the reactive centre loop must be inserted into the A β -sheet in an inhibitory complex. Another group, however, performed similar modeling experiments of AT and α_1 -proteinase inhibitor complexed with trypsin, elastase and thrombin, and predicted a different serpin-enzyme complex (Whisstock *et al.*, 1996). They established criteria that the serpin reactive centre loop be partially-inserted into the β -sheet at residue 12 prior to reaction with its protease, and also that the reactive centre loop between P3 and P3' maintain a canonical conformation similar to that seen with non-serpin inhibitors. Their modeling experiments demonstrated partial insertion of the reactive centre loop into the A β -sheet, but that the protease was flipped not to the opposite side of the serpin, but to the side.

More recently, Stratikos and Gettins (1998) provided biochemical evidence that supported the hypothesis that the protease is flipped to the opposite pole of the serpin during inhibitory-complex formation. They employed α_1 -proteinase inhibitor Pittsburgh, a variant of α_1 -proteinase inhibitor that gains thrombin-inhibitory ability, to create serpins with single cysteine mutations in order to use fluorescence energy transfer measurements to determine the orientation of the serpin-enzyme complex. Specifically, they analyzed the contributions of individually labeled cysteine residues in five α_1 -proteinase inhibitor Pittsburgh variants with respect to fluorescence transfer to tetramethylrhodamine-labeled

trypsin. Detection of fluorescence transfer relies on the fluorescence donor and acceptor being in close proximity to one another. Their study found that a cysteine substitution at residue 121 in the serpin demonstrated the highest fluorescent energy transfer of all the mutants analyzed. In conjunction with the crystal structure obtained for cleaved α_1 -proteinase inhibitor, they were able to determine that the fluorescence energy transfer could only occur efficiently at this residue if the protease was flipped to the opposite side of the serpin, after a covalent complex had been formed between the serpin and protease. The same authors were able to further substantiate their complex conformation hypothesis by repeating the study using several cysteine substitutions in α_1 -proteinase inhibitor Pittsburgh and measuring the fluorescence energy transfer with trypsin, by relying on the intrinsic fluorescence of tryptophan residues present in the protease. This study addressed the possibility that manipulation of trypsin with fluorescent markers might yield non-physiological information about the serpin-enzyme complex (Stratikos and Gettins, 1999). Furthermore, this study demonstrated that an earlier modeling study, which proposed that thrombin was flipped to the side of the serpin (Wilczynska *et al.*, 1995), was incorrect. However, consolidation of clinical and biochemical data of the interactions of the serpin-enzyme complex will only be completely accurate once a serpin-enzyme complex is crystallized and its true structure known.

1.4 Biochemistry of Antithrombin

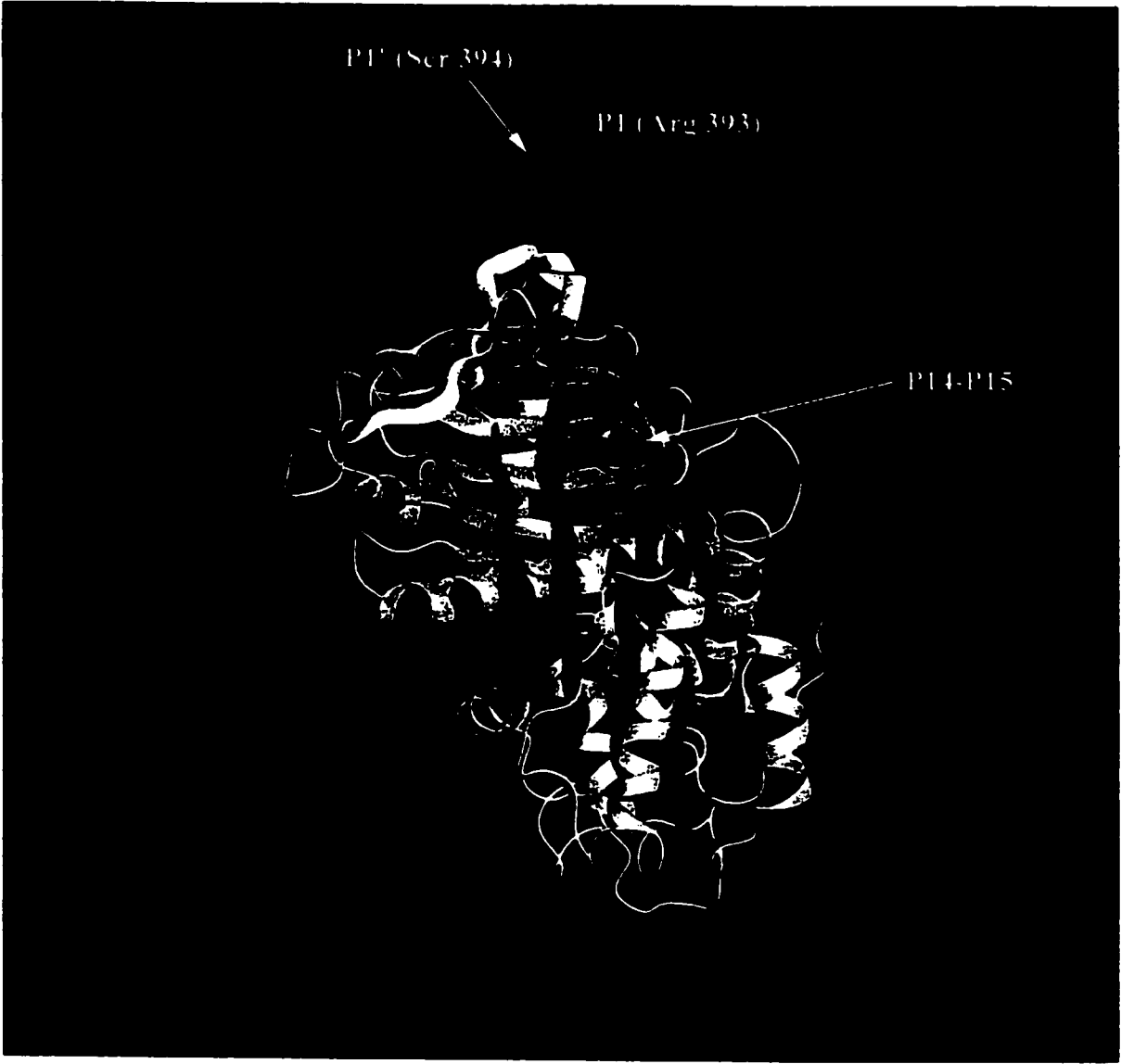
1.4.1 Structure of Antithrombin

Human AT is a 58 kDa single chain glycoprotein (Rosenburg and Damus, 1983; Peterson *et al.*, 1979) consisting of 432 amino acids (Bock *et al.*, 1982) that is produced

and secreted from the liver (Leon *et al.*, 1983), and found circulating at 2-3 μM in plasma (Chan *et al.*, 1979). There are six cysteine residues in the molecule, which contribute to the formation of three disulfide bonds between residues 8 and 128, 21 and 95, and 247 and 430 in the functional protein (Petersen *et al.*, 1979; Sun and Chang, 1989). AT has four sites of N-linked glycosylation on asparagine residues at positions 96, 135, 155 and 192 (Franzén *et al.*, 1980; Mizuochi *et al.*, 1980). While glycosylation always occurs at residues 96, 135, 155 and 192 in the major isoform of AT (α -antithrombin), approximately 10% of AT in plasma is not glycosylated at residue 135 (Carlson and Atencio, 1982; Peterson and Blackburn, 1985; Brennan *et al.*, 1987). This isoform of antithrombin, called β -antithrombin, migrates with slightly greater mobility on SDS-PAGE than the fully glycosylated protein. In addition, β -antithrombin has been shown to have a higher affinity for heparin binding than α -antithrombin (Brennan *et al.*, 1987).

A three-compartment model has been used to describe the *in vivo* metabolism of AT in both humans (Knot *et al.*, 1986) and rabbits (Carlson *et al.*, 1984). The half-life of AT has been found to be approximately 2.8 and 2.3 days in humans and rabbits, respectively (Collen *et al.*, 1977; Knot *et al.*, 1986; Vogel *et al.*, 1979). The half-life of AT appears to be unaffected in individuals with AT deficiency (Menache *et al.*, 1990).

The tertiary structure of AT was initially deduced by analogy to the three-dimensional crystal structure of α_1 -proteinase inhibitor (Loeberman *et al.*, 1984; Carrell *et al.*, 1987a,b) and by peptide modeling of the amino acid sequence of the protein (Whisstock *et al.*, 1996) (Figure 2). These analyses suggested that AT is made up of 31% α -helix, 16% β -sheet, 9% β -turn and 44% random coil. Subsequently, x-ray



crystallographic data has been obtained for the structures of bovine AT (Mourey *et al.*, 1990), a human AT dimer (Schreuder *et al.*, 1994; Carrell *et al.*, 1994;), and a human AT/heparin-derived pentasaccharide dimeric complex (Carrell *et al.*, 1997), all of which support the previous modeling data on the structure of AT. Based upon these recent structural analyses of various AT preparations, the three-dimensional structure of AT is believed to have 9 α -helices, designated from A to I, and three β -sheets, designated A to C.

1.4.2 Molecular Biology of Antithrombin

The human AT complementary DNA (cDNA) was isolated and characterized from liver cDNA libraries in several laboratories (Bock *et al.*, 1982; Prochownick *et al.*, 1983; Stackhouse *et al.*, 1983). The human AT cDNA encodes an open reading frame of 1392 nucleotides, followed by an 84 base pair (bp) untranslated region. In addition, the reading frame is preceded by 70 untranslated nucleotides. From this cDNA, the primary structure of the mature AT protein was deduced. The primary protein structure of human AT consists of a 32-residue secretory signal sequence followed by a mature plasma protein of 432 amino acids. The human AT cDNA was used to isolate the gene encoding AT. The human AT gene is located on chromosome 1, and consists of seven exons and six introns spanning 13480 bp from the transcriptional start site to the last nucleotide in the polyadenylation signal (Kao *et al.*, 1984; Bock *et al.*, 1985; Olds *et al.*, 1993). In addition to the human molecule, molecular cloning has elucidated the rabbit (Sheffield *et al.*, 1992), mouse (Wu *et al.*, 1992), sheep (Niessen *et al.*, 1992) and chicken AT (Tejada and Deeley, 1995) cDNAs. The rabbit AT cDNA is 1359 bp encoding a mature protein

of 433 amino acids, while the mouse cDNA is 1398 bp in length encoding a 433 residue protein. The sequence of the bovine and porcine AT proteins was determined by protein sequencing (Petersen, *et al.*, 1979; Tokunaga *et al.*, 1994).

1.4.3 Species Homology of AT Between Species

The primary amino acid sequences for human, rabbit, murine, sheep, and chicken AT have been elucidated from the cDNAs encoding these proteins, while the AT protein sequence from bovine and porcine species (Medjoub *et al.*, 1991; Tokunaga *et al.*, 1994) has been determined by amino acid sequencing. Compared to the human molecule, other mammalian AT have 84 to 89% amino acid sequence identity (Sheffield *et al.*, 1996), while chicken AT has 69% identity to human AT (Tejada and Deeley, 1995). Rabbit AT, like human AT, exists in two isoforms in plasma: 90% α -AT and 10% β -AT, which differ in glycosylation at Asn 135 (Carlson and Atencio, 1982; Brennan *et al.*, 1987). Interestingly, the six cysteine residues that all contribute to disulfide bond formations are conserved amongst all antithrombins identified to date, indicating the importance of these residues in maintaining the function of the molecule.

1.4.4 The Physiological Function of AT

AT functions to inhibit various serine proteases of the coagulation cascade by forming an irreversible 1:1 stoichiometric complex with its target. While AT is the principal natural inhibitor of thrombin in plasma, it also inhibits the other serine proteases in the coagulation cascade, including factors VIIa, IXa, Xa, XIa, XIIa as well as plasmin and trypsin. Despite the ability of AT to inhibit several proteases, its ability to inhibit thrombin and factor Xa is believed to be the most physiologically relevant. While the

reaction of AT with thrombin and factor Xa occurs slowly in the absence of heparin, the inhibitory reaction is accelerated several thousand-fold when the glycosaminoglycan is present (Olson *et al.*, 1992). This characteristic of heparin is thought to be responsible for its widespread clinical use as an anticoagulant and antithrombotic agent (Hirsh, 1991).

Individuals who are deficient in AT have been shown to be susceptible to thrombosis (Egeberg, 1965; Thaler and Lechner, 1981). Many instances of genetic AT deficiency have been documented, and inherited AT deficiency is usually observed as an autosomal dominant genetic disorder (Lee and Blajchman, 1995). The prevalence of AT deficiency has been reported to be approximately 2 cases per 1000 persons in the general population (Wells *et al.*, 1994). AT deficiency usually presents as venous thrombosis that can occur at an early age, although most thrombotic episodes occur in individuals between 15 and 40 years of age (Hirsh *et al.*, 1989). In addition, persons with AT deficiency tend to have a family history of venous thrombosis (Lee and Blajchman, 1995). AT deficiency has been classified into two categories. Type I AT deficiency describes conditions where AT circulates at levels approximately half of those seen in normal individuals. The defects associated with type I AT deficiency are usually associated with insertions (Olds *et al.*, 1990), deletions (Bock and Prochownik, 1987; Fernandez-Rachubinski *et al.*, 1992) or frameshift mutations (Gandrille *et al.*, 1991) within AT genes, that result in premature terminations in the protein. Type II AT deficiency describes individuals who have normal antigenic levels of AT, but reduced functional levels (Lee and Blajchman, 1995). Type II AT deficiencies are generally divided into three categories, namely mutations that cause defects in heparin binding,

defects in thrombin binding, and pleiotropic mutations. All type II AT variants identified to date have resulted from missense mutations, resulting in the substitution of amino acids at critical residues (Lee and Blajchman, 1995). Homozygous AT deficiency has only been described in one instance, in identical consanguinous twins who died within one month of birth (Hakten *et al.*, 1989), a pattern which suggests that the complete loss of AT is incompatible with life.

1.4.4.1 Interaction of AT with α -Thrombin

AT inhibits thrombin by forming an irreversible 1:1 molar complex with thrombin through an interaction between the reactive centre of AT and the active site of thrombin (Rosenberg and Damus, 1973). AT inhibits thrombin by providing an ideal substrate to the protease on an exposed region of the molecule called the reactive centre loop. Specifically, the reactive centre loop, which includes the Arg393-Ser394 scissile bond, is involved in reacting with thrombin, and initiating the inhibitory mechanism of AT (Jörnvall *et al.*, 1979; Björk *et al.*, 1981; Björk *et al.*, 1982b) (discussed further in section 1.4.2). The active site residues, Arg393 and Ser394, are also known as P1 and P1', respectively, using the nomenclature of Schechter and Berger (1967). In this labeling system, the reactive centre is designated P1-P1', with P1 (and Pn) being amino terminal to the scissile bond and P1' (and Pn') being carboxy-terminal to this bond (with n representing the number of amino acids away from the reactive centre). Complex formation between AT and thrombin is initiated when thrombin cleaves the P1-P1' bond of AT (Björk *et al.*, 1982).

After this initial cleavage occurs, an acyl ester bond forms between the carboxyl group of Arg393 of AT and the hydroxyl group of the serine residue present in the active site of thrombin (Fish and Björk; 1979; Cooperman *et al.*, 1993; Olson *et al.*, 1995). In order to successfully inhibit thrombin, however, AT must undergo a major conformational change, whereby the reactive centre loop inserts into the underlying A β -sheet, in order to prevent hydrolysis of the ester bond between AT and thrombin (discussed in more detail in section 1.4.2). If this conformational change in AT does not occur in a timely fashion, the covalent interaction between AT and thrombin is broken before the reactive centre loop inserts into the body of the protein, and AT becomes a substrate of thrombin (Caso *et al.*, 1991; Stein and Carrell, 1995). When this occurs, AT is cleaved and inactivated by thrombin, leaving an active thrombin molecule capable of further activity. The covalent nature of the AT/protease interaction is supported by the observation that AT reacts minimally with active site mutants of thrombin, or anhydrotrypsin, a trypsin derivative that contains a non-functional active site (Olson *et al.*, 1995). In addition, the inhibitory TAT complex is resistant to denaturation, as evidenced by its stability to both SDS and high temperatures.

The importance of the residues at the reactive centre has been demonstrated by analyses of naturally occurring and engineered reactive site mutants. While some plasticity in residue substitution is observed at P1' (Stephens *et al.*, 1987; Stephens *et al.*, 1988; Olson *et al.*, 1995b), there appears to be a strict requirement for the presence of arginine at P1 (Erdjument *et al.*, 1988; Owen *et al.*, 1988; Lane *et al.*, 1989). Other mutations within the reactive centre loop also affect the thrombin-inhibitory activity of

AT. Different substitutions of amino acids at P2 can impair or enhance AT function (Sheffield and Blajchman, 1994; Chuang *et al.*, 1999), while some other mutations within the reactive centre loop impair the inhibitory activity of AT. Mutations that restrict the mobility of the reactive centre loop have been shown to affect severely AT function (Carrell and Evans, 1992). In addition, mutations occurring outside of the reactive centre loop have also been shown to contribute to impaired thrombin-inhibitory activity of AT (Perry, 1994), demonstrating that a strong relationship exists between the structure and function of AT.

The interaction of AT and thrombin has been extensively studied both *in vivo* and *in vitro*. It has been discovered, *in vitro*, that various reaction conditions can affect the ability of AT to inhibit thrombin. When AT is reacted with thrombin under conditions whereby thrombin is in molar excess, AT proteolysis and cleavage at the reactive centre occurs (Björk, *et al.*, 1981). Excess thrombin that is present in the reaction and does not participate in any interaction with AT is able to proteolyze AT or TAT complexes. In addition, under conditions of low ionic strength, AT is more susceptible to participating in a substrate reaction with thrombin (Olson, 1985).

The *in vivo* interaction between AT and thrombin has also been extensively studied. After AT interacts with thrombin to form TAT, the complex interacts with vitronectin, a 78 kDa glycoprotein that circulates in plasma (Tomasini and Mosher, 1986). The interaction of vitronectin with TAT complexes has recently been shown to be facilitated by platelet protein disulfide isomerase (Essex *et al.*, 1999), an enzyme that catalyzes the formation of disulfide bonds between vitronectin and TAT. Vitronectin has

been shown to stabilize the TAT complex (Podack *et al.*, 1986; Preissner and Muller-Berghaus, 1987), an interaction which is believed to facilitate the rapid clearance of TAT complexes from the circulation before the complex can be degraded. The formation of a ternary complex between AT, thrombin and vitronectin occurs through disulfide bond formation between thrombin and vitronectin, as shown by the failure of vitronectin to bind to TAT in the presence of N-ethylmaleimide, a thiol-alkylating compound (Ill and Ruoslahti, 1985). The vitronectin-TAT complex is cleared from the circulation via binding to a receptor on the endothelial cell surface. The nature of this receptor that is responsible for TAT complex clearance is still not known, but studies have suggested that several receptors may be responsible for complex removal from the circulation. Examples of receptors with TAT-binding ability include the serpin-enzyme complex receptor (SECR) (Perlmutter *et al.*, 1990), low density lipoprotein receptor-related protein (LRP) (Strickland *et al.*, 1995), and cytokeratin 18 (Wells *et al.*, 1997). Despite the fact that the receptor (or receptors) responsible for TAT clearance have not been conclusively elucidated, it is known that TAT complexes are cleared from the circulation faster than their constituent proteins (Pizzo, 1989).

1.4.4.2 Acceleration of AT Activity by Heparin

Heparin is a highly sulfated, negatively charged polymer of alternating hexuronic (D-glucuronic or L-iduronic) acid and D-glucosamine residues. Variable sulfation results in extensive structural heterogeneity of this polymer (Lane and Lindahl, 1989; Lane *et al.*, 1992). A similar glycosaminoglycan, heparan sulfate, varying primarily in the extent of sulfation, is found on the surface of most cells, including endothelial cells lining blood

vessels (Lane and Lindahl, 1989; Lane *et al.*, 1992). Heparin greatly accelerates the inhibition by AT of target proteases. This property of heparin is what has made it such a useful clinical anticoagulant and antithrombotic drug (Hirsh, 1991). In the absence of heparin, AT inhibits thrombin at a rate several thousand-fold lower than when it is present (Jordan *et al.*, 1980; Olson and Björk, 1991).

A subfraction of approximately one third of heparin preparations binds with high affinity to AT (Lam *et al.*, 1976). The subfraction of heparin that binds to AT contains a specific pentasaccharide sequence and binds to a specific site on AT (Casu *et al.*, 1981; Atha *et al.*, 1984). The heparin-binding site on AT consists of several positively charged residues in helices A and D. The residues that have been shown, by site-directed mutagenesis or chemical modification, to be important in heparin binding in AT are Arg-24, Lys-107, Lys-114, Lys-136 and Arg-145, which have all been localized to the A/D helix region (Liu and Chang, 1987; Chang, 1989; Sun and Chang, 1990; Lane *et al.*, 1993;). More recently, other residues in the A/D helix region have also been shown to contribute to heparin binding, including Arg-46, Arg-47, Arg-129, Arg-132 and Lys-133 (Meagher *et al.*, 1996; Ersdal-Badju *et al.*, 1997; Arocas *et al.*, 1999; Desai *et al.*, 2000; Meagher *et al.*, 2000).

Heparin exerts its pharmacological effects by binding to AT and exerting its effects by one of two possible mechanisms. In the inhibition of thrombin by AT, heparin acts via a bridging mechanism to bring AT and thrombin into approximation with each other, where AT can inhibit the protease. For this process to be efficient, a long chain of heparin, with at least 18 monosaccharide units containing the high affinity pentasaccharide

sequence is required for thrombin inhibition (Choay *et al.*, 1983; Atha *et al.*, 1984). In the case of factor Xa inhibition, the pentasaccharide sequence binds to AT and causes a conformational change in the reactive centre loop, enhancing the interaction, and inhibition, of factor Xa by AT (Lane *et al.*, 1984; Olson *et al.*, 1992). Inhibition of factor Xa by AT is minimally affected by the bridging mechanism of heparin. The heparin-catalyzed inhibition of factor Xa by AT is significantly decreased under conditions of increased ionic strength, which demonstrates the importance of ionic interactions between AT and heparin (Nordenman and Björk, 1981).

The precise conformational change of AT that is induced by heparin is still controversial. Crystal structures of AT that have recently been elucidated have provided some clues as to how this process occurs. The positively charged heparin-binding residues located on helices A and D are spatially located just below the A β -sheet (Schreuder *et al.*, 1994; Carrell *et al.*, 1994). These crystal structures also demonstrate that there is partial insertion of the reactive centre loop into the A β -sheet. These observations are consistent with a proposed model of heparin activation of AT. This model proposes that heparin binding to the A/D helices removes a source of positive electrostatic charge, which causes the reactive centre loop to be expelled from the underlying A β -sheet, thereby altering AT into a more appropriate inhibitory conformation (van Boekel *et al.*, 1994). Fluorescence studies that show increased tryptophan fluorescence, in a P14 serine to tryptophan AT variant, after heparin binding, indicate the exposure of a previously “hidden” tryptophan residue, presumably hidden by its insertion into the A β -sheet (Huntington *et al.*, 1996). In addition, the creation of the

serine to glutamate substitution at P14 of AT increases the basal progressive rate of factor Xa inhibition to that observed in the presence of pentasaccharide while leaving anti-thrombin activity unaffected (Futamura and Gettins, 2000). Fluorescence studies also confirm that this P14 mutation expels this region of the reactive centre loop from the β -sheet in the absence of pentasaccharide.

1.5 Biochemistry of Heparin Cofactor II

1.5.1 Structure of Heparin Cofactor II

Human HCII is a 66.5 kDa single chain glycoprotein that is made up of 480 amino acids (Tollefsen, *et al.*, 1982; Ragg, 1986; Blinder *et al.*, 1988). Like most other proteins associated with coagulation, HCII is produced in, and secreted from the liver (Tollefsen and Pestka, 1985). HCII circulates in the plasma at approximately 1.2 μ M (Tollefsen and Pestka, 1985), a level approximately 3-fold lower than that observed with AT. There are three cysteine residues in HCII, but disulfide bonds are not formed in the mature protein (Church *et al.*, 1987). HCII is glycosylated at 3 sites: Asn-30, Asn-169, and Asn-368 and the carbohydrate moieties contribute approximately 10% of the mass of the plasma-derived protein (Tollefsen *et al.*, 1982; Kim *et al.*, 1988). In addition, two tyrosine residues near the N-terminus are sulfated during hepatic biosynthesis (Horton *et al.*, 1986). HCII is approximately 30% identical to AT, as well as other serpins, with respect to the primary amino acid sequence (Church *et al.*, 1995). The highest homology is observed at the C-terminus of HCII. Interestingly, the first 80 N-terminal residues in HCII do not share any homology with any other known serpins. The N-terminus of HCII consists of two repeated acidic domains that are highly homologous to those found in

hirudin (Church *et al.*, 1995), a specific inhibitor of thrombin that is produced in the salivary glands of the medicinal leech.

Several studies have been conducted in several species to analyze the *in vivo* metabolism of HCII. A three-compartment model was used to determine that the half life of HCII is approximately 1.6 and 2.2 days in rabbits and baboons, respectively (Hatton *et al.*, 1997; Sié *et al.*, 1986). A similar metabolism study was completed using a two-compartment model to determine that the half life of HCII is approximately 2.5 days in humans (Sié *et al.*, 1985b). Interestingly, Hatton *et al.*, (1997) have shown that HCII accumulates at an arterial site of vascular injury in rabbits less rapidly than that observed for AT, and that AT is a more active inhibitor of thrombin at the injured blood vessel wall. These authors suggest that HCII may play a minor role in thrombin inhibition given its lower activity compared to AT in both intact and de-endothelialized vessels.

The three-dimensional crystal structure of HCII has not yet been determined, but significant information has been gathered regarding its structure. The reactive site of HCII is Leu444-Ser445 (Griffith *et al.*, 1985). The presence of leucine at P1 is unusual in that all other thrombin-inhibitory serpins contain a P1 arginine, but it explains why HCII, in addition to inhibiting thrombin, also inhibits chymotrypsin and cathepsin G (Church *et al.*, 1985; Pratt *et al.*, 1990). Furthermore, HCII does not inhibit any of the other trypsin-like serine proteases in the coagulation cascade (Parker and Tollefsen, 1985). Creation of a P1 substitution of arginine for leucine resulted in an HCII molecule which gained the ability to inhibit other serine proteases involved in coagulation, but one that also lost the ability to inhibit chymotrypsin and cathepsin G (Derechin *et al.*, 1990).

HCII, like AT, also binds heparin and heparan sulfate, but with a lower affinity than AT (Tollefsen *et al.*, 1982; Tollefsen *et al.*, 1983). In addition, and in contrast to AT, HCII binds to dermatan sulfate and its activity is accelerated by this glycosaminoglycan similarly to that observed with heparin (Tollefsen *et al.*, 1983). Based on its homology to AT, the heparin-binding site on HCII was predicted and identified. A positively charged region in helix D has been identified as the heparin and dermatan sulfate binding sites, however, mutagenesis studies of this region have determined that the binding sites of these two glycosaminoglycans, although not identical, are overlapping (Blinder and Tollefsen, 1990; Ragg *et al.*, 1990; Whinna *et al.*, 1991).

1.5.2 Molecular Biology of Heparin Cofactor II

The human HCII cDNA was isolated and characterized from a liver cDNA library (Ragg, 1986; Inhorn and Tollefsen, 1986). The human HCII cDNA encodes an open reading frame of 1525 nucleotides, followed by a 654 bp untranslated region. From this cDNA, the primary structure of mature HCII was deduced (Blinder *et al.*, 1988). The primary protein structure of human HCII consists of 480 amino acids, plus a 19-residue secretory signal sequence. Recombinant HCII has been successfully expressed in *Escherichia coli* (Blinder *et al.*, 1988), mammalian and insect cell culture (Ragg *et al.*, 1990; Ciaccia *et al.*, 1995), as well as in a cell-free expression system employing mRNA-dependent rabbit reticulocyte lysate (Sheffield *et al.*, 1994c). The human HCII cDNA was used to isolate the gene encoding HCII. The human HCII gene has been localized to chromosome 22 and mapped to 22q11 (Herzog *et al.*, 1991). The gene is approximately 16000 bp in length and contains five exons and four introns in addition to 1749 bp and

476 bp untranslated regions at the 5' and 3' ends of the gene, respectively (Herzog *et al.*, 1991).

1.5.3 Species Homology of Heparin Cofactor II Between Species

The primary amino acid sequences for human, rabbit, mouse, rat, dog, frog, and chicken HCII have been elucidated from the cDNAs encoding these proteins (Blinder *et al.*, 1988; Zhang *et al.*, 1994; Westrup and Ragg, 1994; Sheffield *et al.*, 1994c; Colwell and Tollefsen, 1998). Compared to the human molecule, murine, rat and rabbit HCII are approximately 85% identical with respect to amino acid sequence. The frog and chicken cDNA sequences of HCII are approximately 60% analogous to their mammalian counterparts, and indicate that the HCII structure has been subjected to substantial evolutionary conservation. The regions of HCII that show the highest conservation amongst species are the glycosaminoglycan-binding site, the reactive site, and the N-terminal acidic domain. The high degree of conservation is suggestive that the physiological function of HCII is similar in the different species.

1.5.4 The Physiological Function of Heparin Cofactor II

HCII inhibits thrombin *in vivo* (Andersson *et al.*, 1992; Liu *et al.*, 1995), as demonstrated by the presence of HCII-thrombin complexes in the circulation. However, cultured endothelial cells do not accelerate the thrombin-inhibitory activity of HCII, whereas cultured fibroblasts and vascular smooth muscle cells do increase HCII activity (McGuire and Tollefsen, 1987; Hiramoto and Cunningham, 1988). In addition, individuals with AT deficiency are susceptible to thrombosis despite normal levels of HCII in plasma (Griffith *et al.*, 1983). This had led investigators to wonder if HCII has a

significant intravascular role in regulating coagulation. It has also been shown that fibroblasts synthesize both heparan sulfate and dermatan sulfate (Tollefsen, 1997), suggesting that HCII might have a physiological function in the extravascular space.

HCII is found circulating in plasma at levels approximately 33% of those seen with AT (2.3 μ M versus 1.2 μ M, for AT and HCII, respectively). Individuals have been described who have a heterozygous deficiency in HCII expression but their susceptibilities to thrombosis appear to be no greater than that observed in the general population (Sié *et al.*, 1985; Tran *et al.*, 1985; Weisdorf and Edson, 1991; Matsuo *et al.*, 1992). Kindreds have been described in which HCII deficiency correlated with thrombotic disease, but whether or not it was causal could not be determined (Tran *et al.*, 1985; Sie *et al.*, 1985; Simioni *et al.*, 1990). Interestingly, no homozygous HCII-deficient individuals have been described. This observation could be a result of the low incidence of homozygous HCII deficiency in the general population, the possible incorrect assumption that a homozygous HCII-deficient individual would present with thrombosis, or that homozygous HCII deficiency is lethal *in utero* (Tollefsen, 1997). These observations suggest that the main physiological function of HCII may not be associated with maintenance of intravascular hemostasis, and the protein may have another role.

Indirect evidence exists, based on the detection of dermatan sulfate proteoglycans, to suggest that increased HCII activity is involved in maintaining maternal/fetal circulation across the placenta (Andrew *et al.*, 1992). Dermatan sulfate levels have been detected in both maternal and fetal circulations that increase HCII activity. The placenta

has also been shown to be a rich source of dermatan sulfate, and it has been suggested that the placenta is the source of the glycosaminoglycan detectable in both mother and fetus (Brennan *et al.*, 1984). Moreover, HCII levels, as well as HCII-thrombin complexes are reportedly increased in pregnant women further suggesting a role of HCII in maintaining pregnancy (Massouh *et al.*, 1989).

In addition to being an integral protein in the coagulation cascade, thrombin has other functions that are independent of coagulation (Coughlin *et al.*, 1992). Thrombin is involved in several physiological activities including promoting fibroblast proliferation, inducing monocyte chemotaxis, and promoting neutrophil adhesion to the endothelium. It is possible that HCII-mediated inhibition of thrombin may be more important in these processes associated with inflammation or wound repair, especially when they occur in the extravascular space where dermatan sulfate is present and able to accelerate HCII activity. In addition, HCII may itself be involved in regulating the inflammatory response to injury. Cleavage of HCII by elastase or cathepsin G from activated neutrophils produces a 66 amino acid peptide, corresponding to the N-terminus of HCII, that displays leukocyte chemotactic activity (Pratt *et al.*, 1990). It is possible that HCII recruits leukocytes to sites of vascular injury, thus contributing to the regulation of inflammation and wound repair.

1.5.4.1 Interaction of Heparin Cofactor II with α -Thrombin

Like AT, HCII inhibits thrombin by forming an irreversible 1:1 molar complex by an interaction between an ideal substrate on the reactive centre loop of HCII and the active site of thrombin (Church *et al.*, 1995a). This inhibitory complex, like the TAT

complex, is resistant to denaturation by either SDS or high temperatures, and in it, both thrombin and HCII are inactive. Inhibition of thrombin by HCII is believed to occur by a typical inhibitory serpin-protease reaction, although this opinion is based indirectly from determinations of inhibitor-serpin structures employing other thrombin-inhibitory serpins (Matheson *et al.*, 1991). The reactive centre of HCII, unlike the Arg393-Ser394 bond in AT, consists of a Leu444-Ser445 sequence (Ragg, 1986; Blinder *et al.*, 1988; Griffith *et al.*, 1985). This reactive centre bond is an unexpected substrate for thrombin, but is also responsible for the ability of HCII to inhibit chymotrypsin and cathepsin G. This has been shown by the substitution of arginine for leucine at the P1 residue (L444R) of human HCII (Derechin *et al.*, 1990). Although the L444R variant demonstrated enhanced thrombin-inhibitory activity, it lost the ability to inhibit both chymotrypsin and cathepsin G. In addition, α_1 -antichymotrypsin, another serpin with a Leu-Ser reactive site, is able to inhibit chymotrypsin, but interestingly, does not inhibit thrombin (Huber and Carrell, 1989). With respect to coagulation, HCII is unlike AT in that it is a specific inhibitor of thrombin and does not inhibit any other of the serine proteases in the coagulation cascade (Parker and Tollefsen, 1985). The N-terminal acidic domain of HCII is important in the heparin-catalyzed inhibition of thrombin and binds with high affinity to exosite I of thrombin (Ragg *et al.*, 1990; Van Deerlin and Tollefsen, 1991). The activity of HCII is accelerated by up to 1000-fold in the presence of heparin, heparan sulfate, dermatan sulfate (Tollefsen *et al.*, 1983) and several other polyanions (Pratt and Church, 1989; Church *et al.*, 1989; Church *et al.*, 1987; Scully *et al.*, 1986; Yamagishi *et al.*, 1984), and will be described in detail in the following section.

1.5.4.2 Acceleration of Heparin Cofactor II Activity by Glycosaminoglycans

The activity of HCII is increased approximately 1000-fold in the presence of heparin and dermatan sulfate (Tollefsen *et al.*, 1983). It has also been observed that HCII activity can be significantly increased in the presence of several other polyanions. HCII binds to heparin with an affinity that is approximately 10-fold lower than that of AT (Tollefsen *et al.*, 1982; Tollefsen *et al.*, 1983), although HCII does not require the presence of a specific pentasaccharide structure in heparin to be effective. Conversely, HCII binds to most heparin oligosaccharides that are four monosaccharide units or longer regardless of oligosaccharide composition (Maimone, 1990). However, heparin chains that are longer than twenty monosaccharide units in length are required in order to support maximal HCII acceleration of thrombin inhibition (Maimone and Tollefsen, 1988; Sié *et al.*, 1988; Bray *et al.*, 1989).

HCII is a unique thrombin-inhibitory serpin in that its activity is accelerated by dermatan sulfate, in addition to heparin. Dermatan sulfate is a similar molecule to heparin, consisting of repeating polymers of D-glucuronic or L-iduronic acid and N-acetyl-D-galactosamine (Conrad, 1989). It is still unclear if HCII acceleration by dermatan sulfate is dependent only on the charge density of the glycosaminoglycan or if specific sulfation at discrete sites on the polymer is required (Tollefsen, 1997). HCII binds with high affinity to a subpopulation of dermatan sulfate molecules containing at least six monosaccharide units (Maimone and Tollefsen, 1990). In addition and as mentioned previously, several other non-specific natural and synthetic polyanions also

show the ability to accelerate HCII activity but their possible physiological roles remain to be elucidated.

Like AT-mediated inhibition, thrombin inhibition by HCII appears to rely on a ternary complex mechanism for its heparin-accelerated reaction (Griffith, 1983). However, the contribution of a ternary complex between HCII, thrombin and glycosaminoglycan is not as extensive as that observed between AT, thrombin and heparin. As mentioned previously, the N-terminus of HCII contains two sulfated acidic domains that are highly homologous to hirudin, a natural anticoagulant with very high affinity for thrombin (Blinder *et al.*, 1988). Hirudin has been shown to bind with high affinity to exosite I of thrombin (Rydel *et al.*, 1990), and in addition, an N-terminal peptide of HCII, corresponding to residues 54 to 75, competitively inhibits hirudin binding to thrombin without blocking the active site, as evidenced by the ability of thrombin to hydrolyze tripeptide p-nitroanilide substrates (Horton *et al.*, 1989). Furthermore, mutant HCII molecules lacking the N-terminal acidic domains have been constructed and expressed which demonstrate the importance of the hirudin-like domain of HCII in the inhibition of thrombin. An N-terminal deletion mutant of HCII was constructed, lacking the first 67 amino acids, which had an approximately 100-fold reduction in thrombin-inhibitory activity in the presence of heparin (Van Deerlin and Tollefsen, 1991). In contrast, when this HCII truncation mutant was incubated with thrombin in the absence of heparin, its activity was essentially the same as that observed for the wild-type molecule. Deletion of both acidic domains by truncating the first 74 residues from HCII caused HCII to lose all of its heparin-catalyzed thrombin-inhibitory

activity, but it retained its progressive activity at levels similar to that seen with the wild-type protein (Van Deerlin and Tollefsen, 1991).

Experiments suggest that glycosaminoglycan binding to HCII liberates the hirudin-like N-terminus and that it is the binding of this region of HCII that is responsible for the heparin-catalyzed inhibition of thrombin. This is referred to as the proposed model of glycosaminoglycan acceleration of HCII-mediated thrombin activity. The N-terminus of HCII appears to bind to the glycosaminoglycan-binding site of HCII in the absence of heparin or dermatan sulfate. Mutagenesis of the acidic domains increases the affinity of HCII for heparin-Sepharose, which suggests that the N-terminus of HCII binds to this site (Ragg *et al.*, 1990a; Ragg *et al.*, 1990b; Van Deerlin and Tollefsen, 1991). In addition, altering the positively-charged glycosaminoglycan binding site on HCII to one made up of neutral amino acids produces an HCII molecule which demonstrates accelerated anti-thrombin activity in the absence of heparin or dermatan sulfate (Liaw *et al.*, 1999). This further suggests that the N-terminus of HCII binds to the glycosaminoglycan-binding site of HCII and that it is the competitive displacement of the acidic domains from this site by heparin or dermatan sulfate that liberates this thrombin-binding moiety of HCII from the body of the protein. Once the N-terminus of HCII is available for binding to exosite I of thrombin, a high affinity interaction between HCII and thrombin occurs (Tollefsen, 1997). It is believed that the interaction of the N-terminal acidic domains of HCII with exosite I of thrombin facilitates the formation of an HCII/thrombin complex by bringing the active site thrombin into approximation with the reactive centre of HCII. Further evidence to support this model is that binding of HCII to thrombin mutants with altered

or defective exosite I moieties results in normal rates of progressive thrombin inhibition, but 100-fold reductions in the heparin-catalyzed reaction (Van Deerlin and Tollefsen, 1991; Rogers *et al.*, 1992). Conversely, inhibition of these thrombin mutants by AT is unaffected, both in the absence and presence of heparin (Sheehan *et al.*, 1993).

When high concentrations of glycosaminoglycans are present, the rate of thrombin inhibition by HCII decreases (Tollefsen *et al.*, 1983), arguing for the ternary complex model of HCII-mediated thrombin inhibition. This model requires that thrombin and HCII bind to a single long glycosaminoglycan chain simultaneously for rapid thrombin inhibition (Griffith, 1983). When high concentrations of heparin or dermatan sulfate are present, thrombin and HCII are sequestered on separate glycosaminoglycan chains, and accelerated thrombin inhibition is obstructed. However, it has been shown that a high-affinity dermatan sulfate hexasaccharide can increase the rate of HCII-mediated inhibition of thrombin (Van Deerlin and Tollefsen, 1991) by 50- to 100-fold, suggesting that HCII can inhibit thrombin through a mechanism that is independent of the formation of a ternary complex, since the hexasaccharide would be unable to bind to both HCII and thrombin simultaneously. This effect is not observed in an HCII protein lacking the N-terminal acidic domain (Van Deerlin and Tollefsen, 1991), suggesting that the hexasaccharide merely liberates the N-terminus of HCII, facilitating its interaction with thrombin exosite I. As mentioned previously, however, maximal HCII inhibition of thrombin occurs with oligosaccharides of greater than 20 units in length, demonstrating the importance of the ternary complex in thrombin inhibition by HCII (Tollefsen *et al.*, 1986).

Liaw *et al.* (1999) showed that while heparin follows the ternary model of thrombin inhibition by HCII, dermatan sulfate appears to work through the bridging mechanism, and by another mechanism. Instead of exclusively forming ternary complexes with thrombin and HCII, dermatan sulfate also can induce a greater conformational change in HCII than that seen with heparin, as shown by a change in intrinsic fluorescence of the serpin, which may liberate the N-terminus of HCII and increase its ability of to bind to thrombin.

The binding of glycosaminoglycans to thrombin appears to contribute very little to its inhibition by HCII (Sheehan *et al.*, 1994). Thrombin mutants with altered exosite I domains and decreased glycosaminoglycan binding have minimal influence on the rate of progressive and glycosaminoglycan-catalyzed inhibition by HCII. In contrast, thrombin exosite II mutants decrease the rate of heparin-catalyzed inhibition by AT over 100-fold (Sheehan and Sadler, 1994). These observations demonstrate that AT and HCII interact with thrombin dissimilarly, in that the HCII/glycosaminoglycan complex interacts with thrombin exosite I, whereas the AT/heparin complex interacts with thrombin exosite II.

1.6 Biochemistry of Thrombin

Thrombin is a 36 kDa glycoprotein (Magnusson *et al.*, 1975) and is the central proteolytic enzyme in the coagulation cascade that culminates in the formation of a fibrin clot (Roberts and Tabares, 1995). Thrombin is made up of four distinct domains: the Gla domain (residues 1 to 40), a kringle1 domain (residues 41 to 155), a kringle 2 domain (residues 156 to 271), and a serine protease domain (residues 272 to 579) (Mann, 1994). The serine protease activity of thrombin is homologous to other members of the

chymotrypsin protein family of serine proteases (Mann, 1994). Thrombin exists predominantly in zymogen form, circulating in plasma at concentrations between 1 and 2 μM (McDuffie *et al.*, 1979). Prothrombin is converted to thrombin by the enzymatic removal of an N-terminal propeptide, which can be accomplished by the activity of the prothrombinase complex, as well as by thrombin. Moreover, an additional internal cleavage of prothrombin by factor Xa or thrombin is required for the formation of α -thrombin (Downing *et al.*, 1975). Three particularly important internal structures within the thrombin protein have been described. The active site in thrombin is responsible for the cleavage of target proteases (Friezner Degen, 1995). Anion-binding exosite 1, which is made up of several surface residues on thrombin, has high affinity for fibrinogen, as well as hirudin, an anticoagulant protein present in the saliva of the medicinal leech (Stone *et al.*, 1987; Braun *et al.*, 1988; Chang JY *et al.*, 1990). In addition, the N-terminal region of HCII has high affinity for exosite 1 in the presence of heparin and dermatan sulfate (VanDeerlin and Tollefsen, 1991). Anion-binding exosite 2, which also consists of several surface residues on thrombin, has high affinity for heparin (Church *et al.*, 1989).

Thrombin cleaves fibrinogen to form fibrin, the main protein constituent present in a fibrin clot. Additionally, thrombin is involved in the activation of several coagulation cofactors involved in the coagulation cascade, including factors V and VIII, and factor XIII, which acts to crosslink link fibrin to form an insoluble fibrin clot (Roberts and Tabares, 1995). The serine protease activity of thrombin has been extensively characterized (reviewed by Friezner Degen, 1995). Thrombin preferentially

cleaves proteins C-terminal to arginine residues and this cleavage occurs via the well-characterized activity of the catalytic triad of serine-195, histidine-57, and Aspartic acid-102 residues.

Thrombin also has anticoagulant activity. When thrombin binds to thrombomodulin present on a vessel wall surface, it is capable of activating protein C, another serine protease which inactivates factors Va and VIIIa (Kisiel *et al.*, 1977; Walker, 1981; Esmon and Owen, 1981; Esmon CT, 1989). This results in the inhibition of the prothrombinase and intrinsic tenase activities, respectively. In addition to coagulant and anticoagulant activities, thrombin is involved in several physiological processes including platelet activation, cell growth and proliferation, and cellular chemotaxis (reviewed in Mann, 1994).

1.7 Biochemistry of Neutrophil Elastase

1.7.1 Structure of Neutrophil Elastase

Neutrophil elastase (NE) is a 29.5 kDa glycoprotein that consists of 218 amino acids, which is released, along with a plethora of other potent enzymes, from activated neutrophils during an inflammatory response or a response to injury or microbial invasion (Sinha *et al.*, 1987; Farley *et al.*, 1988). There are eight cysteine residues in the protein, all of which contribute to the formation of four disulfide bridges that are necessary for NE function. NE is heterogeneously glycosylated, with three potential glycosylation sites (Bode *et al.*, 1986; Sinha *et al.*, 1987). To date, three isoforms of NE have been described, differing only in the extent of glycosylation (Baugh and Travis, 1976). Variable glycosylation appears not to affect the catalytic activity of NE.

NE is a serine protease and is homologous to several other serine proteases that have been identified in several organisms, including human and rat pancreatic elastase (Shen *et al.*, 1987; MacDonald *et al.*, 1982), bovine chymotrypsin (Cohen *et al.*, 1981; Tsukuda and Blow, 1987), and human cathepsin G (MacDonald *et al.*, 1982). The tertiary structure of NE has been predicted by the analysis of a similar serine protease, porcine pancreatic elastase (PPE) (Meyer *et al.*, 1988). While PPE crystallization has been achieved relatively easily with its native conformation, production of a crystal structure for NE has been problematic. Native NE produces small crystals that are unsuitable for analysis, but the structure of NE complexed with an active site inhibitor has been accomplished (Bode *et al.*, 1986; Wei *et al.*, 1988). However, analysis of this complex must account for possible changes in protease conformation given that an inhibitor is bound to the active site and has been co-crystallized with the protein.

As with most serine proteases, the primary amino acid structure of NE is organized into a tertiary structure containing two interacting antiparallel β -barrel cylindrical domains (Bode *et al.*, 1989). Separating these two cylindrical domains is a crevice that contains the catalytic residues that are responsible for the proteolytic activity of NE. These catalytic residues are known as the catalytic triad and consist of residues His-57, Asp-102, and Ser-195 (Bode *et al.*, 1989). The substrate-binding site is also contained along the crevice and its binding properties are influenced by contributions of part of the tertiary structures of each β -barrel domain. The catalytic triad is conserved amongst all serine proteases, while the specificity of protease cleavage is determined in large part by the substrate-binding crevice present between the two conserved β -barrel

domains (Bode *et al.*, 1989). The largest differences between serine protease amino acid sequences correspond to residues that are exposed to the substrate-binding domain.

1.7.2 Molecular Biology of Neutrophil Elastase

The human NE cDNA was isolated and characterized from a lymphocyte cell line library (Farley *et al.*, 1988) and it was discovered that NE was identical to medullasin, a protease that had been previously isolated and characterized from bone marrow (Okano *et al.*, 1987). The human NE cDNA encodes an open reading frame of 801 bps that encodes the mature protein, and includes a 5' 87 bp which encodes a 29 residue signal peptide, as well as a 3' 60 bp sequence that is present before the in-frame stop codon. This 60 bp sequence is thought to encode a 20 residue C-terminal propeptide that is not found in the mature protein, but its role is not known. The human NE gene has been localized to chromosome 19 in a gene cluster with two other proteases: proteinase 3 and azurocidin (Zimmer *et al.*, 1992). The NE gene consists of five exons and four introns spanning approximately 4000 bp. The significance of this gene cluster is not known but it has been suggested that this region of chromosome 19 is developmentally regulated and may promote high expression of these proteins in the monocyte-myelocyte cell lineage, which are precursor cells to neutrophils (Zimmer *et al.*, 1992). NE is stored and released from activated neutrophils, but interestingly, mRNA for NE has not been detected in neutrophils, while high NE mRNA levels are seen in bone marrow precursor cells (Takahashi, *et al.*, 1988). These observations suggest that NE is produced in neutrophil progenitor cells, and subsequently stored in granules within neutrophils before being secreted after activation.

1.7.3 Proteolytic Cleavage Characteristics of Neutrophil Elastase

The interaction and cleavage of target proteins by NE occurs through a mechanism that is common to all active serine proteases. As previously mentioned, a catalytic triad exists in the substrate binding cleft of NE and this complex of serine, aspartic acid and histidine residues is ultimately responsible for the proteolytic activity of NE. Cleavage of proteins by NE occurs by a mechanism that involves two distinct steps (Steitz and Shulman, 1982). As described previously, an initial non-covalent Michaelis complex forms between NE and its target protease. Then, an acyl bond is formed by a nucleophilic attack of serine-195 of NE on the carbonyl carbon atom on the P1 residue of the substrate. A covalent bond is formed after cleavage of the ester bond that exists at P1 in the substrate, and is known as an acyl-enzyme intermediate. The histidine-57 and aspartic acid-102 stabilize the interactions between the substrate and enzyme and facilitate cleavage of the substrate between P1 and P1'.

The cleavage of the substrate is completed by deacylation of the acyl intermediate complex between the enzyme and substrate. The deacylation step is the reverse reaction of the acyl step described above, with the exception that water substitutes for the amine group of the substrate that was cleaved in the formation of the acyl intermediate between the enzyme and substrate. In this step, histidine-57 draws a proton away from the water molecule, which permits the remaining hydroxyl group to react with the carbonyl group present in the acyl-enzyme complex. The result of this reaction is that serine-195 is reconstituted by the substitution of the hydroxyl group for the carbonyl group of the acyl-

enzyme complex, with the cleaved substrate free to diffuse away from the binding cleft of the enzyme.

The substrate-binding pocket of serine proteases determines the specificity of these enzymes towards particular substrates (Bode *et al.*, 1989). Based on the interactions between NE and synthetic peptides, NE preferentially cleaves C-terminal to small, non-polar, alkyl amino acids. Specifically, NE cleaves peptide bonds on the carboxy-terminal side of amino acids with small, alkyl side chains with the following preference: Ile=Val>Leu>Ala. Based on the resolved crystal structure of an NE-turkey ovomucoid inhibitor complex, the NE substrate binding site makes direct contact with eight residues of the inhibitor, with an additional five secondary interactions made up of electrostatic associations (Bode *et al.*, 1989). Residues P1 to P3 of the substrate form an anti-parallel β -sheet that fits in a canonical manner into the hydrophobic substrate-binding cleft. Peptide mapping studies have demonstrated that the P1 residue interacts with the catalytic serine to provide approximately one-third of the binding energy that exists between enzyme and substrate (Bode *et al.*, 1989). The other sites within the octapeptide sequence that makes contact with the substrate-binding cleft of NE are of less importance than the P1 residue, but do contribute to the protease specificity of NE. For example, medium-sized hydrophobic residues at P2 are preferred (Bode *et al.*, 1989), while bulky residues are not well tolerated at P3 and P4 (Yasutake and Powers, 1981).

1.7.4 Reaction of Neutrophil Elastase with Antithrombin and Heparin Cofactor II

NE reacts with AT and HCII at sites close to their reactive centres in a manner that is different from the interaction of thrombin, another serine protease. While

thrombin cleaves AT and HCII, and forms inhibitory complexes, NE cleaves AT and HCII without becoming trapped. It has been hypothesized that non-target protease cleavage of these serpins is a means of regulating the physiological activities of these molecules (Carrell and Owen, 1985).

AT is cleaved by NE at residues 389 (valine) and 390 (isoleucine) (Carrell and Owen, 1985), which correspond to residues P5 and P4, respectively. Cleavage and inactivation of AT by NE has been shown to be a heparin-dependent reaction (Jordan *et al.*, 1987; Jordan *et al.*, 1989). Interestingly, an octasaccharide of heparin, containing the high-affinity pentasaccharide sequence, had little effect on NE-mediated inhibition of AT. Only longer polysaccharides catalyzed the inhibition of AT by NE, suggesting that heparin facilitates its catalytic effects by providing a bridge between AT and NE that can approximate the two proteins in the vicinity of one another and promote their interaction (Jordan *et al.*, 1989). In addition, crystal structure analysis of NE demonstrated the existence of a positively charged heparin-binding site on the surface of NE made up of several arginine residues (Baici *et al.*, 1980), further supporting the bridging role of heparin. The NE cleavage sites of AT appear to be equally susceptible to NE cleavage, with no one site preferred by NE over the other (Carrell and Owen, 1985; Jordan *et al.*, 1987). This conclusion was drawn from the observation that amino acid sequencing yielded equal amounts of two AT cleavage products, one with valine-389 as the new N-terminus, and the other with isoleucine-390 as the first amino acid of the peptide.

Like AT, HCII is susceptible to cleavage by NE at two internal sites (Pratt *et al.*, 1990). Similarly, to AT, one NE cleavage site in HCII occurs in the reactive centre loop,

C-terminal to the valine-439 residue (P6). However, in contrast to AT, the other NE cleavage site is not within the reactive centre loop, but rather exists near the N-terminus of the serpin. This N-terminal NE cleavage site in HCII occurs on the C-terminal side of the isoleucine-66 residue. NE cleavage of HCII at residue 66 is the preferred site of NE cleavage in the absence of heparin, although some cleavage at P6 does occur at reduced efficiencies (Pratt *et al.*, 1990). Thus, after prolonged incubations of NE and HCII, in the absence of heparin, cleavage of both target residues occurs. When heparin is present, the preferred NE cleavage site is the P6 residue within the reactive centre loop (Pratt *et al.*, 1990).

Cleavage of HCII by NE, in the absence of heparin, at isoleucine-66 decreases its anti-thrombin activity by approximately 100-fold (Pratt *et al.*, 1990). This reduction in thrombin-inhibitory activity of NE-cleaved HCII is similar to that observed with HCII mutants that lack the N-terminal acidic domains (Van Deerlin and Tollefsen, 1991; Liaw *et al.*, 1999), indicating that, in the absence of heparin, only a small fraction of NE-cleaved HCII molecules are inactivated by reactive centre loop cleavage. In contrast, when heparin is present, NE preferentially cleaves HCII at P6 within the reactive centre loop and inactivates the protein.

In the reaction of NE with both AT and HCII, heparin acts to bridge the serpins with the protease. The reaction of AT or HCII with NE in the presence of pentasaccharide failed to demonstrate NE-cleaved serpins (Jordan *et al.*, 1989; Pratt *et al.*, 1990). The maximal heparin effects of AT and HCII inactivation by NE are observed at heparin concentrations much lower than that needed to attain saturable binding to AT

and HCII, where maximal anticoagulant activity of these thrombin-inhibitory serpins is achieved (Jordan *et al.*, 1989; Pratt *et al.*, 1990). The reasons for this apparent disparity are unclear (Jordan *et al.*, 1989). At high heparin concentrations, heparin decreases the inactivation of AT and HCII by NE. It is believed that NE and serpin substrates bind to individual heparin molecules, a separation which serves to sequester the protease from its substrate. These observations further suggest that heparin acts to bridge NE with AT and HCII.

An association between NE-mediated thrombin-inhibitory serpins and sepsis has been proposed (Carrell and Owen, 1985). It is hypothesized that the cleavage and inactivation of AT and HCII by NE occurs in sepsis and that these interactions contribute to the development of disseminated intravascular coagulation, a thrombotic condition often observed in the course of this disease. A common consequence of sepsis is the consumption of clotting factors and their inhibitors faster than they can be resynthesized. In addition, neutrophils are activated in this syndrome with their concomitant degranulation to release proteolytic enzymes (Mammen, 1998). Use of high doses of AT concentrates in animal models of sepsis has improved survival rates (Emerson, 1994; Dickneite and Paques, 1993), and plasma AT levels of patients at ICU admission correlate strongly with survival in humans (Mammen, 1998) which further suggest an association between levels of functional AT levels and severity of disease.

1.8 Objectives and Rationale of the Current Study

AT and HCII are serpins which interact with several serine proteases. While AT and HCII both react with thrombin to form inhibitory complexes, they react with NE

without becoming trapped. The importance of the reactive centre loop in both kinds of serpin-protease interactions is illustrated by the existence of variant serpins with reactive centre loop mutations that see thrombin as a non-target protease. Because much of the reactive centre loop is not conserved amongst the serpins, particularly at the sites of NE cleavage, the hypothesis that formed the basis of this project was proposed. The hypothesis that was tested in this study was that amino acid substitutions within the reactive centre loop of the thrombin-inhibitory serpins (specifically AT and HCII) would generate molecules that retained function and would allow differentiation of the structural requirements for efficient interaction with thrombin or neutrophil elastase. Put another way, the aim of the study was to determine the limits of reactive centre loop mutagenesis that would reduce or eliminate NE cleavage without losing thrombin-inhibitory activity in both AT and HCII.

Several lines of evidence provided a sound rationale for the implementation of this study. Firstly, the reactive centre loop is not highly conserved amongst the inhibitory serpins, especially in the P4, P5, and P6 residues, where the single and double point mutations were created. In addition, no naturally occurring mutations at P4, P5, or P6 have been reported in either AT or HCII (Stein and Carrell, 1995). It therefore, seemed possible that mutagenesis at these amino acids might not have serious deleterious effects. Furthermore, Eldering *et al.*, (1993) conducted a site-directed mutagenesis study with C1 inhibitor, another serpin that inhibits two serine proteases in the complement cascade and is also susceptible to inactivation by reactive centre loop cleavage by NE. In their study, they mutated the NE cleavage sites at P3 and P5 and demonstrated an 18-fold decrease in

susceptibility to NE cleavage while leaving the inhibitory function of this serpin essentially unaffected. Lastly, previous work was conducted in the laboratory that demonstrated the importance of the P2 residue in influencing the inhibitory activity of AT (Sheffield and Blajchman, 1994a; Sheffield and Blajchman; 1994b). While most amino acid substitutions significantly impaired the thrombin-inhibitory activity of AT, the substitution of proline for wild-type glycine at P2 enhanced AT function. The effects of mutagenesis at P4, P5, and P6 had not been previously described and this study sought to determine if mutagenesis at these three residues would yield similar results to those observed with P2 mutagenesis. The following pages describe, in detail, the studies that were conducted on AT and HCII, and the effects of amino acid substitutions at the primary NE cleavage sites in these serpins on the inhibitory activity of these serpins towards thrombin.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Source of Chemicals and Reagents

Human α -thrombin (factor IIa) (>3300 NIH U/mg;) was the generous gift of Dr. John Fenton (New York State Department of Health, Albany, NY), while purified human plasma α_1 -proteinase inhibitor and antithrombin were donated by Bayer Canada Inc. (Etobicoke, ON). Plasmid pHCI7.2 was the generous gift of Dr. Douglas Tollefsen (Washington University, St. Louis, MI).

Chemicals were purchased from the following suppliers: Alexis Biochemicals (La Jolla, CA), human neutrophil elastase; Promega Biotec (Unionville, ON), restriction and DNA modifying enzymes, 1 kb and λ /HindIII DNA ladders for the determination of nucleic acid molecular size, RNase inhibitors, ribo- and deoxyribonucleotides, mRNA CAP analog 7-methylguanosylguanosine triphosphate $m^7G(5')ppp(5')G$, T7 and SP6 forward DNA primers, rabbit reticulocyte lysate, factor Xa, β -D-isopropyl-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-gal), and plasmid pGEM5zf+; Canadian Life Technologies (Burlington, ON), *Escherichia coli* (*E. coli*) DH5 α competent cells, Lipofectin reagent, penicillin/streptomycin, cell culture grade 1X trypsin-ethylenediamine-tetraacetic acid (EDTA) (0.05% trypsin, 0.53 M EDTA), cell culture grade fetal bovine serum, electrophoresis grade agarose, N,N,N',N'-tetramethylethylenediamine hydrochloride (TEMED), and plasmid pUC19; Fisher

Scientific (Unionville, ON), Millipore centrifugal concentrator units, Whatman #3 chromatography paper; Pharmacia LKB Biotechnology (Baie d'Urfe, PQ), T7 DNA sequencing kits, Q-Sepharose Fast Flow, pGEX-5X-1 GST gene fusion vector, glutathione-Sepharose; Qiagen (Chatsworth, CA), plasmid DNA isolation kits, Ni-NTA agarose; American Type Culture Collection (Rockville, MD), COS-1 cells; Clontech (Palo Alto, CA), Transformer Site-directed mutagenesis kit; Invitrogen (Carlsbad, CA), pBAD(B) bacterial expression vector, *E. coli* TOP10 and LMG194 competent cells; Stratagene (La Jolla, CA), plasmid pSG5 and *Pfu* polymerase; New England Biolabs (Bethesda, MD), peptide:N-glycosidase F; Calbiochem (Mississauga, ON), phenylalanyl-L-propyl-arginine chloromethyl ketone (PPACK), phenylmethylsulfonyl fluoride (PMSF); Sigma (St. Louis, MO) arabinose, N-paranitroguanidinobenzoate (NPGB), polybrene, sodium heparin; Wellmark Diagnostics (Guelph, ON), alkaline phosphatase-conjugated sheep anti-rabbit immunoglobulins; Affinity Biologicals (Hamilton, ON), human heparin cofactor II ELISA kit and affinity-purified human heparin cofactor II, BioRad Laboratories (Oakville, ON), protein molecular weight standards, Silver Stain Plus kit; Ortho Diagnostics (Mississauga, ON), heparin adsorbent; Helena Laboratories (Guelph, ON), chromogenic substrate S-2238 (H-D-phenylalanyl-L-pepecolyl-L-arginine-p-nitroaniline dihydrochloride); and Boehringer Mannheim (Burlington, ON), EDTA-free protease inhibitor tablets, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets. Dulbecco's modified Eagle Medium (DMEM), α -minimal essential medium (α MEM) and phosphate-buffered saline (PBS) were purchased

from the Department of Pathology, McMaster University. All other reagents and chemicals not specifically listed above were of the highest quality available.

2.1.2 Radiochemicals

Translation grade [³⁵S]methionine (>1000 Ci/mmol, 10 μ Ci/ μ l) and α -[³⁵S]dATP (>1000 Ci/mmol, 12.5 μ Ci/ μ l) were purchased from New England Nuclear (Lachine, PQ). Carrier free ¹²⁵Iodine (sodium iodide in 0.1 M NaOH, 99.5% radiochemically pure, 629 GBq/mg) was purchased from Dupont Canada, Inc. (Mississauga, ON).

2.1.3 Cell Lines

COS-1 African green monkey kidney carcinoma cells, purchased from the ATCC (Rockville, MD), were grown in DMEM, supplemented with 10% fetal calf serum and 100 units/ml of penicillin/streptomycin.

2.1.4 Oligonucleotides

The oligonucleotides listed in Table 1 were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, ON). Primers that were complementary to the cDNAs encoding rabbit AT, human AT, and human HCII are listed under the headings "Rabbit AT", "Human AT", and "Human HCII", respectively. Underlined bases indicate a mismatch with the wild type DNA sequence, while bases in parentheses indicate degenerate positions. Other primers used to manipulate DNA sequences for insertion into various vectors are listed under the heading "flanking primers". The term "MCS" represents the multiple cloning site present in the vector.

<u>Primer</u>	<u>Oligonucleotide Sequence</u>	<u>Annealing Position, Mutation, or Description</u>
<u>Rabbit AT</u>		
AB5134	5'-AGCACTGTCATCGGGGGTGTGCTGGCCGTTTCGCTG-3'	1294 – 1326, I390G
AB5250	5'-AGCACTGTCATCGGGAGTGTGCTGGCCGTTTCGCTG-3'	1294 – 1326, I390S
AB5251	5'-AGCACTGTCATCGGGGTTTGTGCTGGCCGTTTCGCTG-3'	1294 – 1326, I390F
AB5307	5'-AGCACTGTCATCGGGG(GA)CG)GCTGGCCGTTTCGCTG-3'	1294 – 1326, I390D, I390E, I390G
AB5308	5'-AGCACTGTCATCGGGA(AG)GC)GCTGGCCGTTTCGCTG-3'	1294 – 1326, I390D, I390K, I390R, I390S
AB6657	5'-AGCACTGTCATCGGGTGGGCTGGCCGTTTCGCTG-3'	1294 – 1326, I390W
<u>Human AT</u>		
AB8623	5'-AGTACCGCTGTTGTGTGGGCTGGCCGTTTCGCTA-3'	1318 – 1351, I390W
AB8624	5'-GCTGCAAGTACCGCTGTTTGGTGGGCTGGCCGTTTCGTAAAC-3'	1312 – 1354, I389W, I390W
<u>Human HCII</u>		
AB9191	5'-ACCACTGTGACCACGTGGGGGTTTCATGCCGCTG-3'	1385 – 1417, V439W
AB9192	5'-ACCACTGTGACCACG(ACT)(CGT)(TCG)GGGTTTCATGCGCTG-3'	1385 – 1417, V439C, V439F, V439I, V439L, V439M, V439P, V439R, V439S, V439T, V439W
AB12872	5'-ACCACTGTGACCACGT(CGT)(TCG)GGGTTTCATGCCGCTG-3'	1385 – 1417, V439C, V439F, V439L, V439S, V439W
AB13383	5'-ACCACTGTGACCACGGAGGGGTTTCATGCCGCTG-3'	1325 – 1341, V439E

<u>Primer</u>	<u>Oligonucleotide Sequence</u>	<u>Annealing Position, Mutation, or Description</u>
<u>Sequencing Primers</u>		
AB9410	5'-GCCATCGACCTGTTCAA-3'	1325 – 1341, RCL of HCII
AB14876	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	pGEX5 5' to MCS
AB14877	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	pGEX5 3' to MCS
AB15673	5'-ATGCCATAGCATTITTTATCC-3'	pBAD 5' to MCS
AB15674	5'-AAATTCTGTTTTATCAGACC-3'	pBAD 3' to MCS
<u>hHCII Deletion Primers</u>		
AB14053	5'-ATGAATTCTTCAGTGAAGACGACGACTAC-3'	412 trunc., + <i>EcoRI</i> (antisense)
AB15533	5'-GATCCATGGGGTCTCATCACCATCACCATCACTTCAGTGAAGACGACGACTACATC-3'	283 – 307, d66 deletion, +H6, + <i>NcoI</i>
<u>Flanking Primers</u>		
AB4899	5'-CTCAACTTTGGCGGATCCACCATGGGGG-3'	<i>BglII</i> site elimination in pSV3-rATIII
AB5775	5'-GAGTCGACCAGACATGATAA-3'	3' to MCS in pSG5 (antisense)
AB14052	5'-ATGAATTCGGATCCCTAGGACCTGCTGGGGTGGTGC-3'	hHCII + <i>EcoRI</i> (antisense)
AB14986	5'-GATCCATGGGGTCTCATCACCATCACCCTACGGGAGCAAAGGGCCGCTGGATCAT-3'	hHCII 1 – 27, + H6, + <i>NcoI</i>
T7	5'-TAATACGACTCACTATAGGG-3'	vector-specific
SP6	5'-GATTTAGGTGACACTATAG-3'	vector-specific

2.2 Analysis of Nucleic Acids

2.2.1 Extraction and Purification of DNA

(a) Small Scale Preparation of Plasmid DNA from Bacteria

Purification of plasmid DNA was achieved using QIAprep minipreps (Chatsworth, CA) using a modification of the method of Birnboim *et al.* (1979). A 2.5 ml aliquot of Luria-Bertani (LB) broth supplemented with 100 µg/ml of sodium ampicillin was inoculated with transformed cells containing the plasmid of interest, and the culture was grown at 37°C in an environmental shaker (200 RPM). Following overnight incubation, 1.5 ml of bacterial culture was centrifuged for 1 minute at 14000 RPM in a microcentrifuge. The supernatant was aspirated and discarded, and the bacterial pellet was resuspended in 250 µl buffer P1 (50 mM Tris-Cl, 10 mM EDTA, pH 8.0, 100 µg/ml RNase A). The cells were lysed using 250 µl buffer P2 (200 mM NaOH, 1% SDS) and protein, RNA and chromosomal DNA were precipitated by the addition of 350 µl buffer N3 (3.0 M KAc, pH 5.5). The mixture was then centrifuged for 10 minutes at 14000 RPM, and the supernatant added to a QIAprep centrifugation column. The centrifugation column was then centrifuged at 14000 RPM for 1 minute and the filtrate discarded. The column was then washed twice with 750 µl of buffer PE (1 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100) by adding the washing buffer to the column and centrifuging for 1 minute at 14000 RPM. Plasmid DNA was eluted by the addition of 50 µl buffer EB (10 mM Tris-Cl, pH 8.5) by adding it to the column, incubated at room temperature for 1 minute and collected in an Eppendorf tube by centrifugation.

(b) Large Scale Purification of Plasmid DNA

Large-scale purification of plasmid DNA was performed using the Qiagen plasmid maxi kit, using the method of Birnboim *et al.* (1979). A 200 ml culture was inoculated with transformed cells harbouring the plasmid of interest, and grown to stationary phase by incubation at 37°C with shaking (200 RPM). LB supplemented with 100 µg/ml sodium ampicillin was the medium. Cells were collected by centrifugation at 6000 RPM for 20 minutes using a Sorvall GSA rotor. The bacterial pellet was resuspended in 10 ml buffer P1 (100 µg/ml RNase A, 50 mM Tris-Cl, 10 mM EDTA, pH 8.0) and lysed by the addition of 10 ml of buffer P2 (200 mM NaOH, 1% SDS) for 5 minutes. Then, 10 ml of chilled (4°C) buffer P3 (3.0 M KAc, pH 5.5) was added and the mixture was incubated on ice for 20 minutes before being centrifuged at 12000 RPM at 4°C. While the mixture was centrifuged, a Qiagen-tip 500 column was prepared for use by the addition of 10 ml QBT equilibration buffer (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100). The supernatant of the bacterial lysate was passed over the column by gravity flow and washed twice with 30 ml buffer QC (1M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). Plasmid DNA was eluted from the column resin with 15 ml buffer QF (1.25 M NaCl, 50 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Plasmid DNA was then precipitated with 10.5 ml isopropanol, and collected by centrifugation at 12000 RPM at 4°C for 30 minutes. The precipitated DNA pellet was then washed using 5 ml of 70% ethanol and centrifuged for 30 minutes at 12000 RPM and 4°C. The plasmid DNA was subsequently air-dried for 5 minutes and resuspended in 250 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

2.2.2 Gel-based Analysis of DNA

Agarose gels were prepared, as described by Sambrook *et al.* (1989), by the addition of 1% (weight/volume) electrophoresis grade agarose in 1X TAE (89 mM Tris base, 89 mM acetic acid, 2 mM EDTA, pH 8.0) and heated in a microwave oven for 90 seconds. Once the solution cooled to 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml, and the entire mixture was poured into a casting tray containing sample well combs. The agarose solution was allowed to solidify for 20 minutes at room temperature. DNA samples, containing loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 6X TAE) were loaded into the wells and electrophoresed in 1X TAE at 80V for 30 minutes. Subsequently, the DNA bands were visualized using a Fotodyne Chromato-Vue transilluminator (model TM-20; UVP, San Gabriel, CA).

2.2.3 Quantification of DNA

Nucleic acid samples were quantified by the infrared spectrophotometric absorbance at OD₂₆₀. 1 µl of DNA was diluted to 1 ml in distilled, deionized water and the absorbance at 260 nm was determined. Nucleic acid concentration was determined by employing Beer's law, assuming that one OD₂₆₀ unit corresponded to 50 µg/ml of double-stranded DNA (Sambrook *et al.*, 1989). For estimation of dilute DNA solutions, an aliquot was electrophoresed and its abundance was gauged by comparing the relative ultraviolet fluorescence intensities of the sample and standard DNA markers that had been electrophoresed on the same gel.

2.2.4 Transformation of Competent Bacteria

The procedure described below was used to transform competent *E. coli* DH5 α , LMG194, BMH and TOP10 cells with plasmid DNA or ligation reaction mixtures. Competent cells were thawed slowly on ice for 20 minutes and 100 μ l of the cell suspension was added to chilled microcentrifuge tubes. 50 ng of DNA (either plasmid or ligation mixture) was added to the cell suspension and chilled on ice for 20 minutes. The cells were then heat-shocked at 37°C for 20 seconds and returned to ice for an additional 2 minutes. 900 μ l of LB medium was then added and the cells were incubated in a 37°C shaker for 1 hour. The cells were then collected by centrifugation (14000 RPM, 2 minutes) and resuspended in 200 μ l of LB medium. The cells were then plated in aliquots of 20 μ l and 180 μ l onto LB agar plates supplemented with 100 μ g/ml ampicillin, and incubated overnight in a 37°C incubator. Colonies of ampicillin-resistant transformed bacteria were then picked and used to inoculate overnight cultures. For long-term storage, stationary phase cell suspensions were made 16% in glycerol and frozen at -70°C.

Commercially-available plasmids that had been engineered to demonstrate, via colour change, the successful insertion of DNA sequences into the vector were used in some subcloning experiments. In these instances, LB plates containing 100 μ g/ml ampicillin were treated with 10 μ l of 100 mM IPTG and 40 μ l of a 2% (w/v) of X-gal, and bacterial transformants subsequently plated. Successfully ligated constructs appeared as white colonies due to the disruption of codons specifying the β -galactosidase α -peptide.

2.2.5 DNA Sequencing

DNA for sequencing was obtained by the purification techniques described above. Plasmid DNA was denatured before sequencing reactions were performed. 5 µg of double-stranded plasmid DNA was incubated in denaturing buffer (0.2 M NaOH, 0.2 mM EDTA, pH 8.0) for 15 minutes at 37°C. The denatured DNA was then neutralized by the addition of one tenth volume of 3 M NaOAc, pH 5.2 and 2 volumes of ice cold absolute ethanol. Denatured DNA was recovered by precipitation in ethanol and centrifugation at 14000 RPM for 15 minutes. The DNA pellet was washed once with 100 µl 70% ethanol and then centrifuged and dried in a SpeedVac centrifuge. The lyophilized DNA was then resuspended in 10 µl distilled, deionized water.

DNA sequencing was then conducted using the dideoxy method initially described by Sanger *et al.* (1977) using a T7 sequencing kit (Pharmacia). 2µl annealing buffer and 2 µl 0.8 µM sequencing primer were added to 10 µl denatured DNA. The reaction was incubated at 65°C for 5 minutes, followed by an incubation at 37°C for 10 minutes, and another room temperature incubation for 10 minutes. The extension reaction was conducted as follows: 6 µl enzyme premix (1 µl ddH₂O, 3 µl labeling mix, 2 µl T7 polymerase (1.5U/µl) and 1 µl α-[³⁵S]-dATP) were added to the reaction containing the denatured DNA and annealed primers and incubated at room temperature for 4 minutes. 4.5 µl aliquots were then removed and added to each of the four dideoxy nucleotides, and incubated at 37°C for 5 minutes. The reactions were stopped by the addition of stop solution (10 mM EDTA, 97.5% formamide, 0.3% bromophenol blue, 0.3% xylene cyanol) to each tube. Each reaction was boiled for 2 minutes before adding

2.5 μ l to a pre-made polyacrylamide gel, in which separation of the DNA bands was resolved.

The sequencing gel was prepared to a final concentration of 8% (w/v) acrylamide, as follows: 30 ml 40% acrylamide (38% (w/v) acrylamide, 2% (w/v) bis-acrylamide), 20 ml 5X TBE (0.089 M Tris-Cl, 0.089 M boric acid 0.002 M EDTA, pH 8.0), 50 g urea (8 M final concentration) and 20 ml ddH₂O. Once the solution had been filtered through Whatman paper and degassed, 1 ml 10% (w/v) APS and 15 μ l TEMED were added to facilitate polymerization of the solution once it had been poured. After the samples had been loaded onto the polymerized gel, the apparatus was operated under a constant power of 60 W for approximately 2 hours. The sequencing gel was then transferred to Whatman paper and dried under vacuum at 80°C for 2 hours on a BioRad slab gel dryer. The dried gel was then exposed to Kodak X-OMAT XK-1 film overnight at room temperature, and subsequently developed in the Department of Radiology, McMaster University using a Kodak automated film developer.

2.3 Site-directed Mutagenesis and DNA Manipulations

2.3.1 “Megaprimer” Protocol

The previously described plasmid pSV3-rATIII (Sheffield *et al.*, 1992)) served as the template for mutagenesis of rabbit AT cDNA, using either a megaprimer (Chen and Przybyla, 1994) or restriction site elimination (Ray and Nickoloff, 1992) protocol. The megaprimer protocol is a two step PCR process that uses a mutant AT- or HCII-specific primer, and two vector-specific flanking primers to perform site-directed mutagenesis. The following protocol creating the I390G mutation in rabbit AT describes a typical

mutagenesis reaction employing this technique. Creation of other AT mutants was achieved by substituting different mutagenic primers in similar reactions.

In the first round, the following reagents were added to a sterile 500 μ l tube in either the given amount or to the given final concentration: 1.5 mM MgCl₂, 0.2 mM tri-phosphated nucleotides, 1 μ M phosphorylated mutagenic primer AB5134, 1 μ M T7 primer, 20 ng of plasmid pSV3-rATIII (template DNA), a 10-fold dilution of cloned *Pfu* buffer, and 5U *Pfu* DNA polymerase. The reaction was incubated in a thermocycler for 40 cycles under the following conditions: 1 minute at 95°C, followed by 90 seconds at 54°C, followed by 2.5 minutes at 72°C. The amplified DNA from the PCR reaction was electrophoresed on a 1% agarose gel. A 246 base pair DNA fragment was then excised from the gel and purified from the agarose using a Sephaglas kit (Pharmacia LKB).

The 246 base pair product from the first PCR reaction was used as a “megaprimer” in the second round of DNA synthesis. Specifically, 1.5 mM MgCl₂, 0.2 mM tri-phosphated nucleotides, one fifth volume of the gel-purified megaprimer reaction, 1 μ M SP6 primer, 20 ng of plasmid pSV3-rATIII (template DNA), a 10-fold dilution of 10X cloned *Pfu* buffer, and 5 units of *Pfu* DNA polymerase, were added to a sterile 500 μ l tube. The tube was incubated in a thermocycler for 40 cycles using the same conditions as those described above and the 1604 base pair product was detected and excised from a 1% agarose gel. The PCR product was again purified from the agarose gel using a Sephaglas kit.

High-fidelity *Pfu* polymerase was used to ensure fidelity of the final 1604 bp product, which was subcloned into the SmaI site of pUC19 by standard ligation

procedures (Sambrook, *et al.*, 1989). Table 1 lists all of the mutagenic primers that were used in the course of this study. Candidates for appropriately mutated transcription plasmids were verified by restriction endonuclease digestion (Sambrook, *et al.*, 1989) and double-stranded DNA sequencing (Sanger, *et al.*, 1977), and designated pSV3-rATIII₁₋₄₃₃(I390X), where X designates the new amino acid encoded. To add back the AT secretory signal sequence lacking in the above constructs that were used for cell-free expression, a 930 bp *EcoRI-NcoI* restriction fragment of pSG5(rab)WT (Sheffield *et al.*, 1995) was combined with a 546 bp *NcoI-EcoRI* restriction fragment of pSV3-rATIII₁₋₄₃₃(I390X), where X was the substituted residue, in a three part ligation. Standard subclone analysis and verification by DNA sequencing were employed as above. Human AT mutagenesis was conducted as above by using the vector pSG5-AT(WT) (Sheffield, *et al.*, 1994) as a template. In addition, site-directed mutagenesis was conducted on human HCII using the pSG5-HCII(WT) vector as a template. The construction of this expression vector is described in section 2.6.

2.3.2 Restriction Site Elimination Protocol

When megaprimer mutagenesis was not employed, site-directed mutagenesis was achieved by a restriction site elimination protocol using the Transformer site-directed mutagenesis kit (Clontech). The mutagenic primer contained the mutation of interest within either the AT or HCII cDNA, and the selection primer that was used removed an *XbaI* site from the pSG5 vector. A typical reaction creating the I390S mutation in rabbit AT was performed as follows. A sterile microcentrifuge tube containing 100 ng of pSV3-rATIII (template DNA), 100 ng of primer AB4899 (selection primer), 100 ng of

primer AB5250 (mutagenic primer) and a 10-fold dilution of 10X annealing buffer (200 mM Tris, pH 7.5, 100 mM MgCl₂, 500 mM NaCl) was boiled for 3 minutes, and then immediately chilled for 5 minutes at 0°C. The mutant DNA strand was synthesized by adding 4 units of T4 DNA polymerase, 6 units of T4 DNA ligase, and a 10-fold dilution of 10X synthesis buffer (5 mM dNTPs in 200 mM Tris, pH 7.5) to the reaction tube and incubating at 37°C for 2 hours. The reaction mixture containing both parental and mutagenic DNA strands was then digested with *Xba*I for 3 hours at 37°C. Transformation of mutS competent *E. coli* cells was then achieved using the digested DNA mixture. Since circular DNA transforms bacterial cells far more efficiently (Conley *et al.*, 1984) than linear DNA, the mutated DNA is expected to transform cells preferentially. Transformed cells were amplified in selective growth media (LB/ampicillin) overnight at 37°C and then rapid mini-preparations of plasmid DNA were made (described previously). The plasmid DNA was then digested with *Xba*I for 3 hours at 37°C, and DH5α competent cells were transformed using the digested DNA mixture. The transformed DH5α cells were then grown overnight at 37°C on LB agar plates supplemented with 100 µg/ml ampicillin and individual bacterial colonies were selected and amplified in selective growth media (LB/ampicillin). Rapid mini-preparations of plasmid DNA containing the mutated plasmid were then made after overnight incubation in an environmental shaker.

Again, appropriate mutagenesis was confirmed by restriction enzyme digestion and double-stranded DNA sequencing. Creation of other point mutants involved a similar procedure whereby the DNA template and/or mutagenic primers were altered.

2.4 Cell-Free Expression of AT

2.4.1 *In vitro* Transcription of mRNA

Cell-free expression of AT variant proteins was accomplished *in vitro* as described by Krieg and Melton (1984). Briefly, SP6 or T7 RNA polymerase was used to produce mRNA from a linearized plasmid containing the mutated AT cDNA of interest. Specifically, 2 μg of linearized template DNA was added to a microfuge tube containing transcription buffer, 10 mM DTT, 5 μg acetylated BSA, 0.5 mM NTPs, 0.5 mM CAP analog and 40 U of SP6 polymerase. The 50 μl reaction was incubated at 37°C for a total of 120 min; however after 15 minutes, 5 μl of 4 mM guanosine triphosphate (GTP) was added to force the incorporation of the CAP analog early in transcription. The reaction was stopped by the addition of 1 U RQ1 RNase-free DNase for 15 min at 37°C.

2.4.2 *In vitro* Translation of AT Variants

The DNase-treated AT mRNA template was then added to the necessary reagents to produce radiochemically pure protein in a procedure first described by Pelham and Jackson (1976). Briefly, in a 50 μl reaction, 35 μl nuclease-treated rabbit reticulocyte lysate, 5 mM glutathione, 40 U RNase inhibitor, 40 μM amino acid mixture (minus methionine), and ^{35}S -methionine were added in addition to 4 μl of RNA produced *in vitro*. The reaction was incubated at 30°C for 60 min and then diluted 2-fold with Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 150 mM NaCl). The reaction mixture was then dialyzed overnight at 4°C against TBS prior to analysis of protein products.

2.5 Expression of AT Variants in COS-1 Cells

2.5.1 Maintenance of Cells in Growth Media

COS-1 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin . Cells were grown in a tissue culture incubator at 37°C and in a humidified air mixture containing 5% carbon dioxide. Cells were permitted to grow on 100 mm plates until confluent, at which time they were enzymatically lifted from the plates and diluted as follows. Cells were washed once with 5 ml PBS and 2 ml trypsin/EDTA was added for 5 minutes at 37°C to remove the cells from the bottom of the plates. 8 ml Dulbecco's medium containing serum was then added to neutralize the trypsin and the cells were diluted as desired. The diluted cells were then added in 10 ml aliquots to new 100 mm plates.

2.5.2 Transient Transfection of COS-1 Cells Using Liposomes

Transfections were performed essentially as described initially by Felgner *et al.*, 1987. Cells were grown to 80% confluence in DMEM containing 10% FBS and 100 units/ml penicillin/streptomycin and then washed twice with 5 ml PBS. For each plate that was transfected, 10 µg plasmid DNA was incubated with 60 µl Lipofectin reagent in 5 ml DMEM lacking FBS and penicillin/streptomycin for 5 minutes to permit the association of DNA with liposomes. 5 ml of media containing the DNA/liposome mixture was then added to the cells, and incubated at 37°C for 4 to 6 hours. The medium was then removed from the cells and replaced with 10 ml serum and penicillin/streptomycin-containing medium for 36 hours. Subsequently, the cells were washed twice with 5 ml PBS and 10 ml serum-free medium was added for 24 hours. The

serum-free medium was then collected and residual cellular material was removed by centrifugation at 3000 RPM for 10 minutes. The medium was subsequently analyzed, by ELISA, to determine the immunological concentration of AT. Cells used for transient transfection were killed by incubation in 4% sodium hypochlorite for 10 minutes in accordance with MRC Biosafety guidelines.

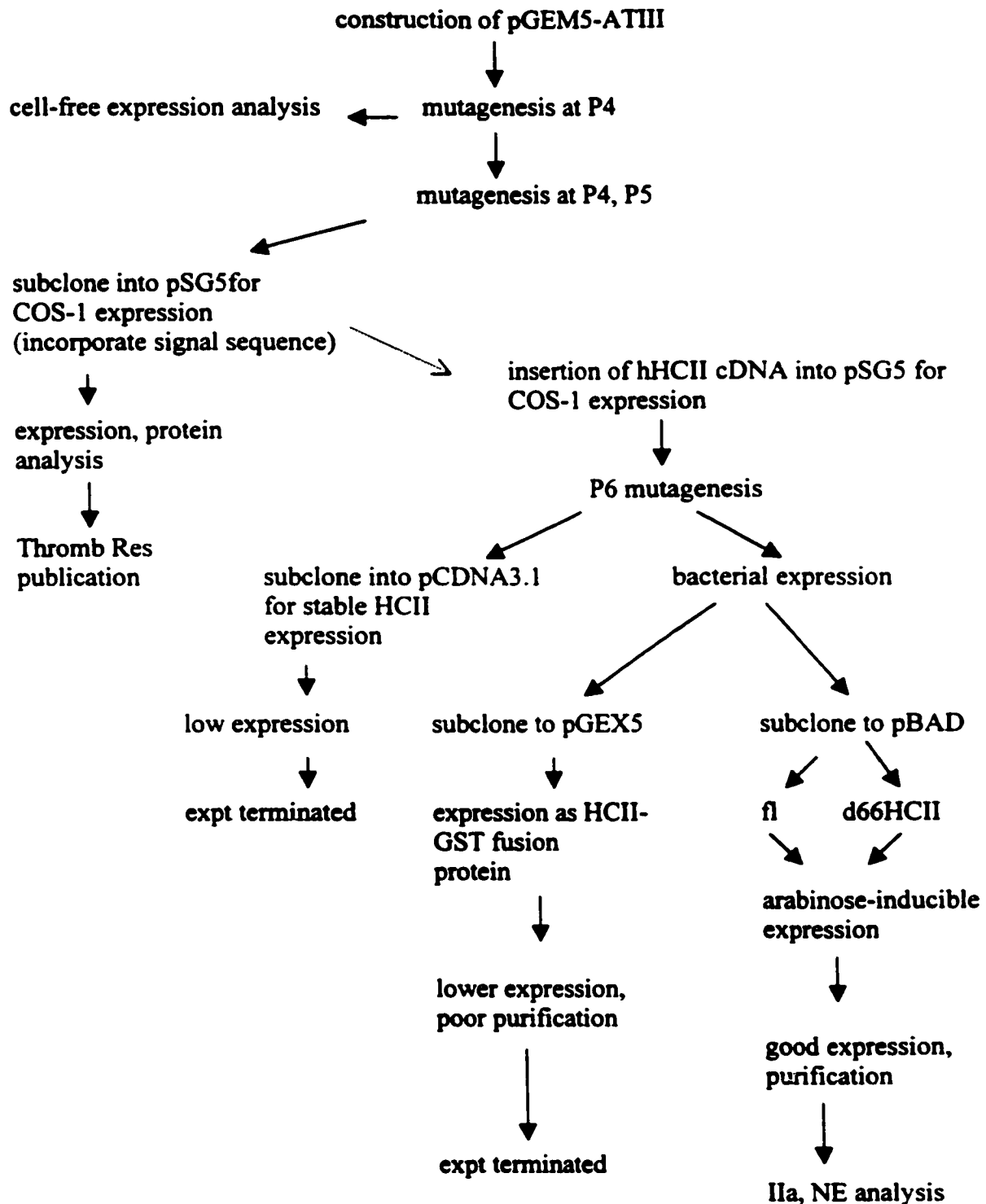
2.6 Expression of Human HCII Variants

The human HCII cDNA was initially subcloned into the pSG5 vector by restricting the plasmid pHCI7.2 with *EcoRI* and ligating the HCII cDNA into pSG5 which had been restricted with the same enzyme. Mutagenesis at the P6 residue was conducted using the restriction site elimination site-directed mutagenesis protocol described previously. The pSG5-HCII(WT) and pSG5-HCII[(V439X), where X designates the new amino acid encoded] expression plasmids served as the templates for further creation of both prokaryotic and eukaryotic expression constructs. Figure 3 summarizes the various expression systems used to complete this study.

2.6.1 Expression of HCII in Baby Hamster Kidney (BHK) Cells

Digestion of the plasmid pSG5-hHCII with *EcoRI* was performed to liberate the human HCII cDNA, which was subsequently inserted into the pCDNA3.1 expression vector. Appropriate insertion of the cDNA was confirmed by DNA sequencing and a large scale purification of plasmid DNA was performed, as described previously.

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BHK cells were grown to 80% confluence in alpha-minimum essential medium (α -MEM) supplemented with 10% FBS and 100 units/ml penicillin/streptomycin and then washed twice with 5 ml PBS. For each plate that was transfected, 10 μ g plasmid DNA was incubated with 20 μ l Lipofectin in 4 ml α -MEM lacking FBS and penicillin/streptomycin, for 10 minutes to permit the association of DNA with liposomes. 4 ml of media containing the DNA/liposome mixture was then added to the cells and incubated at 37°C for 5 hours. The transfected media was then removed and the cells were trypsinized and split 4-fold in α -MEM, supplemented with 10 % FBS, 100 U/ml penicillin/streptomycin and 1mg/ml gentamycin.

BHK cells transfected with the pCDNA3.1-hHCII construct were selected by growth in α -MEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 1 mg/ml gentamycin for 2 weeks at 37°C in a humidified incubator. The cells were washed with 2 ml PBS, and the selective media replaced every 4 days. After two weeks, gentamycin-resistant cell colonies were isolated and grown to confluence in multi-well dishes, still using α -MEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 1 mg/ml gentamycin. The cells were then washed twice with 2 ml PBS and grown in α -MEM supplemented with 1 mg/ml gentamycin, but lacking FBS and penicillin/streptomycin. After incubation for 24 hours, the serum-free media was collected and analyzed by ELISA to determine the immunological concentration of HCII. The serum-free media was replaced by serum-containing α -MEM, supplemented with 1 mg/ml gentamycin and returned to the 37°C humidified incubator.

2.6.2 Construction and Bacterial Expression of the pGEX- Δ 66HCII Expression Vector

Creation of this construct produced a glutathione-S-transferase (GST) fusion protein (Smith and Johnson, 1988), in which Δ 66HCII had been attached to the C-terminus of GST, with a factor Xa cleavage site present between the protein moieties. Plasmid pGEX-5X-1- Δ 66HCII was constructed from full-length HCII (pSG5-HCII(WT)) by PCR employing oligonucleotides AB10453 and AB14052. The primers used incorporated an *EcoRI* restriction site at both ends of the Δ 66HCII DNA fragment, which allowed insertion into the pGEX-5X-1 vector, which had been restricted with the same enzyme. Appropriate insertion of the PCR products was verified by restriction enzyme digestion and by DNA sequencing. The pGEX- Δ 66HCII expression vector was used to transform *E. coli* BL-21 competent cells, as described previously.

Expression of the GST- Δ 66HCII fusion protein was accomplished by diluting an overnight culture of BL-21 cells transformed with pGEX- Δ 66HCII 100-fold in 100 ml of YT media supplemented with 100 μ g/ml sodium ampicillin, and growing in an environmental shaker at 30°C until the OD₆₀₀ of the culture reached 0.6. β -D-isopropylthiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM to induce the expression of the GST- Δ 66HCII fusion protein for 6 hours. Bacterial cells were then collected by centrifugation for 15 minutes at 6000 RPM in a Sorvall RC5 centrifuge. The bacterial pellet was then suspended in 5 ml ice cold PBS and the cells disrupted in a sonicator for 5 minutes. Triton X-100 was added to a final concentration of 1% (v/v) and incubated at room temperature for 20 minutes on a rocking platform. The

cellular debris from the mixture was then separated by centrifugation at 12000 RPM for 30 minutes at 4°C. The supernatant was then collected and used in the subsequent glutathione-Sepharose affinity chromatographic purification, while the cellular component was discarded.

The supernatant was passed, by gravity, over a 1 ml glutathione-Sepharose column which had been equilibrated with PBS. The column was then washed with 10 ml of PBS. Glutathione-binding proteins were then eluted from the column by adding 2 ml of PBS supplemented with 100 mM glutathione. The elution step was repeated three times.

2.6.3 Construction of pBAD(B)-H₆-Δ66HCII Expression Vector

Plasmid pBAD-H₆-Δ66HCII was constructed from full-length human HCII (pSG5-HCII(WT), or pSG5-HCII(VP6X, where X is the amino acid substitution created by site-directed mutagenesis)) by PCR employing oligonucleotides AB15533 and AB5775, in which the 5' primer incorporated the DNA sequence encoding codons for a hexahistidinyI tag on the 5' end of plasmid pBAD-H₆-Δ66HCII . Two restriction sites were also incorporated into either end of the PCR product: a *NcoI* site at the 5' end, and an *EcoRI* site at the 3' prime end. The pBAD vector was restricted with these enzymes and the PCR products were inserted into the vector by standard restriction enzyme digestion and ligation techniques (Sambrook, *et al.*, 1989). Appropriate insertion of the PCR products was verified by restriction enzyme digestion and by DNA sequencing.

2.6.4 Induction of Protein Expression in Bacteria by Arabinose

A fresh *E. coli* TOP10 colony containing the pBAD-H₆-Δ66HCII plasmid of interest was isolated from an LB agar plate supplemented with 100 μg/ml ampicillin and used to inoculate 2.5 ml of LB medium containing 100 μg/ml ampicillin. This culture was grown in a shaking incubator overnight at 37°C. The culture was then diluted 1 in 100 (v/v) into 250 ml LB containing 100 μg/ml ampicillin and grown for about 2 hours until the OD₆₀₀ was approximately 0.5. 20% arabinose was then added to a final concentration of 0.002% and the culture was grown in a shaking incubator at 37°C for an additional 4 hours. The culture was then centrifuged at 6000 RPM for 10 minutes to pellet the cellular material. The supernatant was discarded and the cellular pellet stored at -70°C until further use.

2.6.5 Preparation of Bacterial Lysates for H₆-Δ66HCII Purification

Bacterial pellets which had been stored at -70°C were thawed slowly on ice and 1 ml of protease inhibitors (Boehringer Mannheim, proprietary product) added. The pellet was resuspended in 25 ml lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole) and sonicated at 4°C for 5.2 minutes (40% amplitude, 6 second pulses). 20% Triton X-100 was then added to a final concentration of 1% to the bacterial mixture and mixed for 20 minutes at room temperature on a rocking platform. The cellular debris from the mixture was then separated by centrifugation at 12000 RPM for 30 minutes at 4°C. The supernatant was then collected and used in the subsequent nickel affinity chromatographic purification, while the cellular component was discarded.

2.6.6 Nickel Chelate Chromatographic Purification of H₆-Δ66HCII

The supernatant from a 200 ml culture was added to a 2 ml column of nickel-NTA agarose, that had been equilibrated with 3 bed volumes of lysis buffer, and passed, by gravity, over the beads at a rate of 0.5 ml/minute. The column was then washed with ten bed volumes of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). Nickel-binding proteins were eluted from the column by three 2 ml additions of elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole). The elution fractions were pooled and dialyzed against 50 mM Tris, pH 7.4 overnight at 4°C.

2.6.7 Q-Sepharose Chromatographic Purification of H₆-Δ66HCII

The dialyzed protein was added to a 2 ml column of Q-Sepharose, that had been equilibrated with 50 mM Tris, pH 7.4, and allowed to flow by gravity. The column was washed with 10 bed volumes of 50 mM Tris, pH 7.4 and protein fractions were eluted using a salt gradient (50 mM Tris, pH 7.4, 0 to 600 mM NaCl). 1 ml (32 drops) elution fractions were collected using a fraction collector (Pharmacia Biotec, Baie d'Urfe, PQ) and fractions that contained the protein of interest, as determined by SDS-PAGE, were pooled and concentrated using a centrifugal concentrator unit (Fisher Scientific, Mississauga, ON). Determination of total purified protein concentration was accomplished by Bradford assay, followed by HCII protein concentration determined by ELISA.

2.6.8 Construction of pBAD(B)-H₆-HCII Bacterial Expression Vector

Plasmid pBAD(B)-H₆-HCII was constructed by PCR, using plasmid pHCII7.2 as the template DNA. Specifically, primers AB14986 and AB14052 were used to mobilize,

by PCR, the full-length hHCII cDNA, lacking the 5' secretory signal sequence, from the plasmid. In addition, a *NcoI* and an *EcoRI* site were engineered onto the 5' and 3' ends, respectively, of the PCR product. The full-length HCII cDNA PCR product was inserted into pBAD(B) which had been restricted by these enzymes, and its proper orientation confirmed by DNA sequencing.

The P6 mutants which had been created in the pBAD-H₆-Δ66HCII were recreated in the pBAD(B)-H₆-HCII construct. Specifically, both the pBAD-H₆-Δ66HCII and pBAD(B)-H₆-HCII constructs were restricted with *SmaI* and *EcoRI* digests, which yielded a 488 base pair DNA fragment of the 3' end of the cDNA. This fragment encoded the C-terminal portion of HCII containing the P6 residue. In addition, fragments of 5193 and 5259 base pairs were produced, which corresponded to the remainder of constructs pBAD-H₆-Δ66HCII and pBAD(B)-H₆-HCII, respectively. The 488 base pair fragments containing the P6 mutations from the pBAD-H₆-Δ66HCII constructs were ligated with the 5259 base pair fragment corresponding to the pBAD(B)-H₆-HCII construct, which had been restricted with *SmaI* and *EcoRI*, and which lacked the 3' end of the cDNA. These DNA manipulations incorporated the P6 mutations created in the pBAD-H₆-Δ66HCII into the pBAD(B)-H₆-HCII constructs. Successful ligations of appropriate DNA bands to create the desired constructs were confirmed by DNA sequencing. All pBAD(B)-H₆-HCII constructs were used to transform *E. coli* LMG194 competent cells and then subsequently expressed via induction by arabinose (described previously).

2.6.9 Purification of Bacterially-Derived H₆-HCII

Nickel-chelate purification of full-length HCII was performed exactly as described previously in sections 2.6.4 and 2.6.5. Purification of full-length HCII differed from that of H₆-Δ66HCII only in the Q-Sepharose chromatographic purification step. Full-length HCII which had been dialyzed against 50 mM Tris, pH 7.4 was added to a 2 ml column of Q-Sepharose, and allowed to flow by gravity. The column was washed with 10 bed volumes of wash buffer (50 mM Tris, pH 7.4, 50 mM NaCl), and protein fractions were eluted using an elution buffer (50 mM Tris, pH 7.4, 150 mM NaCl). 2 ml elution fractions were collected and fractions that contained the protein of interest, as determined by SDS-PAGE, were pooled and concentrated using a centrifugal concentrator unit (Fisher Scientific, Mississauga, ON). Determination of total protein was performed by Bradford assay, and HCII concentration determined by ELISA.

2.7 Characterization of Recombinant AT and HCII Variant Proteins

2.7.1 Quantification of AT and HCII Variants by ELISA

A trapping ELISA was used to quantify both AT and HCII variants. Microtiter wells were coated with 1 µg/ml sheep anti-human AT or HCII IgG using a coating buffer containing freshly made 0.1 M sodium carbonate, pH 9.6. After the wells had been coated for a minimum of 24 hours at 4°C, the coating buffer was removed and the wells were washed once with TBST (10 mM Tris base, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The sites of non-specific protein binding were blocked by the addition of 200 µl of blocking buffer (1% skim milk powder in TBST) for 60 minutes at room temperature. 100 µl of either protein samples, or standards of known HCII concentrations, using

dilutions of protein from 180 to 5.625 ng, were then added to the wells. Each sample was added in triplicate. The samples were incubated at 37°C for 90 minutes and the wells were subsequently washed three times with TBST.

Captured protein was detected by the addition of 100 µl biotinylated sheep anti-human AT or HCII diluted 1 in 1000 in blocking buffer to each well and incubated for 60 minutes at 37°C. The wells were again washed three times with TBST, and 100 µl streptavidin/alkaline phosphatase diluted 1 in 5000 in TBST containing 0.4% BSA added to each well. The plate was incubated again at room temperature for 60 minutes. 100 µl of developing solution (1 mg/ml paranitrophenyl phosphate, 9.7% diethanolamine, 0.5 mM MgCl₂, pH 9.8) was prepared fresh and added to each well. Colour development was monitored over 30 minutes and then quantified at 405 nm using an ELISA plate reader. Dilutions of protein samples falling within the linear portion of the standard curve were used to determine AT or HCII concentration in the sample.

2.7.2 SDS-Polyacrylamide Gel Electrophoresis of AT and HCII

Protein samples were analyzed by SDS-PAGE employing the following procedure, described by Laemmli (1970). Polyacrylamide gels were cast into plates separated by 0.75 mm spacers in a two step process. Firstly, the resolving gel (9.7% acrylamide, 0.3% bis-acrylamide, 25% resolving buffer (1.5 M Tris-Cl, pH 8.8), 0.1% SDS, 0.1% APS, 0.1% TEMED) was added to the precast plates (approximately 4 ml) and a 200 µl top layer of isopropanol was added to the solution while the gel polymerized. The isopropanol was then removed and the separating gel added (3.9% acrylamide, 0.1% bis-acrylamide, 25% separating buffer (0.5 M Tris-Cl, pH 6.8), 0.1%

SDS, 0.1% APS, 0.1% TEMED). A 0.75 mm comb containing either 10 or 15 lanes was added to the top of the separating gel and the mixture allowed to polymerize for 20 minutes at room temperature.

One quarter volume of SDS-PAGE sample buffer (250 mM Tris-Cl, pH 6.8, 40% glycerol, 8% SDS, 200 mM DTT, 0.5% bromophenol blue) was added to each sample. When protein samples were electrophoresed under non-reducing conditions, no DTT was added to the sample buffer. Protein samples were boiled for 2 minutes before being added to the gel. After the samples had been loaded, the upper and lower chambers of the apparatus were filled with electrode buffer (0.3% M Tris base, 1.44% glycine, 0.1% SDS) and the proteins were electrophoresed at 200 V for 30 minutes.

Once the dye front reached the bottom of the gel, SDS-PAGE gels were removed from the apparatus and shaken in staining solution (35% methanol, 10% glacial acetic acid, 0.05% Coomassie brilliant blue) for 10 minutes at room temperature. The gels were then destained with shaking to remove background staining in a destaining solution (35% methanol, 10% glacial acetic acid) for 16 hours at room temperature. The destained gels were then dried on Whatman #3 chromatography paper using a BioRad slab gel dryer (model #583) at 80°C for 1 hour.

In gels that contained [³⁵S]-methionine, gels were not stained in staining solution, but were incubated in destaining solution for 20 minutes to fix the proteins to the gel. Subsequently, they were incubated in the fluorographic Amplify reagent (Amersham) for 20 minutes on a shaker at room temperature to enhance the detection of radiolabelled proteins. These gels were then dried for 1 hour at 65°C and then exposed to Kodak X-

Omat XAR-1 film at -70°C . The exposed film was then developed after an appropriate period of time (typically after overnight exposure).

2.7.3 Silver Staining of SDS-PAGE Gels

A silver staining kit (BioRad; Oakville, ON) was used to detect small amounts of proteins in some SDS-PAGE gels. In these instances, the gels were electrophoresed as described previously. They were then fixed for 20 minutes in fixative enhancer solution (50% v/v methanol, 10%v/v acetic acid, 10% fixative enhancer solution). The gels were then rinsed twice for 10 minutes in deionized distilled water, and subsequently stained for 20 minutes in staining solution (5% v/v silver complex solution, 5% reduction moderator solution, 5% image development reagent, 50% development accelerator solution). The staining reaction was stopped by incubating the gels for 15 minutes in a 1% acetic acid solution. The gels were then stored in distilled deionized water until photographed or dried.

2.7.4 Western Blot Analyses of AT and HCII

The Hoeffer Electrobloetter was used to blot proteins from SDS-PAGE gels onto either nitrocellulose or PVDF membranes. After SDS-PAGE, the gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol) for 15 minutes at room temperature by slow rocking. Nitrocellulose and Whatman #3 chromatography paper were equilibrated in the same buffer at the same time. The chromatography paper, nitrocellulose and gel were placed together in the electroblotter filled with transfer buffer according to the manufacturer's instructions, and the proteins within the gels transferred to nitrocellulose at 0.2 A, and 16°C for 2 hours. The membrane was then incubated

overnight in TBST supplemented with 5% skim milk powder at room temperature in order to block non-specific protein binding sites.

Western blots (also known as immunoblots) were incubated for 1 hour at room temperature with the primary antibody in TBST supplemented with 5% skim milk powder. The types and dilutions of antibodies used in each Western blot are given in the appropriate figure legends. The blots were then washed three times (5 minutes each) with TBST by gentle shaking. The alkaline-phosphatase conjugated secondary antibodies were then added to a 1/5000 dilution in TBST supplemented with 5% skim milk powder and were shaken over the blots for 1 hour at room temperature. The blots were again washed three times, as described above, and the secondary antibodies were visualized by the addition of BCIP (0.165 mg/ml final concentration) and NBT (0.33% mg/ml final concentration) in water. Colour development was stopped after approximately 10 minutes by the addition of 100 ml of water. The membranes were then dried overnight on Whatman #3 chromatography paper.

2.7.5 Determination of Functional Thrombin Concentration by Active Site

Titration

The functional concentration of thrombin was determined by active site titration using NPGB (Chase Jr. and Shaw, 1969). 7.7 μM thrombin (determined by measuring the optical density at 280 nm and applying Beer's Law to determine protein concentration) was incubated with 50 μM NPGB in veronal buffer (0.1 M barbitol, pH 8.3, 0.02 M CaCl_2) and the change in absorbance at 405 nm was calculated using an

ELISA plate reader. The OD₄₀₅ was multiplied by 6.025×10^{-5} M to give the functional concentration of thrombin, as described in the report by Chase and Shaw.

2.7.6 Formation of AT-Thrombin Complexes with Cell-Free Derived AT

The conditions whereby AT was inhibited by thrombin, were determined by the source and quantity of the recombinant AT being studied (Austin *et al.*, 1990; Sheffield, *et al.*, 1992, Sheffield, *et al.*, 1994a, Sheffield, *et al.*, 1994b). In the case of cell-free translated AT, once it had been dialyzed against TBS overnight, thrombin was added to a final concentration of 300 nM, representing an estimated 300 to 400-fold molar excess of protease compared to AT. Heparin was also added to a final concentration of 2 U/ml, and the incubation was conducted at 37°C. At specific intervals, aliquots were removed and the reaction stopped by the addition of PPACK to a final concentration of 5 µM.

Formation of denaturation-resistant AT-IIa complexes was observed by electrophoresing an aliquot of the reaction on SDS-PAGE and visualizing the radioactive protein bands by autoradiography.

2.7.7 Cleavage of Cell-Free Derived AT by Neutrophil Elastase

Cell-free translated AT that had been dialyzed overnight against TBS, was included in a reaction with 10 U/ml heparin and 420 nM neutrophil elastase, which provided a 300 to 400-fold molar excess of NE relative to cell-free derived AT. These amount of proteins were similar to those reported in earlier studies (Sheffield *et al.*, 1992; Sheffield and Blajchman, 1994a). At specific intervals, aliquots were removed and the reaction stopped by the addition of 5 µM PMSF. Progressive cleavage of AT was

observed by non-reducing SDS-PAGE, and autoradiography and fluorography of dried gels.

2.7.8 Analysis of Thrombin Inhibition by COS-Derived AT

COS-derived AT protein was incubated with a heparin adsorbent (ECTEOLA-cellulose) on a rocking platform at room temperature for 30 minutes prior to centrifugal removal of adsorbent and 100-fold concentration of the conditioned media. Following determination of AT concentration by ELISA, conditioned media contributing 35 nM AT was incubated with 3.5 nM ^{125}I -IIa in the presence or absence of 2 U/ml heparin, at 37°C for 5 minutes. Formation of TAT complexes was detected by SDS-PAGE and autoradiography of dried 10% reducing gels.

Another assay analyzing the ability of COS-derived AT to inhibit IIa involved the addition of media containing 125 nM AT with 25 nM IIa in the presence of 2 U/ml heparin at 37°C for 5 minutes. The reaction was stopped by a 37.5-fold dilution with 100 μM S-2238 chromogenic substrate. Colour generated by cleavage of this substrate was measured at intervals over a 9 minute period by determination of the absorbance at 405 nm in an UltraspecIII (Pharmacia LKB) spectrophotometer. Linear regression was used to quantify the slopes of the colour generation curves; the reduction of the slope, compared to a “no inhibitor” control, was reported as the percentage thrombin inhibition.

2.7.9 Analysis of Elastase Cleavage of COS-Derived AT

Conditioned media containing 125 nM AT was preincubated with 5 nM NE for 9 minutes in the presence of 10 U/ml heparin. Next, the NE was inactivated by the addition of α 1-proteinase inhibitor to a final concentration of 1 $\mu\text{g}/\text{ml}$ for 5 minutes. 25 nM IIa

was then added for 10 minutes. All these reactions occurred at 37°C. The reaction mixture was then diluted 37.5-fold with 100 μ M S-2238 chromogenic substrate and the colour generated by residual IIa of the substrate was measured at intervals over 9 minutes by determination of the absorbance at 405 nm. Again, linear regression was used to quantify the slopes of the colour generation curves. The percentage thrombin inhibition was reported as the reduction in slope, compared to a “no inhibitor” control reaction.

2.7.10 Formation of HCII-Thrombin Complexes with Bacterially-Derived HCII

Bacterially-derived H₆-HCII and H₆- Δ 66HCII were quantified by ELISA and subsequently reacted with thrombin under the following conditions: 1 μ M HCII was incubated with 200 nM IIa in the absence and presence of 2 U/ml heparin, at 37°C for 5 minutes. To stop the reaction, SDS-PAGE sample buffer was added to the reaction. After boiling for 2 minutes, the samples were electrophoresed on SDS gels and the ability of the HCII variants to form inhibitory complexes with IIa was determined by Coomassie staining.

2.7.11 Kinetic Characterization of HCII Inhibition of Thrombin

Pseudo first-order conditions were used to determine the second order rate constants (k_2) for inhibition of IIa by the various HCII variants in the presence or absence of 1 μ M heparin (Olson, *et al.*, 1993). The same conditions were used for full-length HCII as well as Δ 66HCII. In a 96 well dish, 10 μ l aliquots of thrombin (final concentration 7 nM) were incubated for varying intervals with 140 nM HCII in a kinetics buffer (20 mM Na₂HPO₄, pH 7.4, 0.1% polyethylene glycol 8000, 100 mM NaCl and 0.1 mM EDTA). All reactions were arrested by 10-fold dilution of 100 μ M S-2238

chromogenic substrate in kinetics buffer containing 10 mg/ml polybrene. Residual thrombin activity was determined by measuring the absorbance at 405 nm for 10 minutes using a BioTek Instruments EL808 plate reader. The pseudo-first-order constants (k_{obs}) for thrombin inhibition were determined by fitting the data to the equation $k_{obs} \cdot t = \ln([P_o]/[P_t])$, where $[P_o]$ is the initial thrombin activity, and $[P_t]$ is the thrombin activity at time t . The k_{obs} was calculated as the slope of the line created by plotting $\ln([P_o]/[P_t])$ versus time, and the k_2 was then determined by dividing k_{obs} by the HCII concentration used in the assay.

2.7.12 Determination of Stoichiometries of Inhibition of HCII Variants

The stoichiometries of inhibition (SI) were determined for all HCII variants in both the full-length and $\Delta 66$ HCII backgrounds in the presence and absence of 2 U/ml heparin. A constant amount of IIa (200 nM) was incubated with varying amounts of HCII (corresponding to a molar of HCII:IIa of 1.35, 1.125, 0.9, 0.675, and 0.45) in kinetics buffer (20 mM Na_2HPO_4 , pH 7.4, 0.1% polyethylene glycol 8000, 100 mM NaCl and 0.1 mM EDTA) for at least 15 hours at room temperature to ensure that the inhibitory reaction had gone to completion (Olson *et al.*, 1993). The reaction was then diluted 30-fold with 100 μM S-2238 chromogenic substrate in kinetics buffer, and the colour development due to S-2238 cleavage by IIa measured at 405 nm using a spectrophotometer. Residual IIa activity was measured at intervals over 10 minutes and the residual IIa activity was plotted against the molar ratio of HCII:IIa. Linear regression was used to determine a line of best fit, and this line was extrapolated to determine the

molar ratio of HCII:Ila when residual Ila activity was zero. The molar ratio where zero Ila activity resulted was identified as the SI.

2.7.13 SDS-PAGE Based Analysis of Bacterially-Derived HCII Cleavage by Neutrophil Elastase

Bacterially-derived full-length H₆-HCII and H₆-Δ66HCII which had been quantified by ELISA were reacted with NE under the following conditions: 1 μM HCII was incubated with 20 nM NE in TBS in the presence and absence of 10 U/ml heparin (Pratt *et al.*, 1990). At various intervals, aliquots were removed from the reaction and stopped by the addition of SDS-PAGE sample buffer containing 400 mM DTT. The samples were boiled for 2 minutes and the extent of NE cleavage of HCII were analyzed by SDS-PAGE and Coomassie staining.

2.7.14 Kinetic Characterization of Elastase-Treated Bacterially-Derived HCII

Additional analyses of NE cleavage of full-length H₆-HCII and H₆-Δ66HCII were conducted to quantify and differentiate the extent of NE cleavage of different HCII variants. 1 μM HCII was incubated with 1 μM heparin in the presence and absence of 20 nM NE for 10 minutes at 37°C. α₁-proteinase inhibitor was then added to a final concentration of 100 nM for 5 minutes (at 37°C) to inactivate the NE. The HCII reaction was diluted 4 times with 14 nM Ila and incubated at room temperature over various time intervals. The reaction of Ila with HCII was stopped by 10-fold dilution with 100 μM S-2238 in kinetics buffer. Residual Ila activity was measured at intervals over 10 minutes by determining the absorbance at 405 nm using a spectrophotometer. The pseudo-first order rate constant (k_{obs}) was determined by fitting the data to the equation

$k_{obs} \cdot \ln([P_o]/[P_t])$, where $[P_o]$ is the initial thrombin activity, and $[P_t]$ is the thrombin activity at time t . The k_{obs} was calculated as the negative slope of the line created by plotting $\ln([P_o]/[P_t])$ versus time. Since the residual functional HCII concentration could not be determined after NE incubation, a second order rate constant could not be determined. However, the k_{obs} determined for all HCII variants was compared to the k_{obs} of HCII which had not been incubated with NE.

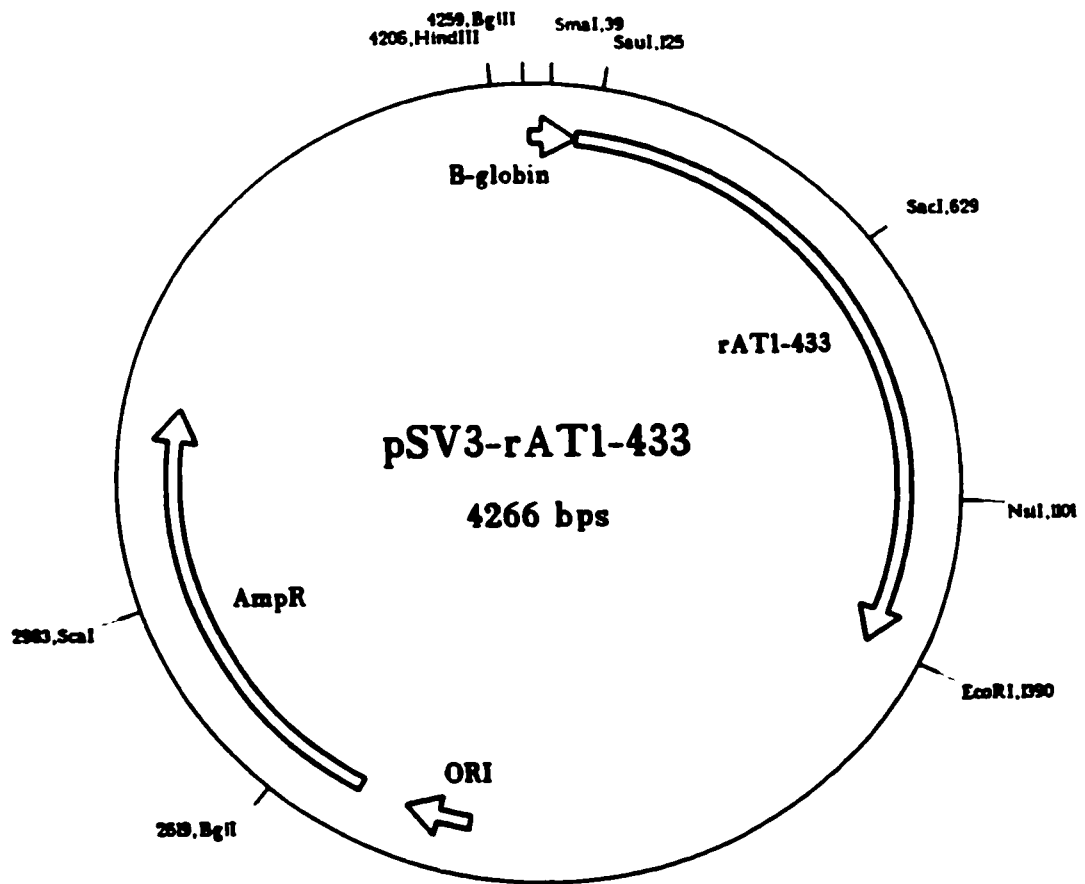
3. RESULTS

3.1 Cell-Free Expression of Rabbit AT P4 Variants

3.1.1 Expression of Rabbit AT Variants

The interactions of rabbit AT with α -thrombin and NE were investigated by expression of wild-type and variant forms of AT in a cell-free expression system, previously established in our laboratory (Austin *et al.*, 1990). The plasmid pSV3-rAT-III₁₋₄₃₃ (Figure 4) was used to create, by site-directed mutagenesis, six variant AT proteins, mutated at amino acid 390 (P4) from wild-type isoleucine (Ile) to serine (Ser), glutamic acid (Glu), arginine (Arg), glycine (Gly), phenylalanine (Phe), and tryptophan (Trp). The substituted residues are representative of all classes of amino acids, from the hydrophobic Ile, to polar uncharged (Gly, Ser), positively charged (Arg), negatively charged (Glu), and bulky hydrophobic (Phe, Trp). DNA sequencing in all cases (data not shown) confirmed successful alteration of the coding regions.

The wild-type and variant AT cDNAs were expressed by *in vitro* translation of the products of the *in vitro* transcription reactions, using an mRNA-dependent rabbit reticulocyte lysate supplemented with [³⁵S]-methionine. Figure 5 shows an autoradiogram of a non-reducing SDS gel of the major radiolabelled peptides synthesized in the presence of wild-type rabbit AT mRNA. All AT variants had an apparent molecular mass of 47 kilodaltons (kDa), which is in agreement with the predicted relative



Control Wild-type

66.4 -

55.6 -

42.7 -

36.5 -

26.6 -



free system was used in the initial characterization of the P4 mutants, with respect to their abilities to inhibit thrombin as well as for their susceptibilities to elastase cleavage.

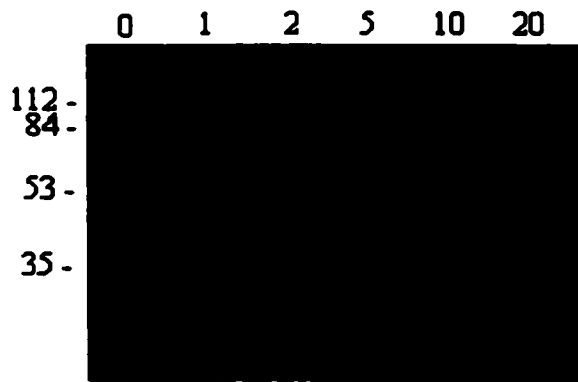
3.1.2 Inhibition of Thrombin by Cell-Free-Derived Rabbit AT Variants

To evaluate the ability of cell-free derived rabbit AT to inhibit thrombin, a previously established protocol was used (Austin *et al.*, 1990). Cell-free translation products were incubated with 300 nM thrombin, an estimated 300 to 400-fold molar excess of protease compared to AT (based on ELISA data), in the presence of saturating (2U/ml) sodium heparin, at 37°C. The reaction was followed over time, with thrombin being instantaneously quenched by excess PPACK, using SDS-PAGE and autoradiography.

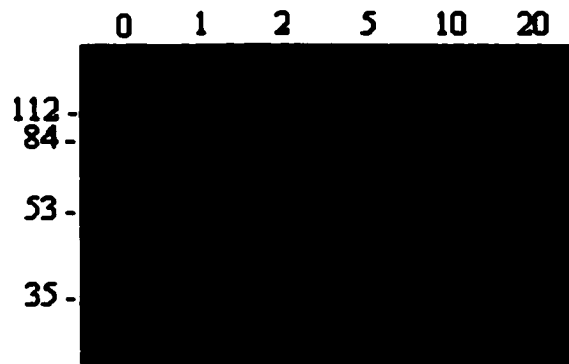
Functional AT forms a denaturation-resistant inhibitory complex with thrombin, in a reaction which is greatly accelerated in the presence of heparin. As shown in Figure 6A, reaction of the 47 kDa cell-free derived rabbit AT with α -thrombin led to the formation of two products: an 84 kDa thrombin-dependent band previously shown to be the thrombin-antithrombin (TAT) complex (Austin *et al.*, 1990); and a 50 kDa band, corresponding to AT cleaved at the reactive centre. The cleaved product migrated with decreased mobility relative to intact AT due to the use of non-reducing electrophoretic conditions.

Identical experiments to those shown in Figure 6A were performed, in triplicate, for both cell-free derived wild-type rabbit AT and the six mutated products. Complex formation with thrombin, as a proportion of intact AT starting material, was determined and compared after 2 minutes of reaction, and plotted relative to wild-type activity (Table

A



B



2). Since complex formation is still increasing linearly with time at this point in the reaction, this value is proportional to the initial rate. Quantitation of [³⁵S]-methionine-labeled AT species was performed using laser densitometry and quantitation software. The Glu and Arg variants exhibited 2-3 fold reductions in thrombin-complexing ability, while the Ser variant failed to form detectable complexes; the Trp variant was unaffected, while Phe and Gly variants showed modest decreases in thrombin reactivity (Table 2).

3.1.3 Reaction of Elastase with Cell-Free Derived Rabbit AT Variants

To evaluate the susceptibility to NE cleavage, cell-free derived rabbit AT was incubated with 420 nM NE, which corresponded to an estimated 300 to 400-fold molar excess of NE compared to AT, in the presence of 10 U/ml heparin. At specific intervals, aliquots were removed and the reaction was stopped by addition of PMSF to 5 μM. All reactions were performed at 37°C. Again, the samples were subsequently electrophoresed by SDS-PAGE, and the radioactive bands visualized on dried gels by autoradiography.

In contrast to the reactions between thrombin and rabbit AT, the reaction of NE with wild-type rabbit AT resulted only in the formation of a cleaved product, which corresponds to AT cleaved in the reactive centre (Figure 6B). Similarly to the cleaved AT product incubated with thrombin, the NE-cleaved product migrated with decreased mobility relative to intact AT, due to the use of non-reducing electrophoresing conditions.

Identical experiments to those shown in Figure 6B were performed, in triplicate, for both cell-free derived wild-type rabbit AT, and the six mutated products. Cleavage of AT by NE, as a proportion of intact AT starting material, was determined

Rabbit AT Mutant	TAT Complex (%)	NE Cleavage (%)
Wild-type	100 ± 15	100 ± 6
I390S	0	13 ± 1
I390E	40 ± 6	78 ± 4
I390R	46 ± 5	96 ± 4
I390G	76 ± 8	160 ± 10
I390F	82 ± 3	78 ± 5
I390W	110 ± 10	80 ± 14

densitometrically after 2 minutes of reaction, and plotted relative to wild-type AT cleavage. The results are shown in Table 2. The proportion of starting AT converted to cleaved AT by NE in 2 minutes was taken as a measure of the initial rate of NE-mediated AT inactivation. The Arg and Trp P4 mutants show cleavage levels similar to wild-type over time, and Glu and Phe variants showed modest decreases in NE sensitivities. The Gly variant exhibited increased susceptibility to NE cleavage, while the Ser variant reacted only minimally with NE. The Trp variant most closely resembled wild-type rabbit AT with respect to its ability to inhibit thrombin and to its susceptibility to NE cleavage, while the Ser variant was largely unreactive with either thrombin or NE. These two variants were subsequently selected for expression in cultured cells.

3.2 Expression of Rabbit AT P4 Variants in COS-1 Cells

3.2.1 Reaction of Plasma-Derived Rabbit AT with Neutrophil Elastase

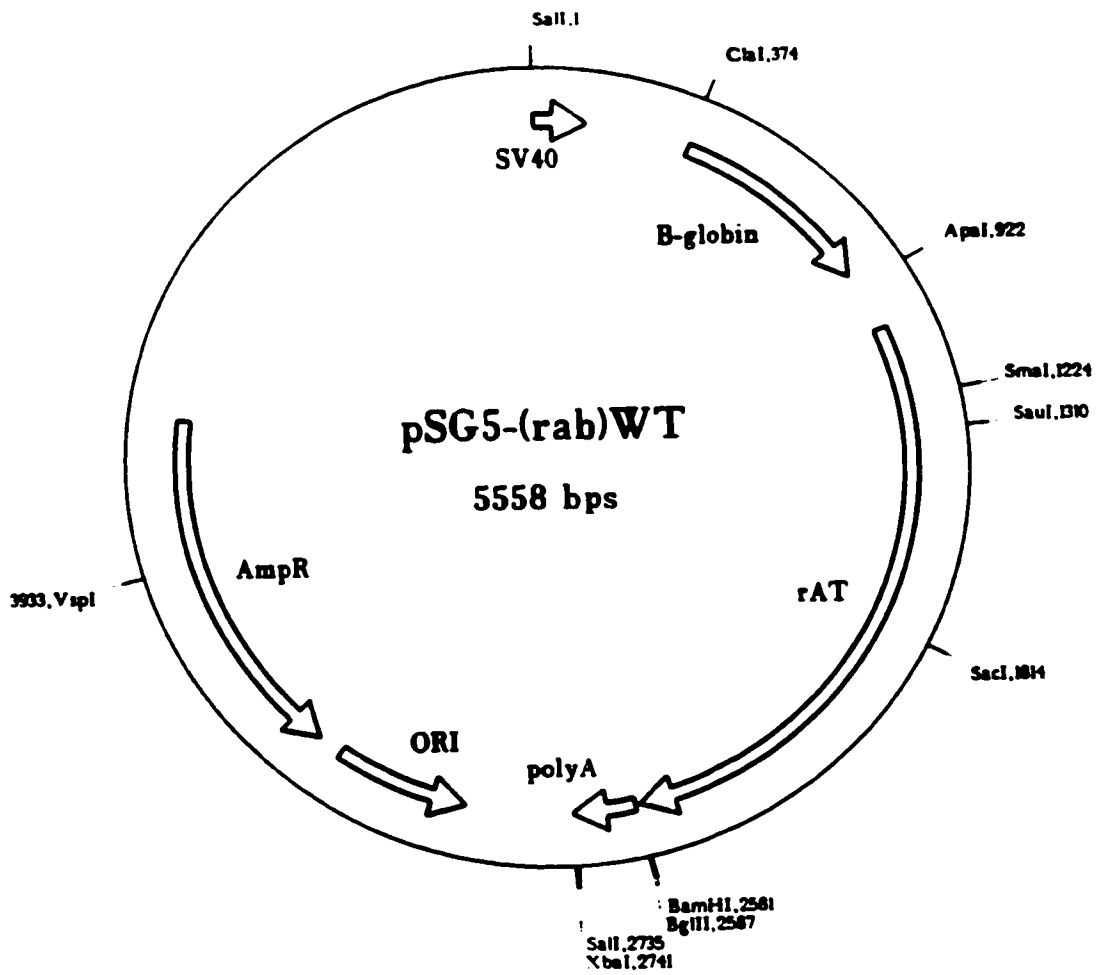
While NE-mediated inactivation of human AT had been well described (Carrell and Owen, 1985, Jordan *et al.*, 1987), and although we had shown some effect of large excesses of NE on recombinant rabbit AT mobility (Figure 6B), no other data were available concerning the reaction of rabbit AT with NE. The control experiments shown in Figure 7 were therefore undertaken. As previously reported, the inactivation of AT by NE was heparin-dependent, since formation of the 94 kDa TAT complex was unaffected by preincubation with NE in the absence of heparin, but abrogated in its presence (Figure 7, left panel). Similarly, rabbit AT was unaffected by incubation with NE pre-inhibited by α_1 -proteinase inhibitor, in that it still formed TAT complexes; complex formation was abrogated, however, by preincubation with functional NE (Figure 7, middle panel). An

increased mobility of both rabbit α - and β -AT isoforms was noted (Figure 7, middle panel, compare lanes 2 and 5), consistent with cleavage of AT and loss of the disulfide bonded chain under reducing conditions; this difference was also seen following removal of the glycans by PNGase F (Figure 7, right panel, compare lanes 2 and 3).

Having demonstrated that plasma-derived rabbit AT, at physiological concentrations, was inactivated by NE in the presence of heparin, we sought to establish conditions to assay NE-reactivity with much lower nM concentrations of the recombinant inhibitor. This strategy was necessary because of the low levels of recombinant AT that were attainable from transfected COS-1 cells. Reducing the AT concentration from 2 μ M to 125 nM plasma-derived AT, while keeping the NE, heparin and time fixed, reduced the magnitude of the NE-mediated AT inactivation. However, a small but statistically significant ($p < 0.01$ (Student's t-test, two-tailed, unpaired)) decrease in the extent of AT-mediated IIa inhibition remained (data not shown).

3.2.2 Expression of Rabbit AT P4 Variants

In order to gain some insight into whether the results in the cell-free system could be extrapolated to glycosylated, optimally-folded AT, expression plasmids were constructed to permit the expression of selected P4 AT mutants in COS-1 cells. The previously described plasmid, pSG5(rab)WT (Sheffield *et al.*, 1995), was employed to create appropriate eukaryotic expression constructs for use in COS-1 cells. Specifically, a 5' 980 bp *EcoRI-NcoI* restriction fragment of pSG5(rab)WT was combined with a 3' 546 bp *NcoI-EcoRI* restriction fragment of pSV3-rAT-III₁₋₄₃₃(I390X), where X was S or W, in a three-part ligation (Figure 8). Again, standard ligation procedures and



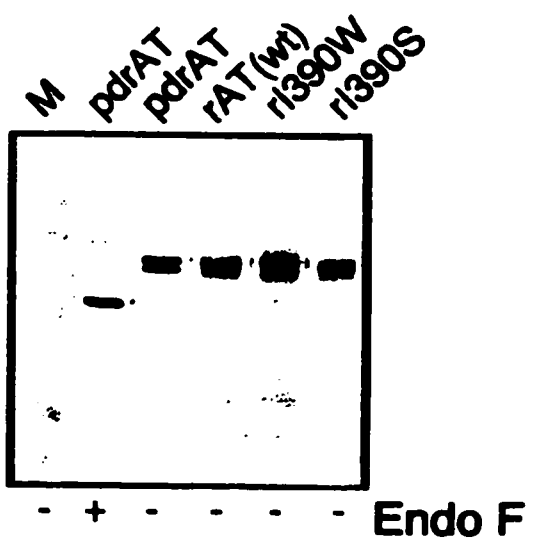
verification by DNA sequencing were conducted, as described previously. This three-part ligation was performed in order to add back the AT secretory signal sequence lacking in the pSV3-AT-III₁₋₄₃₃ constructs, which contained the point mutants at P4.

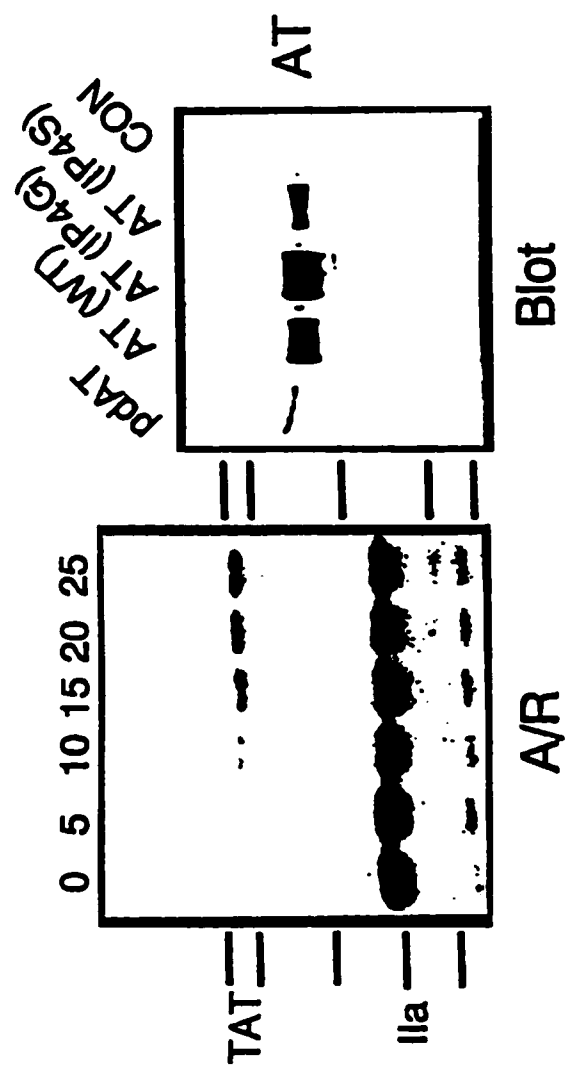
Transfection of COS-1 cells with constructs pSG5(rab)WT, pSG5(rab)I390S, and pSG5(rab)I390W resulted in the appearance in media conditioned by these cells of secreted proteins that co-migrated with plasma-derived rabbit AT on immunoblots employing affinity-purified, monospecific anti-AT antibodies (Figure 9). Conditioned media from non-transfected cells exhibited no immunoreactive products.

3.2.3 Inhibition of Thrombin by Rabbit AT P4 Variants

To evaluate the ability of COS-derived rabbit AT to inhibit thrombin, conditioned media from transfected COS-1 cells was first incubated for 30 minutes with a heparin adsorbent, ECTEOA-cellulose, to remove endogenous glycosaminoglycans that may have been secreted from the cells, and which could potentially affect the ability of COS-derived AT to inhibit thrombin. The conditioned media was then concentrated 100-fold and the concentration of rabbit AT was determined by ELISA. Initially, conditioned media contributing wild-type rabbit AT was incubated with equimolar [¹²⁵I]-IIa in the presence or absence of 2 U/ml heparin, at 37°C for 25 minutes. Reaction of this material led to TAT complex formation over time, such that approximately 50% of the initial thrombin had been consumed after 25 minutes of the reaction (Figure 10), demonstrating the functional expression of wild-type rabbit AT in COS-1 cells.

Subsequently, a more quantitative analysis of the extent of thrombin inhibition by variant rabbit AT was conducted. Conditions that had initially been established



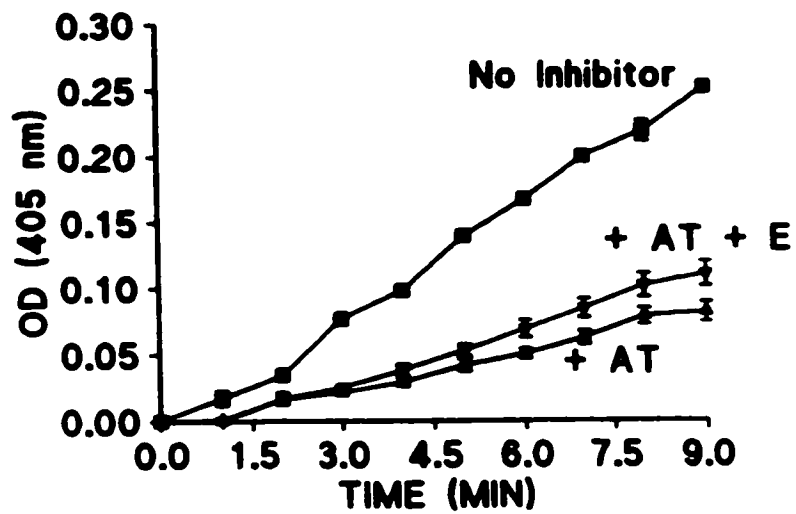
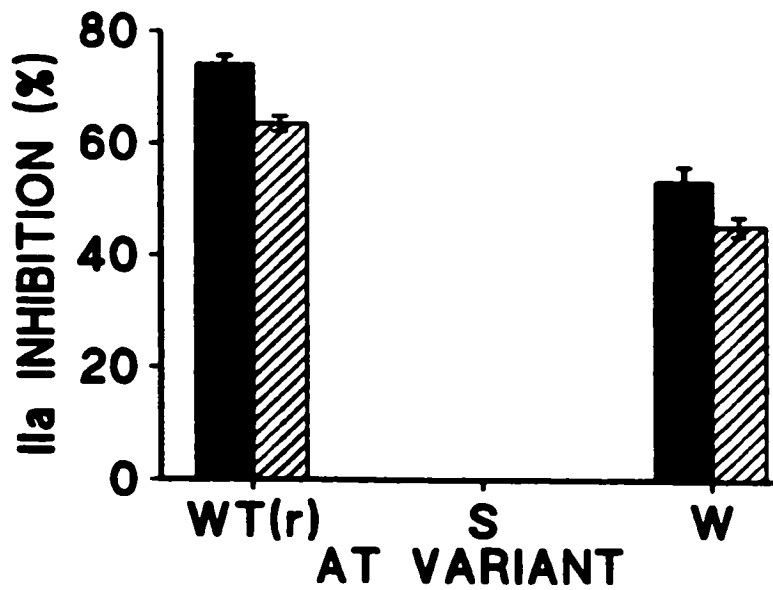


(described above) with plasma-derived AT were used. Specifically, 125 nM COS-derived AT was incubated with 25 nM IIa in the presence of 2 U/ml heparin for 5 minutes at 37°C. The reaction was stopped by a 37.5-fold dilution into 100 µM S-2238 chromogenic substrate. Residual thrombin activity was determined by measuring the spectrophotometric conversion of the chromogenic substrate by thrombin at 405 nm at intervals over 9 minutes (Figure 11A). Linear regression was used to quantify the slopes of the colour generation curves; the reduction of the slope, compared to a control lacking AT, was reported as the percentage thrombin inhibition.

As shown in Figure 11B, incubation of thrombin with both wild-type and I390W rabbit AT resulted in thrombin inhibition of approximately 77 and 49%, respectively. These observations demonstrated a modest decrease in the degree of thrombin inhibition (of $28 \pm 2\%$, $p=0.02$ (Student's t-test, two-tailed, unpaired)) by the Trp variant relative to wild-type. In contrast, the Ser variant showed no ability to inhibit thrombin. Trivial explanations for the differences in reactivity of the Ser and Trp mutants were ruled out by the demonstration that both proteins are intact, and of expected molecular mass (Figure 9).

3.2.4 Reaction of Neutrophil Elastase with Rabbit AT P4 Variants

The reaction of elastase with COS-derived rabbit AT was analyzed using a procedure similar to that used to evaluate the ability of rabbit AT to inhibit thrombin. In this two-step reaction, a 25-fold molar excess of rabbit AT was preincubated with NE for in the presence of 10 U/ml heparin. The NE was then inactivated by the addition of α_1 -proteinase inhibitor. Thrombin was then added and the reaction was stopped by dilution

A**B**

into S-2238 chromogenic substrate. Residual thrombin activity was determined by measuring the spectrophotometric conversion of the chromogenic substrate by thrombin at 405 nm over time. Linear regression was used to quantify the slopes of the colour generation curves; the reduction of the slope, compared to a “no inhibitor” control, was reported as the percentage thrombin inhibition.

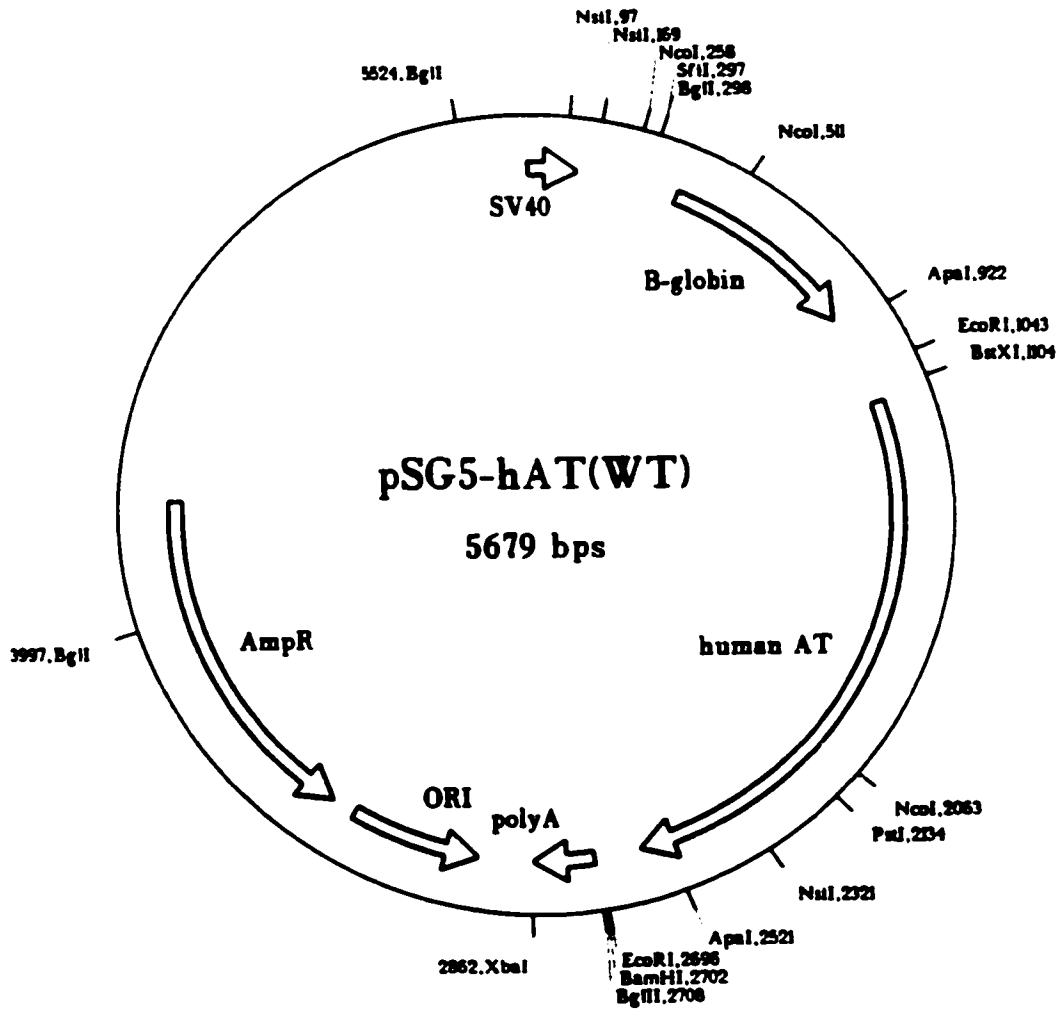
As shown in Figure 11B, the thrombin-inhibitory activities of both wild-type and the Trp variant were reduced by 14 to 16% by NE pretreatment, compared to COS-derived AT which had not been pretreated with NE. However, since the Ser variant lacked any ability to inhibit thrombin, any NE effect could not be determined.

3.3 Expression of Human AT Variants in COS-1 Cells

3.3.1 Expression of Human AT Variants

The retention of NE sensitivity by the rabbit P4 Trp variant suggested the possible role of at least one other site. In human AT, a second site of NE cleavage, C-terminal to Val389 (P5) had been shown to be equally susceptible to NE, *in vitro* (Owen and Carrell, 1985). Unlike P4, P5 is not identical in rabbit and human AT, so subsequently single and double P4 and P4/P5 Trp substitutions mutants were incorporated into recombinant human AT.

The human AT cDNA in plasmid pSG5-AT(WT) (Sheffield *et al.*, 1994b) (Figure 12) was subjected to site-directed mutagenesis, employing a restriction site elimination protocol. The I390W mutation was created using primer AB8623, while the V389W, I390W double mutation was created using primer AB8624. Appropriate mutagenesis



was again confirmed by DNA sequencing and the eukaryotic expression plasmids designated pSG5-AT-(V389W,I390W) and pSG5-AT-(I390W).

Transfection of COS-1 cells with constructs pSG5-AT-(V389W,I390W) and pSG5-AT-(I390W) resulted in the appearance in media conditioned by these cells of secreted proteins that co-migrated with plasma-derived human AT on immunoblots employing affinity-purified, monospecific anti-AT antibodies (Figure 13). Again, conditioned media from non-transfected cells exhibited no immunoreactive products.

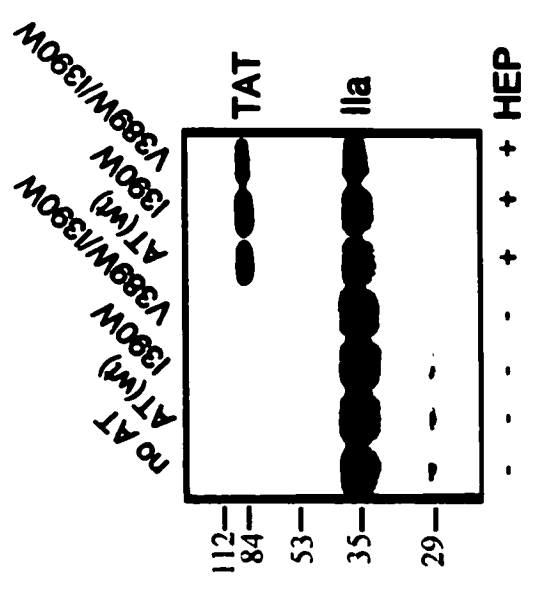
3.3.2 Inhibition of Thrombin by Human AT Variants

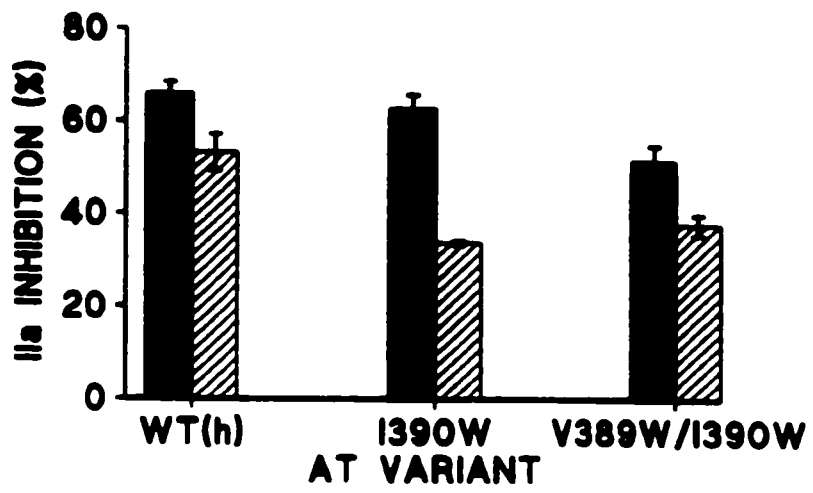
The thrombin-inhibitory activity of COS-derived human AT variants was initially confirmed by demonstrating their abilities to form inhibitory complexes with thrombin (Figure 14). Formation of TAT complexes was detected by electrophoresis and autoradiography of dried SDS-PAGE gels. As shown in Figure 14, the formation of visible TAT complexes is heparin-dependent under the conditions employed. In addition, wild-type, I390W, and V389W, I390W human AT variants all retain thrombin-inhibitory activity.

A more quantitative analysis of thrombin inhibition by COS-derived human AT variants was performed, as described in Section 3.2.2. The extent of thrombin inhibition, compared to a control lacking AT, by the human AT mutants is shown in Figure 15 and compared to the thrombin-inhibitory activity of COS-derived wild-type human AT. Both human AT mutants maintain thrombin-inhibitory activities close to that observed with the wild-type protein. Specifically, incubation of thrombin with wild-type, I390W, and V389W, I390W human AT variants resulted in thrombin inhibition of 66, 63 and 51%,

M Pd AT
AT(wt)
I390W
V389W/I390W







respectively. As predicted by the results of the mutagenesis in the rabbit AT background, human I390W showed no impairment in reactivity with thrombin, although a modest decrease in thrombin-inhibitory activity of 22% ($p=0.02$ (Student's t-test, two-tailed, unpaired)) was observed for the double mutant.

3.3.3 Reaction of Neutrophil Elastase with Human AT Variants

The interactions of NE with COS-derived human AT variants were analyzed using the procedure described in Section 3.2.4. The effect of NE pretreatment on the thrombin-inhibitory activity of the human AT variants is shown in Figure 15. Incubation of wild-type human AT with NE prior to its reaction with thrombin, resulted in a 20% statistically significant ($p\leq 0.05$ (Student's t-test, two-tailed, unpaired)) reduction in thrombin-inhibitory activity, compared to the untreated serpin. Interestingly, the substitution of Trp at P4 appears to have created an apparent increase in NE degradation, given that NE pretreatment of I390W AT resulted in a 46% reduction in thrombin-inhibitory activity, compared to the untreated protein. Conversely, NE pretreatment of the V389W, I390W AT variant caused a 26% reduction in thrombin-inhibitory activity, compared to the untreated double Trp mutant, which was not statistically significantly different from the susceptibility of wild-type human AT to NE cleavage. Therefore, in spite of the alteration of both NE cleavage sites, no reduction in NE sensitivity was observed in the double Trp mutant at P4 and P5 in human AT. The results of the P4 mutagenesis in rabbit AT, and the P4/P5 mutagenesis in human AT are reported in the study by Cunningham, *et al.* (1997).

3.4 Expression of Human HCII Variants

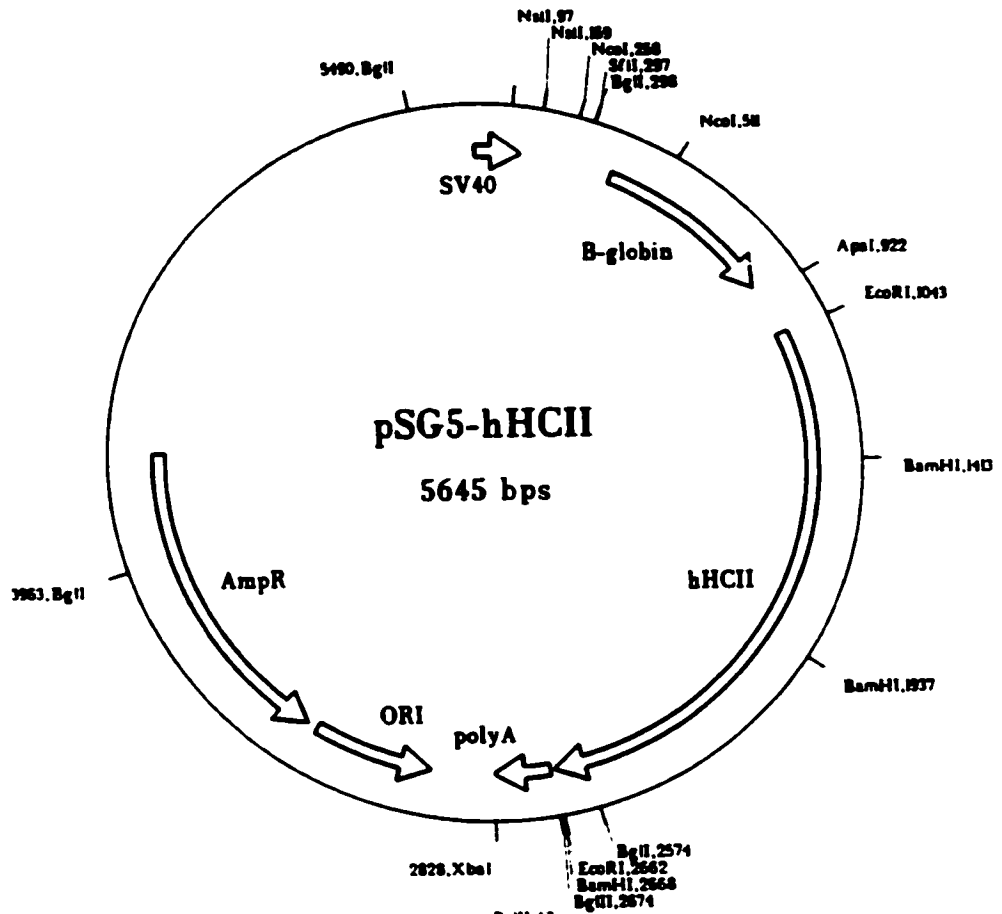
3.4.1 Construction and Expression of Eukaryotic Human HCII Expression Vector

A pSG5-HCII expression vector was constructed initially in order to generate another serpin susceptible to NE inactivation in the same experimental system as that employed for AT, transiently transfected COS-1 cells (Figure 16). Clearer objectives were thought to be possible by working with HCII, which was reported to possess a single NE cleavage site in the reactive centre loop (Pratt *et al.*, 1990), in contrast to the two sites in AT.

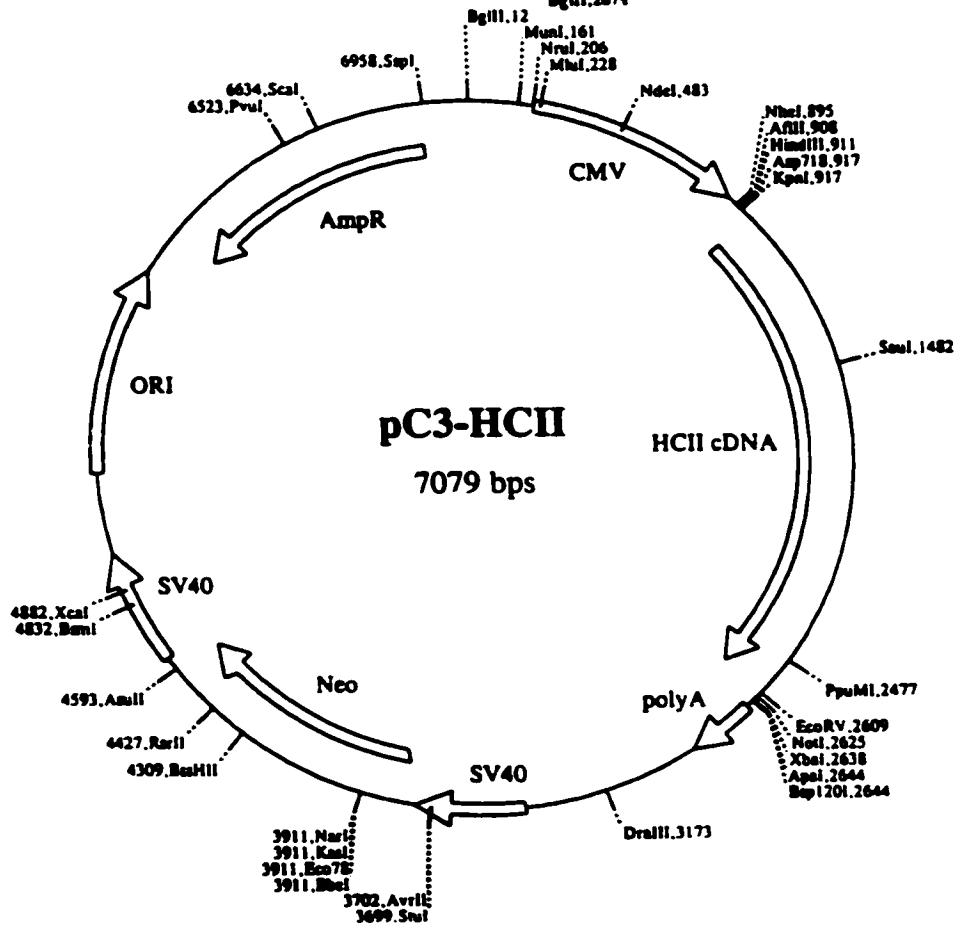
Accordingly, the HCII expression plasmid was constructed by transferring a cDNA fragment containing the entire coding region of human HCII, an *EcoRI* fragment of pHCI7.2 (Blinder *et al.*, 1988), into the corresponding site of pSG5. A subclone with the insert appropriately oriented was designated pSG5-HCII(WT), and was used as the template plasmid for site-directed mutagenesis (Figure 16), where amino acid substitutions of Cys, Glu, Phe, Gly, Leu, Arg, Ser, and Trp for the wild-type Ile were created at residue 439 (P6). As in the AT P4 experiments reported above, the P6 substitutions were representative of all classes of amino acids, and were confirmed by DNA sequencing (not shown).

Because the results with COS-derived AT reported above suggested that larger quantities of recombinant serpin would be required to complete the analysis in a rigorous and quantitative manner, the background vector was altered prior to transfection. To increase potential yields of HCII, we sought to develop permanently transfected cell lines producing HCII and variants. As stable COS-1 cell lines had not been reported, the

A



B



cDNAs encoding wild-type and P6 variants of human HCII were transferred from the pSG5 background to the pCDNA3.1 expression vector, one suitable for permanent transfection of Baby Hamster Kidney (BHK) cells.

Subsequently, the wild-type and P6 variant human HCII cDNAs were restricted by *EcoRI* and isolated from their pSG5-HCII plasmids, and then ligated to the plasmid pCDNA3.1, which had been restricted with *EcoRI*. This was necessary in order to make stably transfected BHK cell lines, which would be resistant to the antibiotic, geneticin (G418), and would also produce human HCII variant proteins. BHK cells were transfected with pCDNA3.1-HCII plasmids, and G418-resistant cells were isolated from BHK cells that did not harbour the expression plasmid. Subsequent analysis of serum-free BHK-conditioned media by HCII ELISA, however, revealed that HCII was expressed at very low levels. Specifically, the G418-resistant cell line that showed the highest level of HCII expression gave only 250 ng/ml (5 pM) of HCII, as determined by ELISA (data not shown). Given the need for larger amounts of protein, HCII was subsequently expressed in bacteria. Previous studies (Blinder, *et al.*, 1988) had demonstrated the expression of large amounts of functional HCII in bacteria, which suggested that expression of human HCII variants in bacteria would yield sufficient protein to conduct an extensive study of HCII and its reaction with both thrombin and NE.

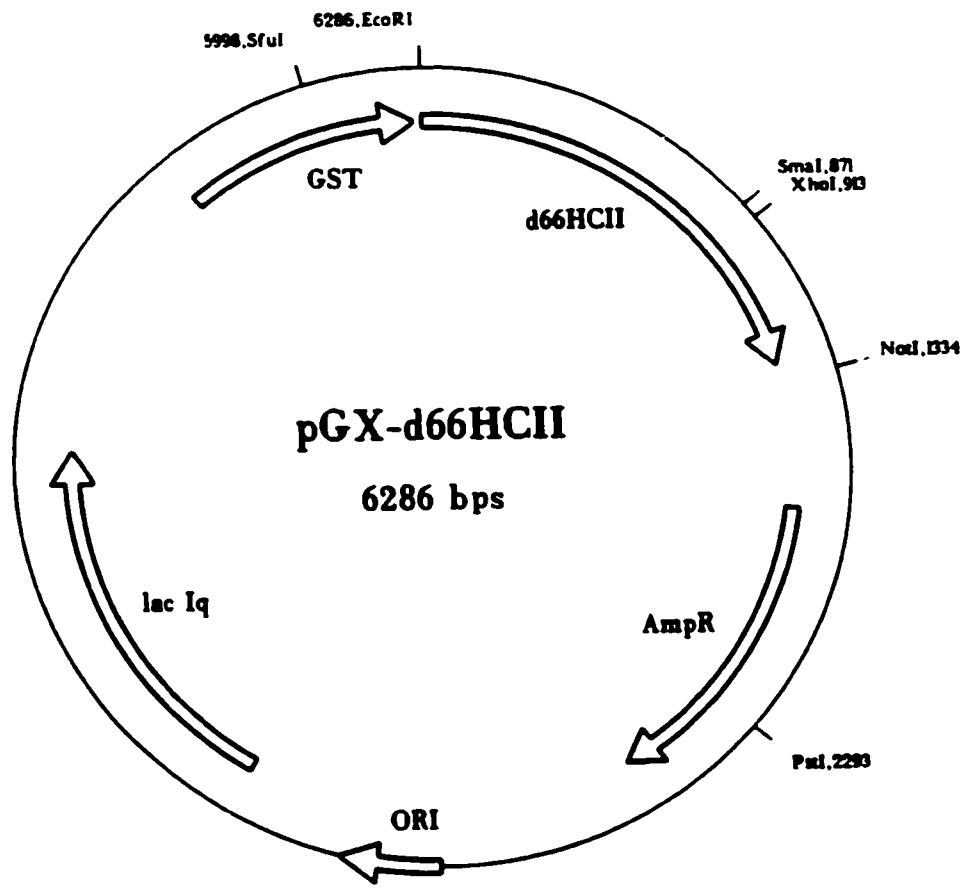
3.4.2 Construction and Expression of the pGEX-5X-1- Δ 66HCII Expression Vector

The commercially available vector, pGEX-5X-1 was the first vector we used to express human HCII variants in bacteria. Insertion of Δ 66HCII into this vector created a

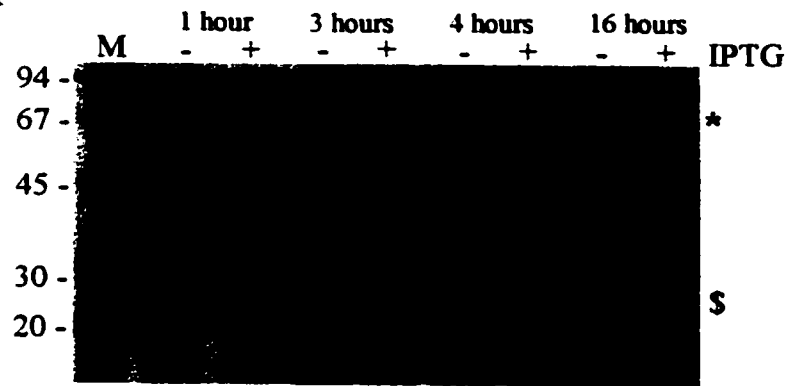
fusion protein whereby GST codons were fused in-frame to codons specifying $\Delta 66\text{HCII}$. Codons specifying a factor Xa cleavage site were incorporated between the two cDNA moieties, in order to permit the potential removal of GST from the resulting fusion protein after purification on glutathione-Sepharose. GST occurs naturally as a 26 kDa protein that can be efficiently expressed in functional form in *E. coli*. Previous studies have shown that fusion proteins that contain the GST moiety retain GST enzymatic activity (Smith and Johnson, 1988; Parker *et al.*, 1990), and that expression of GST fusion proteins have provided an efficient means of bacterial protein expression for studies involving protein-protein interactions (Kaelin, *et al.*, 1991).

$\Delta 66\text{HCII}$ was created from a full-length HCII cDNA by PCR, and inserted into the multiple cloning site of pGEX-5X-1 (Figure 17). Appropriate construction of pGEX-5X-1- $\Delta 66\text{HCII}$ was confirmed by DNA sequencing. After transformation of competent BL-21 *E. coli* cells, the total protein profile of bacterial cultures that had been induced with IPTG was examined (Figure 18A). Two proteins appeared to increase in abundance during induction of 76 and 26 kDa.

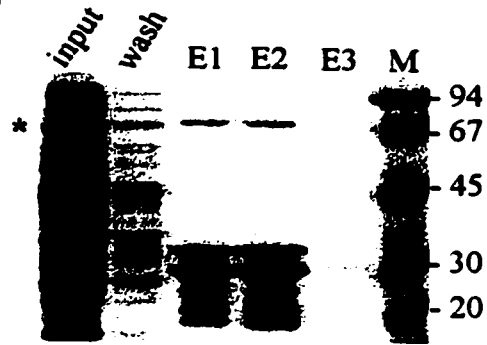
Figure 18B shows that partial purification of the GST $\Delta 66\text{HCII}$ fusion protein was achieved by employing glutathione-Sepharose affinity chromatography. Specific elution of the affinity column with reduced glutathione yielded a fraction containing a 76 kDa protein, as expected for the GST $\Delta 66\text{HCII}$ fusion protein. This 76 kDa band was a minor component of the eluate; as shown in Fig. 18, the majority of the eluate was made up of proteins migrating between the 37 and 25 kDa markers. Immunoblotting with anti-HCII



A



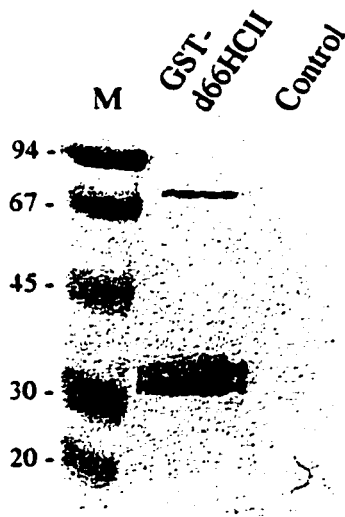
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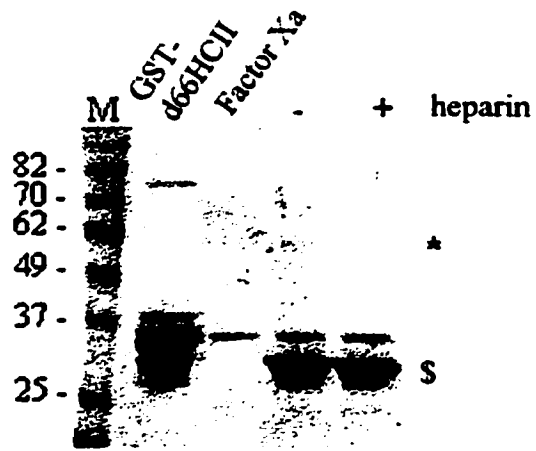
antibodies, as shown in Figure 19, showed that the 76 kDa band and at least the major 37-25 kDa components contained at least portions of d66HCII.

The GST- Δ 66HCII-containing eluate was cleaved by Factor Xa under the following conditions: approximately 1 μ M GST- Δ 66HCII was incubated at 37°C with 3 U of factor Xa for 16 hours in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM CaCl₂. SDS gel analysis revealed conversion of the 76 kDa band to a 50 kDa anti-HCII-reactive band, and conversion of several bands in the upper 37 to 25 kDa range to a single prominent 26 kDa species (Fig. 20A) which co-migrated with unfused GST (not shown) and lacked HCII antigens (Figure 20B). Interestingly, the 50 kDa form of HCII liberated by Xa cleavage was actually a doublet, possibly indicating that Xa cleaved not only its engineered recognition site but also within the HCII protein domain.

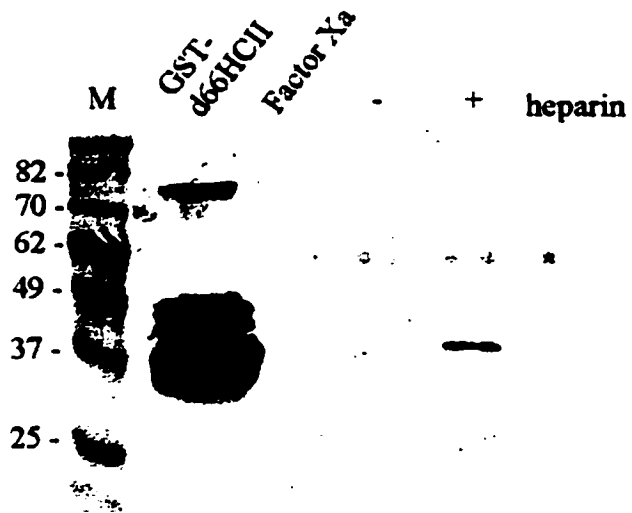
Subsequent analysis of the GST- Δ 66HCII fusion protein was conducted, and its interaction with α -thrombin was characterized. The following reaction was performed at 37°C: 50 nM GST- Δ 66HCII was incubated with 5 nM [¹²⁵I]-IIa in the presence of 2 U/ml heparin for various times. As shown in Figure 21A, GST- Δ 66HCII appears to form an inhibitory complex with thrombin in that a radiolabelled protein band of approximately 112 kDa appears on the autoradiogram. However, the incubation time needed to visualize this 112 kDa thrombin-inhibitory complex was between 60 and 90 minutes and was dependent on the presence of heparin. The interaction of a five-fold molar excess of GST- Δ 66HCII with unlabeled thrombin was analyzed again, in the presence of heparin and visualized by Coomassie-stained SDS-PAGE gels (Figure 21B). A thrombin-



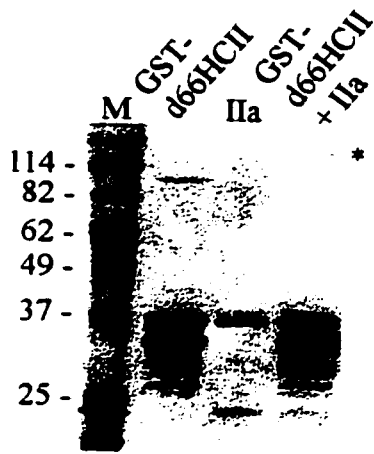
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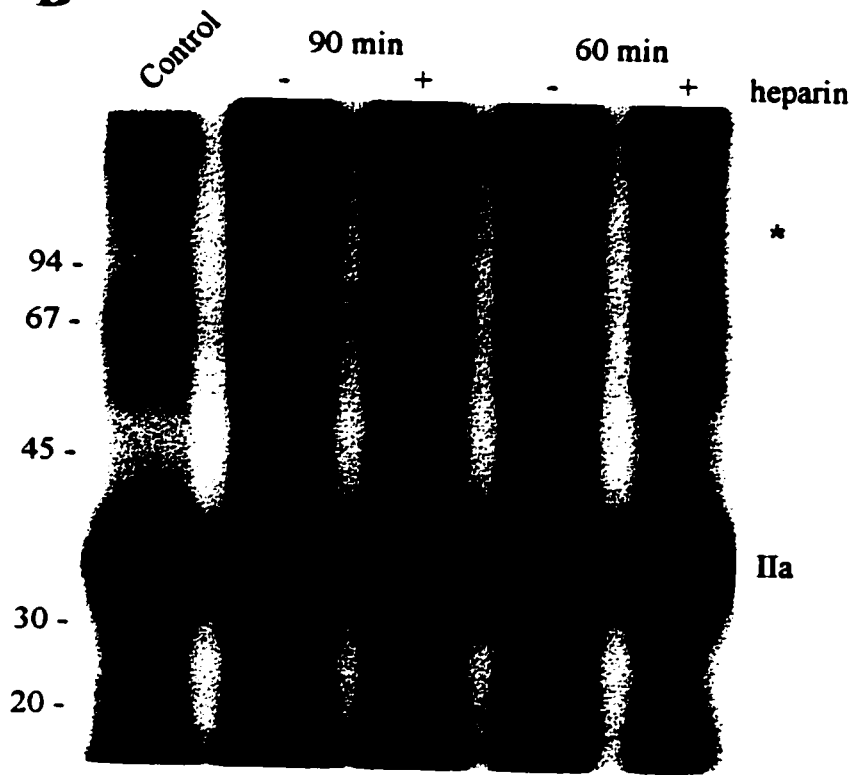
B



A



B



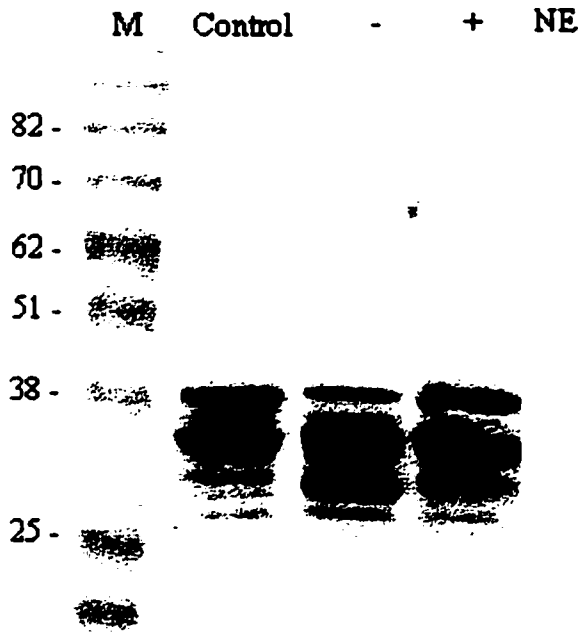
dependent band of approximately 120 kDa was observed, although this band was not abundant on the stained gel.

Next, the reaction of GST- $\Delta 66$ HCII with NE was analyzed under the following conditions: approximately 1 μ M GST- $\Delta 66$ HCII was incubated with 10 nM NE in the presence and absence of 10 U/ml heparin at 37°C for 10 minutes. Aliquots of the reaction were electrophoresed on SDS-PAGE and the products of the reaction were visualized by Coomassie staining. Figure 22 shows that GST- $\Delta 66$ HCII is susceptible to NE cleavage in a heparin-dependent manner. However, extensive proteolysis appeared to occur, and this complicated the detection of any NE cleavage within the reactive centre loop of HCII.

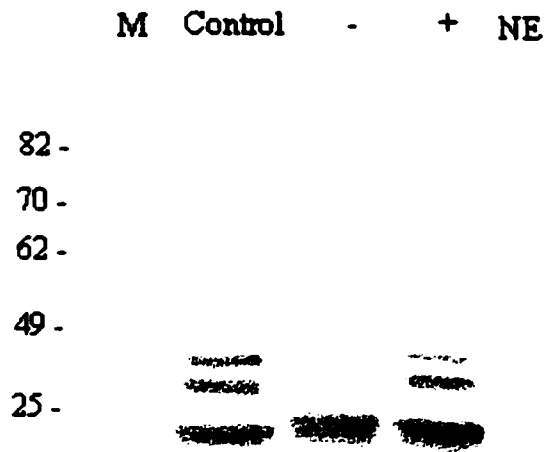
3.4.3 Construction and Expression of pBAD(B)-H₆-HCII and pBAD(B)-H₆- $\Delta 66$ HCII

Another commercially-available prokaryotic expression vector, pBAD(B), was used to try and express significant levels of HCII variants in bacteria. It was hoped that the use of a different bacterial expression system might reduce the proteolytic activity observed using the GST gene fusion system described above and yield larger amounts of functional protein. Insertion of H₆- $\Delta 66$ HCII into pBAD(B), described in Section 2.6.3, created an expression vector with the potential for arabinose-inducible expression of H₆- $\Delta 66$ HCII in bacteria. $\Delta 66$ HCII was created from full-length HCII, by PCR, using plasmid pSG5-HCII(WT), and the PCR product was inserted into the multiple cloning site of pBAD(B). Appropriate construction of pBAD(B)-H₆- $\Delta 66$ HCII (Figure 23A) was confirmed by DNA sequencing (data not shown). After transformation of competent

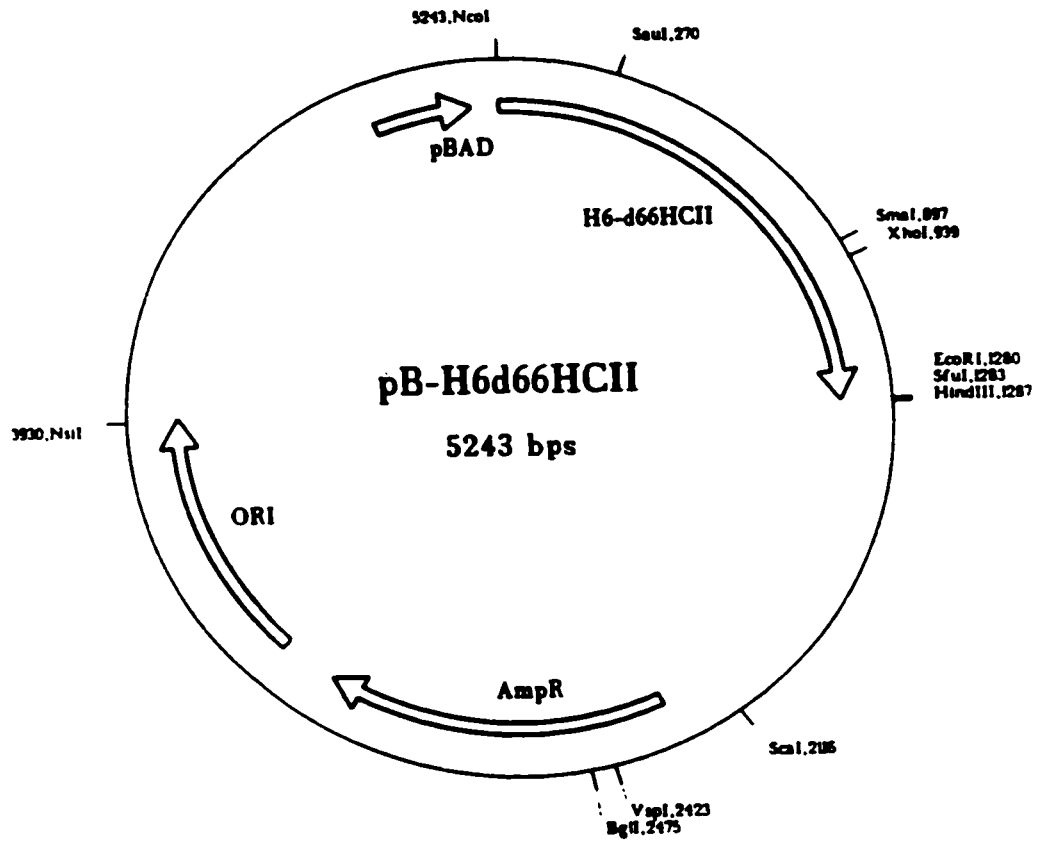
A



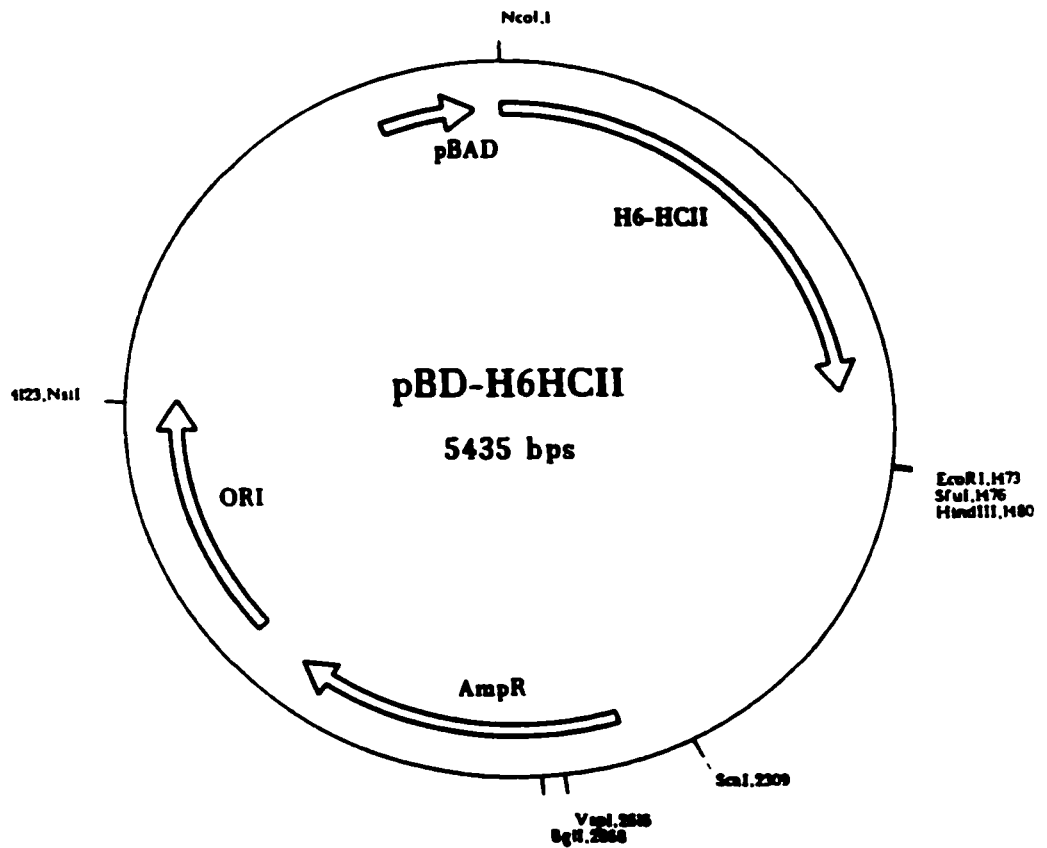
B



A



B



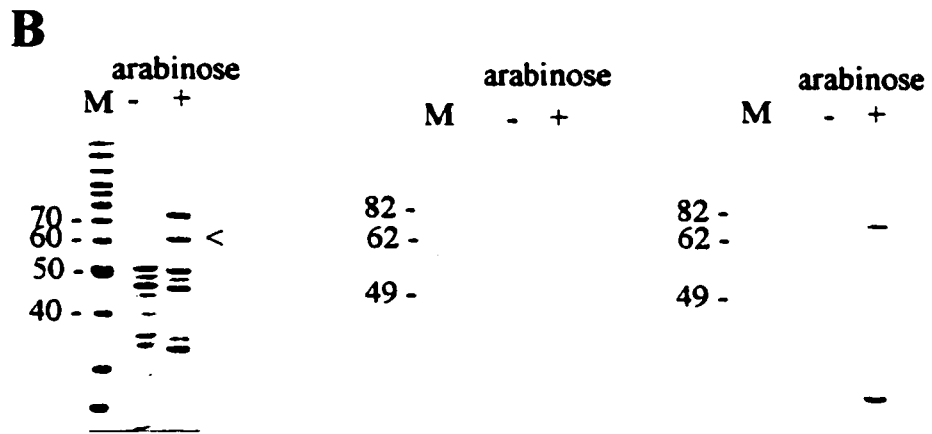
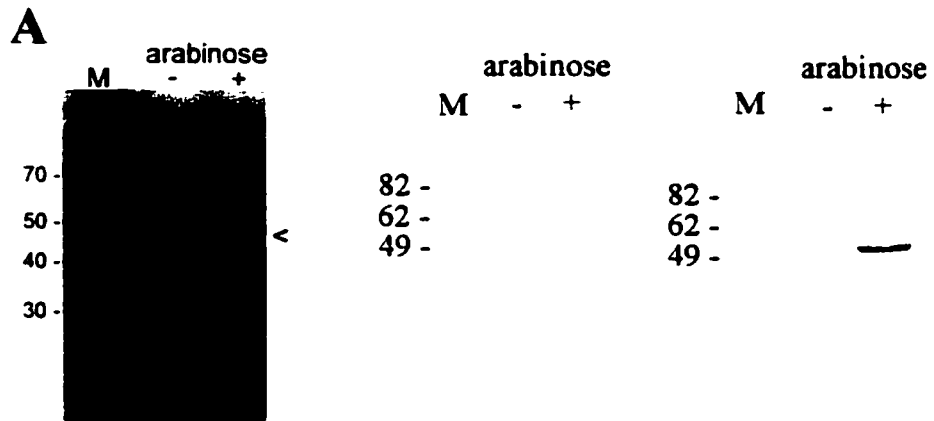
TOP10 *E. coli* cells with the expression plasmid, bacterial cultures inoculated with the transformed cells were induced with arabinose.

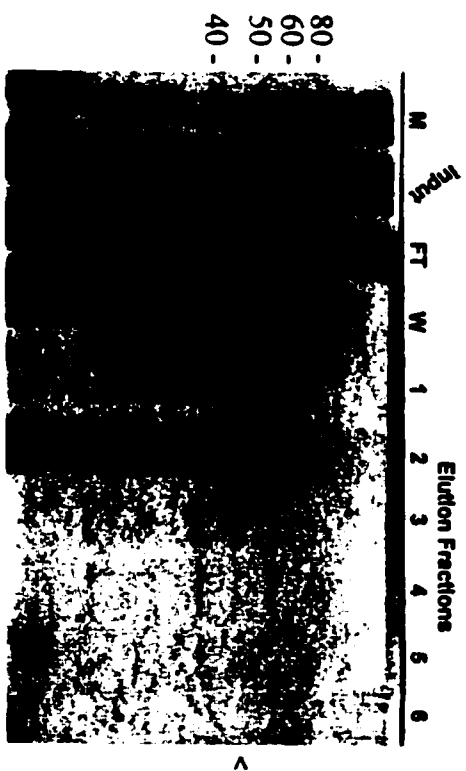
The pBAD(B)-H₆-HCII expression plasmid was created by insertion of H₆-HCII into pBAD(B). Specifically, H₆-HCII was created from full-length HCII, by PCR, again using plasmid pSG5-HCII(WT). The PCR product was inserted into the multiple cloning site in pBAD(B) and the appropriate construction of the plasmid (Figure 23B) was confirmed by DNA sequencing (data not shown). Competent LMG194 *E. coli* cells were transformed by the pBAD(B)-H₆-HCII plasmid, and identical induction conditions as those described for pBAD(B)-H₆-Δ66HCII were used to grow bacterial cultures inoculated with transformed cells containing the pBAD(B)-H₆-HCII expression plasmid. The total protein profile of bacterial cultures that had been induced by arabinose was examined. Figure 24 (left panels) shows that induction of bacterial cultures containing the pBAD(B)-H₆-Δ66HCII and pBAD(B)-H₆-HCII expression constructs results in the arabinose-dependent up-regulation of 50 kDa and 60 kDa proteins, respectively.

Figure 24 (middle panels) shows that the induced proteins in the bacterial cultures react with affinity-purified anti-human HCII antibodies and in addition, both protein species react with affinity-purified anti-hexahistidine antibodies (Figure 24, right panels).

3.4.3.1 Purification of H₆-Δ66HCII

Cells harbouring H₆-Δ66HCII that had been induced by arabinose were lysed as described in Section 2.6.5 and the soluble fraction of the bacterial lysate preparation was initially subjected to nickel-chelate affinity column chromatography using nickel-NTA agarose. Figure 25 shows the various protein fractions collected in the nickel-NTA

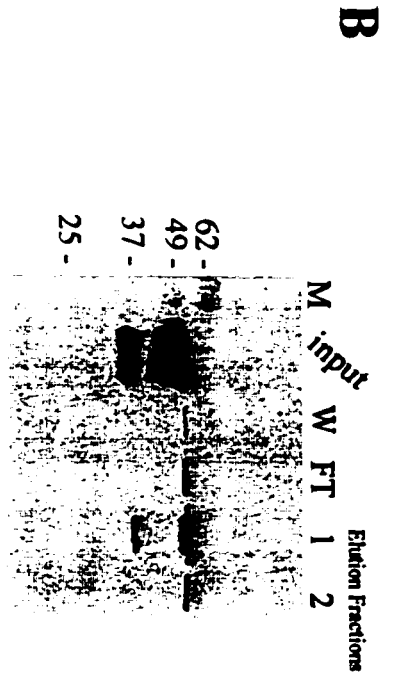
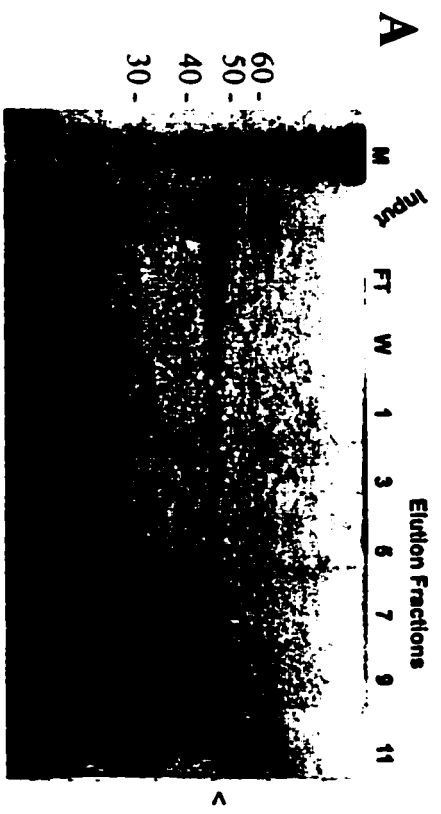




v

agarose purification, and demonstrates that H₆-Δ66HCII can be partially purified using this affinity chromatography step. Elution fractions were collected by passing a buffer containing 250 mM imidazole over the column that competitively removed nickel-binding proteins from the column. The major nickel-binding protein observed in the elution fractions were approximately 50 kDa, corresponding to the expected molecular mass of H₆-Δ66HCII. In addition, several other smaller molecular mass proteins were present in the elution fractions that reacted with the anti-human HCII antibodies (data not shown), indicating either that H₆-Δ66HCII is susceptible to intracellular proteolytic degradation, or that the intracellular translation of the protein is sometimes subject to premature termination of translation.

Subsequent to partial nickel-NTA purification, H₆-Δ66HCII was further purified, employing a Q-Sepharose column, by passing the partially purified H₆-Δ66HCII over the column by gravity. The column was washed with 10 bed volumes of 50 mM Tris, pH 7.4, and the bound proteins were then eluted using a salt gradient. Figure 26A shows the various protein samples collected in the different steps of Q-Sepharose affinity chromatography. The majority of H₆-Δ66HCII failed to bind to the Q-Sepharose column and was collected in the flow through and wash fractions. Figure 26B demonstrates the reactivity of the proteins collected in the various purification fractions with anti-human HCII antibodies and this confirms that H₆-Δ66HCII is the major protein species being isolated in the wash and flow through fractions. In addition, some H₆-Δ66HCII was detected in the early elution fractions containing between 0 and 50 mM NaCl but other protein contaminants also eluted in these fractions.

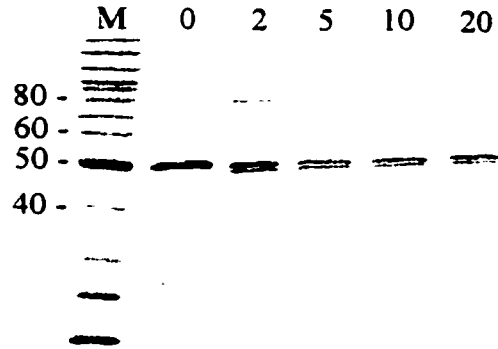


3.4.3.2 Characterization of the Interaction of H₆-Δ66HCII P6 Variants with Thrombin

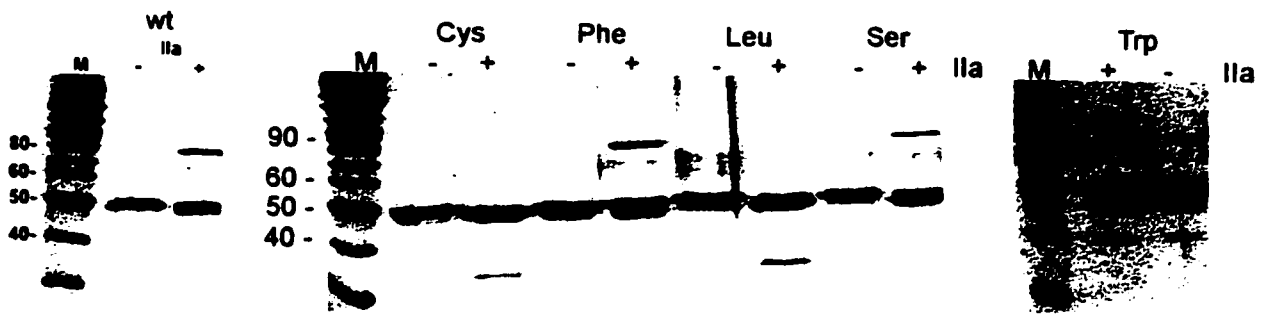
Prior to characterizing the thrombin-inhibitory activity of the bacterially-derived H₆-Δ66HCII P6 variants, the functional concentration of thrombin was determined by active site titration, as described in Section 2.7.5. Then gel-based assays were conducted on wild-type H₆-Δ66HCII to determine if a visible HCII-thrombin complex could be observed on an SDS-PAGE gel. 1 μM H₆-Δ66HCII was incubated with 200 nM thrombin in the presence of 2 U/ml heparin at 37°C. The reaction was stopped at various times by diluting aliquots of the reaction into SDS-PAGE sample buffer supplemented with 500 mM DTT. Aliquots of the reaction were electrophoresed on 10% SDS-PAGE gels under reducing conditions. As shown in Figure 27A, wild-type H₆-Δ66HCII readily forms a visible complex with thrombin by 2 minutes as evidenced by the presence of a thrombin-dependent band of approximately 85 kDa. In addition, a band of slightly greater electrophoretic mobility than intact H₆-Δ66HCII is observed after incubation of H₆-Δ66HCII with thrombin, corresponding to the thrombin-cleaved protein moiety. This gel-based analysis of thrombin-inhibitory activity was repeated with all other H₆-Δ66HCII P6 variants. In subsequent reactions with the other H₆-Δ66HCII P6 variants, the reaction was allowed to proceed for 5 minutes before SDS-PAGE analyses.

Figure 27B demonstrates the ability of some of the H₆-Δ66HCII P6 variants to form inhibitory complexes with thrombin. Specifically, the Phe, Ser, and Trp P6 mutants visibly inhibited thrombin in the presence of 2 U/ml heparin as shown by the presence of

A



B



a thrombin-dependent band of approximately 85 kDa, while the Cys, Glu, Leu, and Arg mutants did not form thrombin-inhibitory complexes under the conditions described above, but were cleaved in a substrate-type reaction.

Subsequently, a more quantitative analysis of the thrombin-inhibitory activities of all of the H₆-Δ66HCII P6 variants was undertaken by performing a kinetic analysis of these proteins. Specifically, second order rate constants were determined under pseudo-first order conditions to characterize the abilities of these bacterially-derived proteins to inhibit thrombin. As described in Section 2.7.11, the pseudo-first order constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 t = \ln([P_o]/[P_t])$, where $[P_o]$ is the initial thrombin activity, and $[P_t]$ is the thrombin activity at time t . The k_1 was calculated as the slope of the line created by plotting $\ln([P_o]/[P_t])$ versus time, and the second order rate constant (k_2) was then determined by dividing k_1 by the H₆-Δ66HCII concentration used in the assay. The second order rate constants were determined both in the presence and in the absence of 1 μM heparin.

The second order rate constants were determined, in triplicate experiments, and are shown in Table 3. Kinetic analysis of wild-type H₆-Δ66HCII revealed second order rate constants of $1.6 \pm 0.2 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ and $7.8 \pm 0.8 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ (\pm SD in both cases) in the absence and presence of 1 μM heparin, respectively. These values are within the published ranges of progressive and heparin-catalyzed activities for similar N-terminally truncated recombinant HCII molecules (Pratt *et al.*, 1990; VanDeerlin and Tollefsen, 1991; Bauman and Church, 1999). Second order progressive rate constant determinations of the H₆-Δ66HCII P6 variants revealed that the Phe, and Ser mutants

P6 Variant	k_2 (- heparin) $\times 10^4$ ($M^{-1} \cdot \text{min}^{-1}$)	k_2 (+ heparin) $\times 10^4$ ($M^{-1} \cdot \text{min}^{-1}$)	sI (- heparin)	sI (+ heparin)	Gel Complex
wt	1.6 ± 0.2	7.8 ± 0.8	1.4 ± 0.03	1.8 ± 0.1	+
Cys	ND	ND	4.4 ± 0.2	17.1 ± 0.3	-
Glu	ND	1.5 ± 0.4	3.9 ± 0.4	15.4 ± 0.4	-
Phe	1.6 ± 0.6	9.0 ± 2.6	1.5 ± 0.2	3.8 ± 0.6	+
Leu	ND	0.01 ± 0.001	3.9 ± 0.3	16.9 ± 0.4	-
Arg	ND	ND	7.2 ± 1.2	16.3 ± 0.9	-
Ser	1.9 ± 0.5	3.6 ± 0.7	1.4 ± 0.4	9.6 ± 1.1	+
Trp	0.57 ± 0.01	4.7 ± 0.3	3.0 ± 0.7	5.9 ± 0.6	+

displayed rate constants similar to that of wild-type H₆-Δ66HCII, while the Trp mutant, although having a discernible progressive rate of thrombin inhibition, had decreased thrombin-inhibitory activity which was statistically significantly different from that of the wild-type protein ($p < 0.05$ (Student's t-test, two-tailed, unpaired)). The Cys, Glu, Leu and Arg P6 variants did not have detectable second order rate constants, consistent with the gel-based results that showed that these mutants did not form inhibitory complexes with thrombin.

When the heparin-catalyzed second order rate constants were determined, a different pattern of thrombin inhibition was observed. The Phe P6 variant displayed a rate constant that was most similar to that determined for the wild-type molecule. The difference between wild-type and the P6 variant was not statistically significant. While the Ser and Trp variants had significant heparin-catalyzed rate constants of $3.6 \pm 0.7 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ and $4.7 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, respectively, they demonstrated some reduced thrombin-inhibitory activities that were statistically significantly different ($p < 0.05$, in both cases (Student's t-test, two-tailed, unpaired)) from wild-type H₆-Δ66HCII. Conversely, the Glu and Leu variants showed more significant reductions in heparin-catalyzed rate constants, while the Cys and Arg P6 variants had no observable rate constants in the presence of heparin.

To eliminate the possibility that the mutations present at P6 were responsible for the observed reductions in heparin-catalyzed rate constants due to a reduction in heparin-binding affinity, heparin-Sepharose affinity chromatography was conducted on three of the H₆-Δ66HCII P6 variants. In addition to wild-type, the Glu and Ser variants were

analyzed, with respect to their heparin-binding affinities, because they both lose and retain thrombin-inhibitory activities, respectively, compared to the wild-type molecule. 20 μ g of protein was passed over a 0.2 ml Hi-Trap heparin-Sepharose column, washed with 10 bed volumes of PBS and proteins eluted with a salt gradient varying from 0 to 1000 mM NaCl in PBS. Elution fractions were collected at each elution step and an HCII ELISA was used to quantify HCII from each 1 ml elution fraction. Analysis of all elution fractions revealed that all three of the H₆- Δ 66HCII P6 variants had similar heparin-binding properties (data not shown), in that all HCII variants eluted after the addition of 500 mM NaCl.

In order to confirm the tendency of some of the P6 mutants to act as substrates instead of inhibitors of thrombin, the stoichiometries of inhibition were determined for all of the H₆- Δ 66HCII P6 variants, both in the absence and presence of heparin. The SI, which is the number of inhibitor molecules consumed before an inhibitor-protease complex forms, indicates the propensity of the inhibitor to behave as a substrate or an inhibitor. As described in Section 2.7.12, a constant amount of thrombin was incubated with various amounts of H₆- Δ 66HCII P6 variant protein in the absence and presence of 1 μ M heparin. The reactions were allowed to proceed at room temperature overnight to ensure that the reactions went to completion. Linear extrapolation on a graph of the rates of thrombin inhibition at different molar ratios of H₆- Δ 66HCII P6 variants to thrombin, to the abscissa yielded the SI. The SIs, determined in duplicate, are shown in Table 3.

Wild-type H₆- Δ 66HCII had SI values of 1.4 and 1.8 in the absence and presence of heparin, respectively, indicating that the wild-type molecule behaves almost

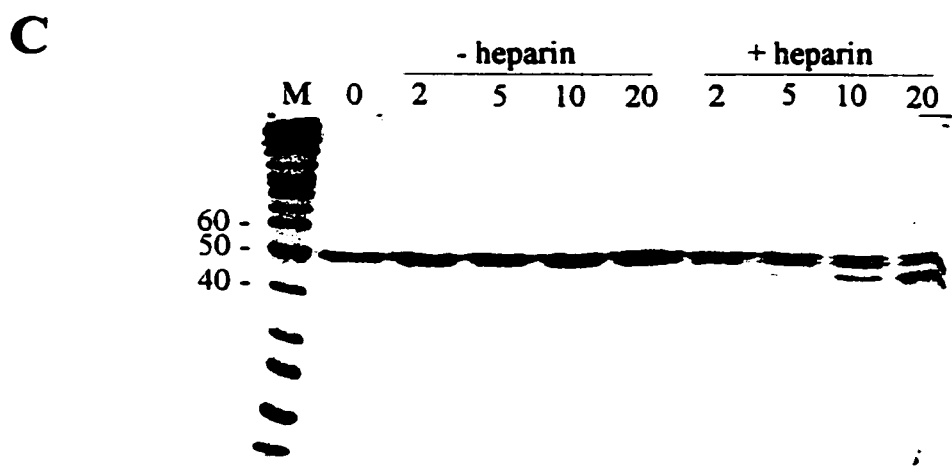
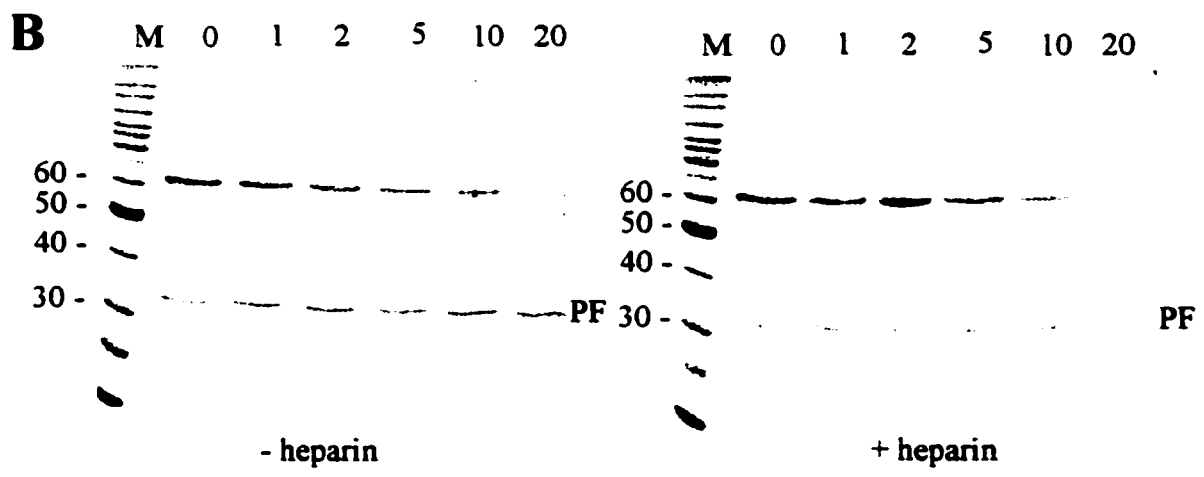
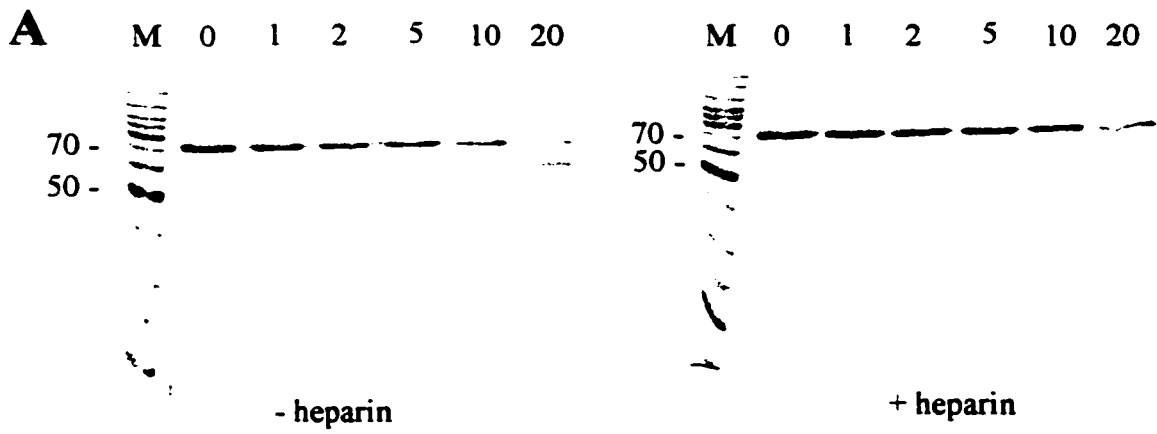
exclusively as an inhibitor of thrombin. An SI value greater than 1 indicates that the inhibitor has some substrate-like activity. As the SI increases, it indicates the increasing tendency of the inhibitor to act as a substrate in the reaction. Of the P6 mutants that formed inhibitory complexes with thrombin in gel-based analyses, the Phe and Ser variants demonstrated similar SI values to wild-type in the absence of heparin. The Trp variant however, had an approximately 2-fold increase in the SI that was statistically significant ($p < 0.05$ (Student's t-test, two-tailed, unpaired)), indicating that it had some substrate activity. In the presence of heparin, all three P6 variants had statistically significant ($p < 0.05$, in all cases (Student's t-test, two-tailed, unpaired)) elevations in SI values. The other P6 mutants that did not visibly inhibit thrombin in gel-based assays had the highest SI values, both in the absence and presence of heparin.

3.4.3.3 Characterization of the Interaction of H₆-Δ66HCII P6 Variants with Elastase

Pratt *et al.* (1990) first described the interaction of plasma-derived HCII with NE and showed that the cleavage pattern of HCII was influenced by whether or not heparin was present in the reaction. Those investigators showed that, in the absence of heparin, cleavage of HCII occurs primarily at residue 66, whereas when heparin was added to the reaction, cleavage at residue 439 (P6) is preferred. Prior to analyzing the effects of P6 mutagenesis in H₆-Δ66HCII, analysis of NE cleavage of plasma-derived HCII was conducted, in the absence and presence of heparin, in order to reproduce these earlier findings. The results of this experiment are shown in Figure 28A.

Plasma-derived HCII was incubated with NE in the presence and absence of heparin under the same conditions used by Pratt *et al.* (1990) and aliquots of timed reactions were electrophoresed on SDS-PAGE. In the absence of heparin, a protein band of approximately 60 kDa appears, and its intensity increases in a time-dependent manner. This 60 kDa band migrates with increased electrophoretic mobility compared to unreacted HCII that is present at approximately 70 kDa at time 0. In the presence of heparin, a band of approximately 60 kDa appears, again, increasing in intensity in a time-dependent manner. However, the intensity of this 60 kDa band appears less than that observed in the same reaction in the absence of heparin at every given time point. Unreacted HCII, with an apparent molecular mass of 70 kDa present at time 0 did not have a 60 kDa band present.

In order to better resolve possible HCII cleavage products, the reaction of HCII with NE in the absence and presence of heparin was repeated, followed by the enzymatic removal of N-linked glycans from the proteins in the reaction (Figure 28B). Pretreatment of HCII with PNGase F resulted in an increased electrophoretic mobility of unreacted HCII (present at time 0) to approximately 60 kDa. In the absence of heparin, PNGase-treated reaction products of 60 kDa and 55 kDa appear on SDS-PAGE, with the 55 kDa protein increasing in intensity over time. In the presence of heparin, unreacted HCII appears to migrate at an apparent molecular mass of 60 kDa, with an additional 55 kDa band appearing over time in increasing intensities. The 55 kDa band present in both sets of reactions is of greater intensity in the reaction lacking heparin at any given time point.



In both sets of gel-based assays, a protein band of approximately 35 kDa is present in all lanes, corresponding to the expected molecular mass of PNGase F.

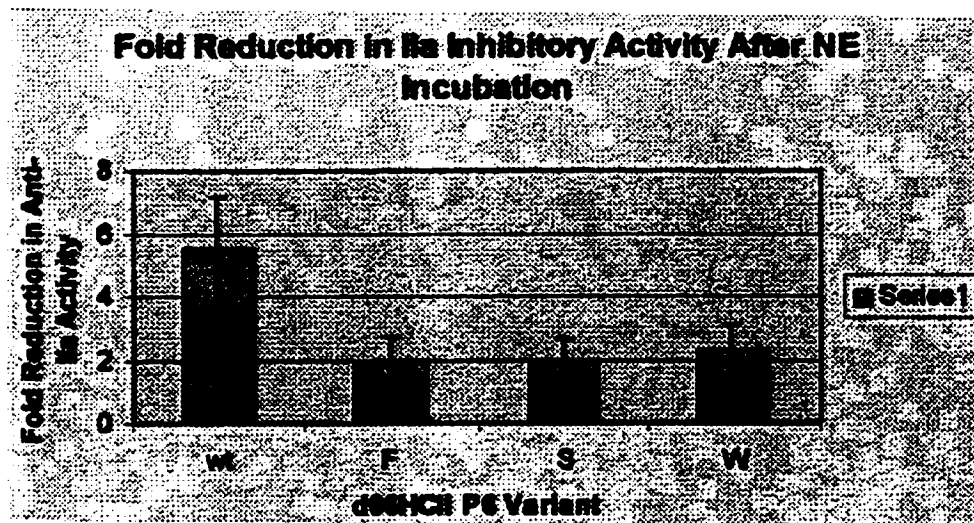
To more completely analyze the NE reaction with plasma-derived HCII, a chromogenic assay was employed in order to quantify the extent of inactivation of HCII by NE in the presence and absence of heparin. HCII was reacted with NE in the presence and absence of heparin, and the residual thrombin activity of HCII was then measured. This analysis was done essentially as described in Section 2.7.14, except that the reactions were performed with and without heparin, with NE present in both reactions. In addition, 1 mg/ml polybrene was added to the thrombin solution after NE inactivation, in order to quench the heparin-accelerated thrombin-inhibitory activity of HCII in the reaction containing heparin. The pseudo-first order rate constants were then determined for both reactions. HCII that had been incubated with NE in the presence of heparin showed an approximately 90-fold decrease in k_{obs} , compared to the reaction that lacked heparin (data not shown).

NE cleavage analysis of H₆-Δ66HCII was then conducted. Given that the upstream NE cleavage site at residue 66 is absent in this molecule, it was expected that minimal cleavage would occur when heparin was absent from the reaction. Conversely, heparin-dependent cleavage at P6 was expected to be unaffected. H₆-Δ66HCII was incubated with NE in the absence and presence of heparin as described in Section 2.7.14, and the reactions were followed over time, with NE being instantaneously inactivated by dilution into DTT-containing SDS-PAGE sample buffer, using SDS-PAGE and Coomassie staining. The results obtained by SDS-PAGE analysis are shown in Figure

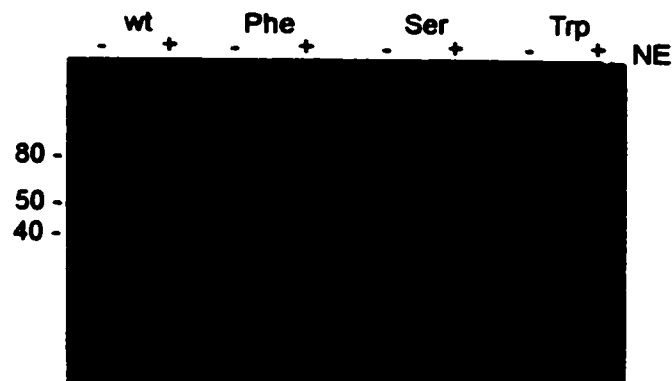
28C. In the heparin-independent reaction, a protein band of approximately 50 kDa, which migrated at the expected molecular mass of H₆-Δ66HCII, is present at all time points. Starting at 5 minutes and increasing in intensity up to 20 minutes, an additional band of approximately 47 kDa is visible. In the presence of heparin, in addition to the 50 kDa band being present at all time points, a 47 kDa band is present by 2 minutes and increases in intensity over the 20 minute time course. Moreover, an additional band of approximately 43 kDa is visible in the heparin-containing reactions by 2 minutes, and this band also increases in intensity over the 20 minute time course experiment. The 47 kDa band that is present in both sets of reactions appears to be visible at earlier time points and at higher intensities in the heparin-containing reactions.

The influence of P6 mutagenesis on the susceptibility of H₆-Δ66HCII to NE cleavage was then analyzed. A quantitative analysis was conducted by determining the observed pseudo first-order rate constants (k_{obs}) of H₆-Δ66HCII P6 variants that had been pretreated with NE in the presence of 10 U/ml heparin. In addition, products of the reaction of these variants with NE were visualized by SDS-PAGE and Coomassie staining. This analysis was limited to the P6 variants that had measurable abilities to inhibit thrombin; namely the wild-type, Phe, Ser and Trp P6 variants. The protocol for this study is described in section 2.7.14. The k_{obs} determined for all four H₆-Δ66HCII P6 variants was compared to the corresponding k_{obs} of the H₆-Δ66HCII variant which had not been incubated with NE. These experiments, conducted on each of the four thrombin-inhibitory P6 H₆-Δ66HCII variants, were conducted in triplicate, and are shown in Figure 29A. Visual products of the reaction are shown in Figure 29B.

A



B



P6 mutagenesis had a significant effect on the susceptibility of H₆-Δ66HCII to NE cleavage. While the wild-type molecule demonstrated a 6.5-fold reduction in thrombin-inhibitory activity after NE pretreatment, the Phe, Ser, and Trp P6 variants showed only 1.45, 1.65, and 1.85-fold reductions in the ability to inhibit thrombin, respectively. These results demonstrated that the Phe, Ser, and Trp variants were all approximately 4 times more resistant to NE inactivation than the wild-type H₆-Δ66HCII molecule ($p < 0.05$, in all cases (Student's t-test, two-tailed, unpaired)). The Coomassie-stained SDS-PAGE gel in Figure 29B demonstrates that NE-dependent cleavage products of the P6 variants are visible, and that wild-type H₆-Δ66HCII appears to be more susceptible to NE cleavage than the other P6 variants.

Another analysis of the NE cleavage products was then conducted on the wild-type and V439S H₆-Δ66HCII proteins. NE was incubated with these molecules in the presence of heparin under identical conditions as those described above. The products of the reactions were then electrophoresed by SDS-PAGE on Tris-Tricine gels and the protein bands were subsequently visualized by silver staining. As shown in Figure 30, electrophoresis of the reactions on Tris-Tricine gels resolved proteins of smaller molecular mass, and this permitted the visualization of a protein band that electrophoresed at an apparent molecular mass of approximately 4.5 kDa that is present after NE treatment but not in the untreated reaction (time 0). This corresponded well to the expected molecular size of 4791 Daltons for this cleavage product. An attempt to visualize this 4.5 kDa product by immunoblotting employing anti-HCII antibodies was

	$\frac{wI + NE}{M}$	$\frac{wI + IIa}{10}$	$\frac{VP6S + NE}{?}$	$\frac{VP6S + NE}{10}$
43 -	0	2	5	10
29 -				
18.4 -				
14.3 -				
6.2 -				
2.85 -				

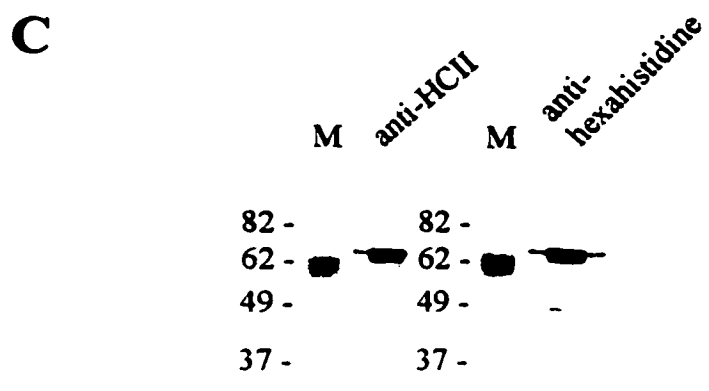
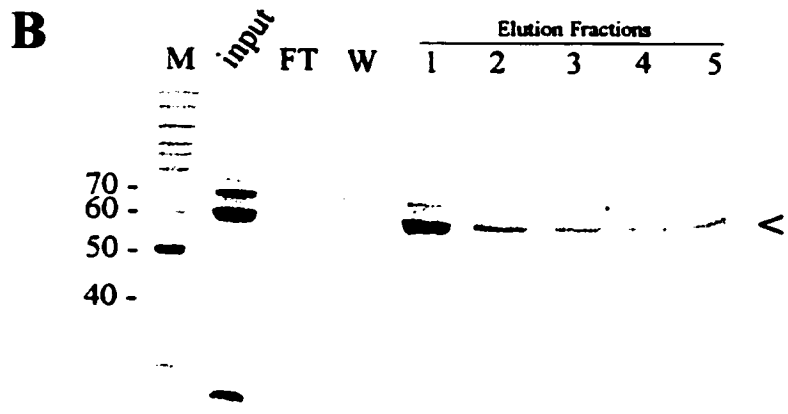
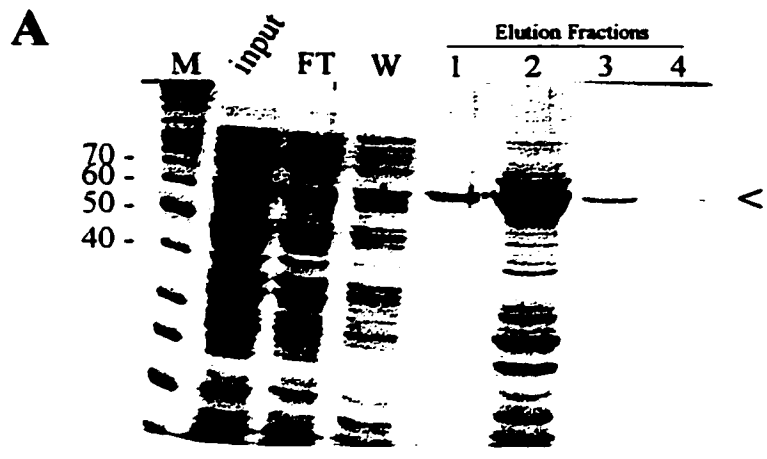
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unsuccessful, likely either due to the too small amounts employed or the specific epitope recognition profile of the antibody.

3.4.3.4 Purification of H₆-HCII

Cells that had been induced by arabinose and were harbouring H₆-HCII were lysed as described in section 2.6.5 and the soluble fraction of the bacterial lysate preparation was initially subjected to nickel-chelate affinity column chromatography using nickel-NTA agarose. As shown in Section 3.4.3, the total protein isolated from bacterial lysates after arabinose induction reacted with both anti-human HCII and anti-hexahistidine antibodies. Figure 31A shows the various protein fractions collected in the nickel-NTA agarose purification, and demonstrates that H₆-HCII can be partially purified using this affinity chromatography step. Elution fractions were collected by passing a buffer containing 250 mM imidazole over the column that competitively removed nickel-binding proteins from the column. The major nickel-binding protein observed in the elution fractions were approximately 60 kDa, corresponding to the expected molecular mass of H₆-HCII. In addition, several other smaller molecular mass proteins were present in the second elution fraction that reacted with anti-human HCII antibodies (data not shown).

Similarly to H₆-Δ66HCII, H₆-HCII was more completely purified by anion exchange chromatography using a Q-Sepharose column. The input protein, which was initially obtained by elution from the nickel-NTA agarose column, was first dialyzed overnight against 50 mM Tris, pH 7.4 to remove the imidazole. The sample was then passed over the Q-Sepharose column by gravity, washed with 10 bed volumes of a buffer

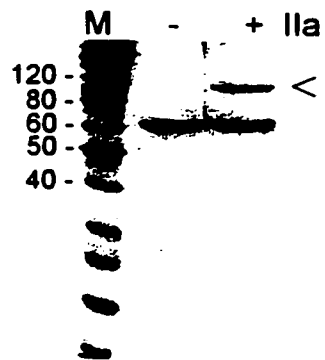


containing 50 mM Tris, pH 7.4 and 50 mM NaCl, and the proteins were then eluted by passing and collecting fractions of a buffer containing 150 mM NaCl. Figure 31B shows the various protein samples collected in the different steps of Q-Sepharose affinity chromatography. The majority of the 60 kDa protein eluted in the first three elution fractions. Proteins present in the first three elution fractions were then pooled and concentrated. An aliquot of the concentrated proteins were electrophoresed by SDS-PAGE and transferred to nitrocellulose, and they were subsequently immunoblotted with anti-human HCII and anti-hexahistidine antibodies (Figure 31C). In both immunoblots employing different antibodies, an intense band appeared at approximately 65 kDa, and in addition, some other smaller molecular mass bands of lesser intensity also reacted with the antibodies used. These observations strongly suggested that major protein moiety that had been purified from bacterial lysates harbouring the pBAD(B)- H₆-HCII was histidine-tagged HCII.

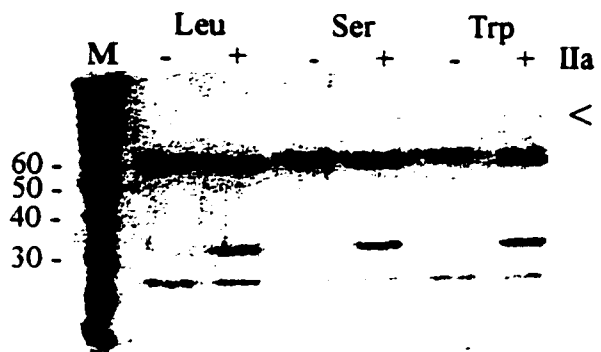
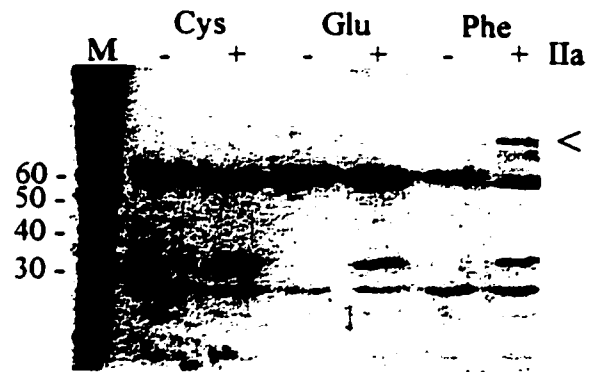
3.4.3.5 Characterization of the Interaction of H₆-HCII P6 Variants with Thrombin

As with H₆-Δ66HCII, gel-based assays were performed with all purified H₆-HCII P6 variants in order to visualize which mutants formed inhibitory complexes with thrombin. These assays were conducted identically to those performed with the H₆-Δ66HCII variants and the results are shown in Figure 32. Initially, wild-type H₆-HCII was incubated in a 5-fold molar excess with thrombin in the presence of 2 U/ml heparin in order to visualize H₆-HCII-thrombin inhibitory complexes. The products of the reactions were visualized by SDS-PAGE and Coomassie staining. Figure 32A

A



B



demonstrates the formation of a thrombin-dependent band of approximately 95 kDa, which corresponds to the expected size of a complex between unglycosylated H₆-HCII and thrombin. In addition, a 60 kDa band is visible in both lanes which migrates at the expected molecular mass (60 kDa) of H₆-HCII. A third band that is present in the thrombin-dependent reaction migrates with an apparent molecular mass of approximately 56 kDa and corresponds to the expected size of H₆-HCII cleaved at the reactive centre.

Gel-based thrombin-inhibitory assays were repeated for all 6 H₆-HCII P6 variants under identical conditions as those described above. The Phe, Ser, and Trp variants all produced a thrombin-dependent protein complex band of approximately 95 kDa, although the intensities of these bands were less than the corresponding band seen in the wild-type reaction with thrombin. All of the P6 variants had bands of 60 kDa in both the thrombin-dependent and independent reactions, which corresponded to the expected molecular mass of intact H₆-HCII, and in addition, all 6 P6 variants had a band of 56 kDa in the thrombin-containing reaction, which was the expected size of H₆-HCII that had been cleaved at the reactive centre.

An extensive kinetic analysis of all six H₆-HCII P6 variants was conducted by the determinations of second order rate constants and inhibition stoichiometries, by identical methods as those used to analyze the H₆-Δ66HCII mutants. The results of the kinetic analyses, performed in triplicate and duplicate experiments for determination of the rate constants and stoichiometries of inhibition, respectively, are shown in Table 4. Wild-type H₆-HCII had second order rate constants of $8.1 \pm 2.1 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ and $2.4 \pm 0.1 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ (\pm SD in both cases) in the absence and presence of heparin.

P6 Variant	\underline{k}_2 (- heparin) $\times 10^4$ ($M^{-1}\cdot\text{min}^{-1}$)	\underline{k}_2 (+ heparin) $\times 10^8$ ($M^{-1}\cdot\text{min}^{-1}$)	\underline{SI} (- heparin)	\underline{SI} (+ heparin)	Gel Complex
wild type	8.1 ± 2.1	2.4 ± 0.03	1.1 ± 0.13	1.1 ± 0.2	+
Cys	ND	ND	7.6 ± 1.1	6.1 ± 1.5	-
Glu	ND	ND	5.5 ± 0.5	7.5 ± 1.9	-
Phe	6.7 ± 1.6	1.3 ± 0.25	3.2 ± 0.3	3.6 ± 0.6	+
Leu	ND	ND	7.8 ± 1.1	12 ± 0.6	-
Ser	4.3 ± 1.4	0.26 ± 0.13	2.8 ± 0.1	9.5 ± 1.6	+
Trp	8.6 ± 1.8	0.6 ± 0.05	1.6 ± 0.1	8.2 ± 1.8	+

respectively. These values are within the published range of activity for recombinant HCII (Blinder, *et al.*, 1988; Derechin and Blinder, 1990; Liaw *et al.*, 1999; Bauman and Church, 1999). Progressive second order rate constant determinations of the H₆-HCII P6 variants revealed that, while the Phe and Trp variants had similar rates to that of the wild-type protein, the Ser variant had a slightly impaired progressive rate, although it was not statistically significant from wild-type. In addition, the Cys, Glu, and Leu variants had no discernable ability to inhibit thrombin in the absence of heparin.

Determination of the heparin-catalyzed second order rate constants demonstrated that the Phe mutant was most similar to wild-type in its ability to inhibit thrombin, while the Ser and Trp variants had significantly reduced rate constants ($p < 0.05$, in all cases (Student's t-test, two-tailed, unpaired)) in the presence of heparin, demonstrating 9 and 4-fold reductions in thrombin-inhibitory activities, respectively, compared to wild-type H₆-HCII. The Cys, Glu, and Leu P6 variants again failed to demonstrate the ability to inhibit thrombin, even when heparin was added to the reaction. The rate constant profiles amongst the various P6 variants appeared to be relatively consistent in both the H₆- $\Delta 66$ HCII and H₆-HCII backgrounds, in both the progressive and heparin-catalyzed reactions.

Inhibition stoichiometries were determined for all of the H₆-HCII P6 variants in both the absence and presence of heparin. The same reaction conditions as those used to analyze the H₆- $\Delta 66$ HCII variants (described in Section 2.7.12) were employed in these analyses. Again, the reactions were allowed to proceed at room temperature overnight to ensure that the reactions went to completion. The SIs, determined in duplicate, are

shown in Table 4. Wild-type H₆-HCII had an SI value of 1.1 in the absence and presence of heparin, suggesting that this molecule had mainly inhibitory activity with thrombin.

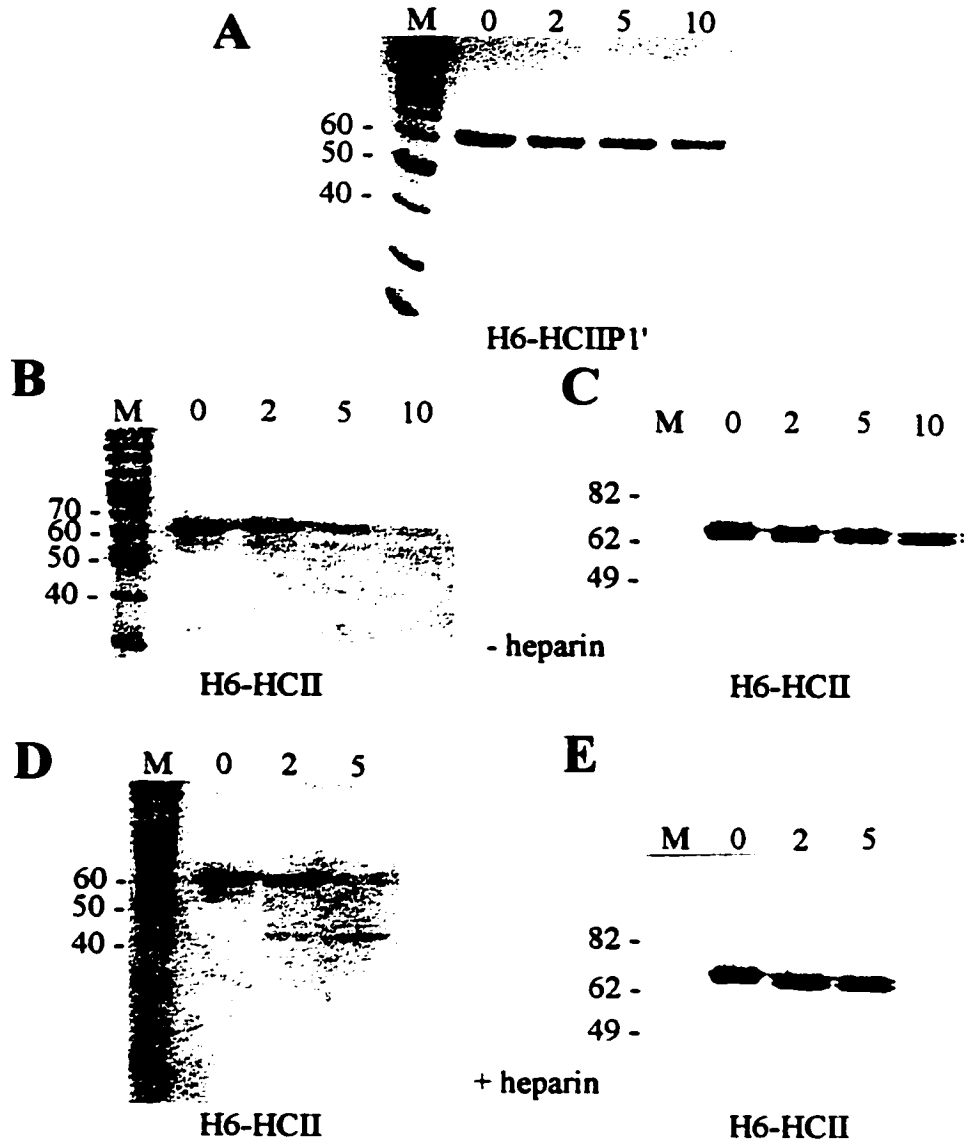
All of the P6 variants of H₆-HCII had elevated SI values that were statistically significant ($p < 0.05$, in all cases (Student's t-test, two-tailed, unpaired)) in both the absence and presence of heparin compared to wild-type. The Trp variant had the most similar SI in the absence of heparin but its SI was considerably elevated when heparin was present. The P6 variants that did not form visible inhibitory-complexes with thrombin and that had the lowest rate constants of thrombin inhibition had the highest SI values, in both the absence and presence of heparin. Conversely, the Phe and Ser P6 mutants, which did visibly inhibit thrombin, also had elevated SI values both in the presence and absence of heparin.

3.4.3.6 Characterization of the Interaction of H₆-HCII P6 Variants with Elastase

In the full-length plasma-derived HCII protein, there are two reported sites of NE cleavage (Pratt *et al.*, 1990). Initially, gel-based assays were conducted to determine if NE reacted at Ile-66 in addition to P6 in HCII. NE cleavage analysis of H₆-HCII was conducted under identical conditions used for the NE analysis of the H₆-Δ66HCII P6 variants. In addition, NE was reacted with H₆-HCIIP1', an HCII molecule that was genetically truncated after Ser-445 (Varsha Bhakta and W.P. Sheffield, unpublished data). The analytical procedure is described in detail in section 2.7.14. A 50-fold molar excess of wild-type H₆-HCII was reacted with NE in the absence of heparin for various times, since NE reportedly preferentially cleaves HCII at Ile-66 when heparin is not added to the reaction (Pratt *et al.*, 1990). After quenching the NE activity by diluting an

aliquot of the reaction into DTT-containing SDS-PAGE sample buffer, the aliquots were electrophoresed by SDS-PAGE under reducing conditions and the products of the reaction were visualized by Coomassie staining of the gels.

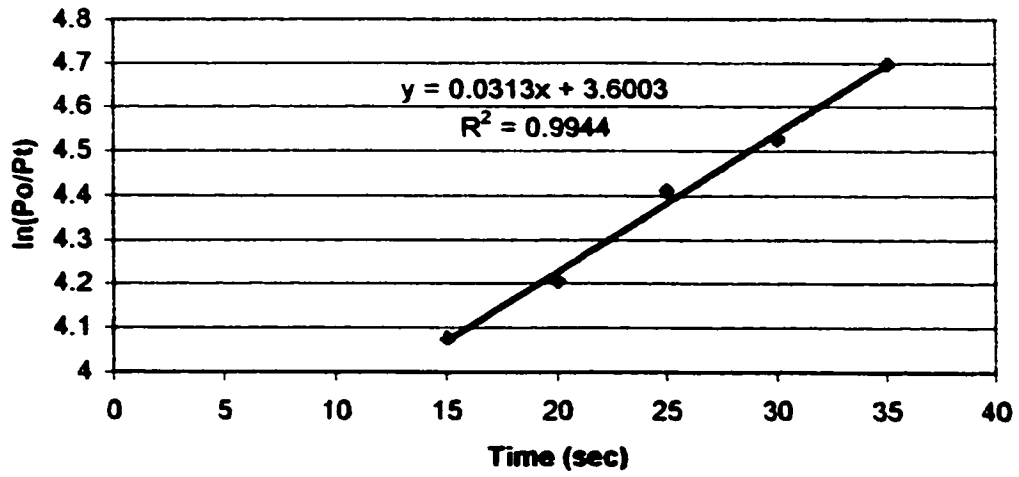
Figure 33 shows the analysis of NE reactions with two bacterially-derived HCII molecules, H₆-HCII and H₆-HCIIP1'. In panel A, the reaction of NE with H₆-HCIIP1' in the absence of heparin is shown. At every time point, the only bands visible are ones that co-migrate with a commercial 60 kDa marker, and which correspond closely to the expected molecular mass of 57 kDa of intact H₆-HCIIP1'. Panel B shows the reaction of H₆-HCII with NE in the absence of heparin. The input protein, shown at time 0, consists mostly of a band of approximately 60 kDa, although some other minor bands are visible around 55 kDa. In the other time points from 2 to 10 minutes, a 60 kDa band is present in each lane, as well as the other minor bands around 55 kDa. However, in these lanes, an NE-dependent band is visible around 58 kDa. This band is not visible at time 0. In the heparin-independent reaction of NE with full-length HCII, a protein band of approximately 55 kDa was expected to appear in increasing intensity over time, but the appearance of this band was not observed in either H₆-HCII or H₆-HCIIP1'. In addition to these gel-based assays, aliquots of the NE/ H₆-HCII reaction were transferred to nitrocellulose and then reacted with anti-hexahistidine antibodies (Panel C). H₆-HCII and N-terminal-containing degradation products would theoretically react with the anti-hexahistidine antibodies. All time points displayed an immunoreactive band of approximately 60 kDa, with an additional band of approximately 58 kDa visible in the NE-containing reactions.



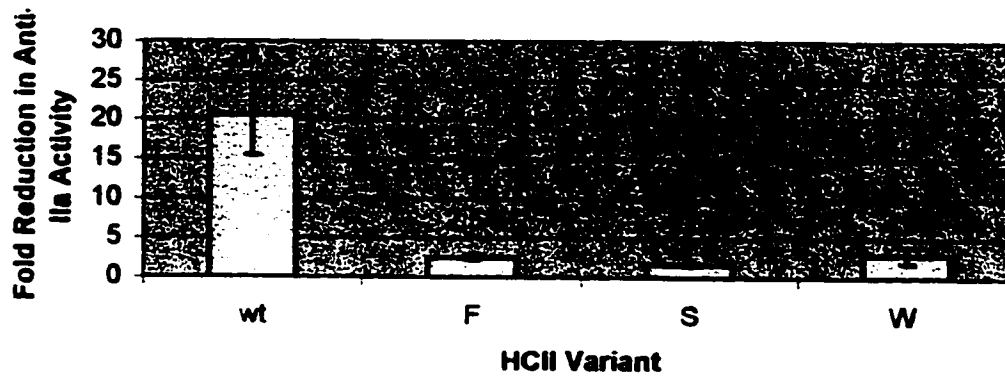
Subsequently, the reaction of NE with H₆-HCII was evaluated in the presence of heparin. In the heparin-accelerated reaction of NE with H₆-HCII, preferred cleavage of H₆-HCII within the reactive centre would be expected to yield an H₆-HCII cleavage product of approximately 56 kDa (Pratt *et al.*, 1990). In the reaction where no NE was added, a band of approximately 60 kDa was observed, consistent with the expected size of intact H₆-HCII. Reaction between NE and H₆-HCII, a band of approximately 58 kDa was observed in addition to a 60 kDa band. Aliquots of these reactions were transferred to nitrocellulose and then reacted with anti-hexahistidine antibodies (Panel E). H₆-HCII and degradation products that had been cleaved at the C-terminus would theoretically react with the anti-hexahistidine antibodies. All time points displayed an immunoreactive band of approximately 60 kDa, with an additional band of approximately 58 kDa visible in the NE-containing reactions.

Like H₆-Δ66HCII, H₆-HCII and the thrombin-inhibitory P6 variants were subjected to quantitative analyses of the effects of NE incubation on thrombin-inhibitory activities. Again, identical analyses, described in Section 2.7.14, were conducted on the P6 variants in H₆-HCII as those conducted on the same variants in the H₆-Δ66HCII background. The k_{obs} values obtained for all the P6 variants that had been incubated with NE in the presence of heparin were compared to the k_{obs} obtained when no NE was present and the residual thrombin-inhibitory activities were determined. The results of these analyses are shown in Figure 34.

As seen in the H₆-Δ66HCII background, P6 mutagenesis had a significant effect on the susceptibility of H₆-HCII to NE cleavage. Wild-type H₆-HCII demonstrated a 20-

A**B**

Fold Reduction in Ila Inhibitory Activity After NE Incubation



fold reduction thrombin-inhibitory activity after NE pretreatment while the Phe, Ser, and Trp P6 variants showed only 2.5, 1.6, and 2.9-fold reductions in the ability to inhibit thrombin, respectively after incubation with NE. These observations demonstrated that all three variants are approximately 10 times more resistant to NE inactivation than the wild-type, recombinant molecule ($p < 0.05$, in all cases (Student's t-test, two-tailed, unpaired)).

3.4.3.7 Characterization of the Interaction of H₆-HCII P6 Variants with Chymotrypsin

Finally, plasma-derived and bacterially-derived full-length HCII were incubated with chymotrypsin in order to determine if P6 mutagenesis affected the protease specificity of HCII towards another serine protease. Gel-based analyses were conducted to see if inhibitory complexes could be observed between HCII and chymotrypsin. Specifically, a 5-fold molar excess of HCII was incubated with chymotrypsin. The reactions were stopped by dilution into DTT-containing SDS-PAGE sample buffer and aliquots of the reactions were electrophoresed by SDS-PAGE under reducing conditions. These samples were electrophoresed concurrently with unreacted HCII controls. The products of the reaction were visualized by Coomassie staining.

Figure 35 shows that the reaction of plasma-derived HCII with chymotrypsin produced a protease-dependent protein band of approximately 95 kDa, which corresponded to the expected size of an HCII-chymotrypsin complex, although this band was not readily visible. A band of approximately 65 kDa was visible in both lanes containing plasma-derived HCII, which corresponded closely to the expected size of

intact plasma-derived HCII. Similarly, a band of approximately 60 kDa is present in all bacterially-derived H₆-HCII reactions lacking chymotrypsin, consistent with the expected size of intact H₆-HCII. However, in all NE-containing reactions with bacterially-derived H₆-HCII P6 variants, unexpected banding patterns appeared. Smaller molecular mass protein bands were present in some reactions, while other reactions had no visible proteins after Coomassie staining.

4. DISCUSSION

AT and HCII are two serpins found in plasma that inhibit thrombin via a unique mechanism that is common to this class of proteins. While AT and HCII inhibit thrombin by forming 1:1 molar complexes with the protease, interactions of NE with AT and HCII have a different outcome. Although NE interacts with these serpins at sites close to the reactive site within the reactive centre loop, NE is not trapped in an inhibitory complex with either of these serpins. The purpose of this study was to provide, via mutational analyses, elucidation of some structural requirements of the reactive centre loop of AT and HCII that determine the inhibitory or substrate behavior of these proteins. An additional aim of the studies conducted was to determine the limits of amino acid substitution plasticity within the reactive centre loop that would allow the proteins to retain thrombin-inhibitory activity, yet be less susceptible to inactivation by NE.

The study of naturally-occurring mutants of AT has provided useful information of the relationship between the structure and function of AT (Blajchman *et al.*, 1992; Stein and Carrell, 1994); however, no such mutants occur between P7 and P2 of the reactive centre loop. This region contains the sites of NE-mediated proteolysis C-terminal to the isoleucine P4 and the valine P5 residues. This region is also of interest because it links the hinge region (P14-P10) with the reactive centre (P1-P1'), the site of thrombin cleavage. The hinge region has been shown to emanate from its partially inserted position in β -sheet A, and transmit an activating conformational change to the reactive centre when AT binds to heparin (Van Boeckl, *et al.*, 1994; Huntington *et*

al., 1996; Gils and Declerck, 1998). Both of these observations provided a sound rationale for conducting the studies described in this thesis.

4.1 Recombinant Expression of AT

Initially, the expression and analysis of rabbit AT and several P4 variants were accomplished using a mRNA-dependent cell-free rabbit reticulocyte lysate system that had been previously established in our laboratory (Austin *et al.*, 1990). The cDNA encoding rabbit AT (residues 1-433) was inserted into the pSV3 cell-free expression vector, under the transcriptional control of SP6 RNA polymerase. mRNA transcripts created from cell-free transcription were used to synthesize mature rabbit AT in a rabbit reticulocyte lysate system that incorporated [³⁵S]-methionine into the proteins that were translated *in vitro*. Analysis of electrophoresed samples following SDS-PAGE and development of film exposed to the radioactive sample yielded a major band of approximately 47 kDa, which corresponded to the expected size of unglycosylated rabbit and human AT. Enzymatic removal of carbohydrate moieties from AT has demonstrated that, although the modified protein is subject to more rapid clearance from the circulation, the thrombin-inhibitory ability of AT is unaffected in both the absence and presence of heparin (Rosenfeld and Danishefsky, 1984; Danishefsky *et al.*, 1978). The rabbit reticulocyte lysate cell-free expression system lacks the cellular components that are necessary for protein glycosylation. In addition, the absence of endoplasmic reticula and Golgi bodies results in heterogeneous folding of the artificially-produced protein products (Austin *et al.*, 1990). The presence of glutathione in the *in vitro* translation reaction maintains an oxidizing environment that permits AT to form intramolecular

disulfide bonds that are necessary for anti-thrombin function. A previous study using cell-free derived AT has demonstrated that the protein produced in this cell-free system mimics, to some extent, plasma-derived AT, with respect to their ability to inhibit thrombin in both progressive and heparin-catalyzed manners (discussed in detail in Section 4.1.1) (Austin *et al.*, 1990). In addition, the recreation and expression of naturally-occurring AT mutants in this system yielded proteins with similar characteristics as their plasma-derived counterparts (Austin *et al.*, 1991a; Austin *et al.*, 1991b).

While both wild-type and mutant AT proteins produced in the cell-free system have reliably reproduced the behavior of their plasma-derived counterparts, expression of the most interesting rabbit AT mutants that were initially characterized using the cell-free system were reproduced in COS-1 cells, which produced more physiological molecules that are appropriately folded and glycosylated. This was done in order to minimize the possibility that the interesting results obtained with AT derived in the cell-free system were an artifact of the artificial cell-free expression system that produced heterogeneous AT. These COS-derived molecules co-migrated with plasma-derived AT on SDS-PAGE gels, and were reactive with affinity-purified monospecific anti-rabbit AT antibodies. Previous work by our laboratory had demonstrated the ability to express functional AT from appropriately transfected COS-1 mammalian cell cultures, which had similar heparin-binding and thrombin-inhibitory activity as that seen with the plasma-derived molecule (Sheffield and Blajchman, 1994a; Sheffield and Blajchman, 1994b).

Because of the potential utility of an elastase-resistant homologous AT molecule in understanding the extent of elastase-mediated inactivation of AT in animal models of inflammation, the site-directed mutagenesis study of the NE cleavage sites in AT was initially conducted in the rabbit AT background. However, while the P4 isoleucine residue is conserved amongst rabbit and human AT molecules, P5 exists as a valine and a glycine residue, in human and rabbit AT, respectively. Moreover, the NE cleavage of AT had been best described, by N-terminal amino acid sequencing, in the human AT background (Owen and Carrell, 1985). Rather than assume that NE would cleave at the P5 glycine in rabbit AT, which seemed to fit the substrate specificity of the protease, the P5 mutagenesis study was conducted on human AT, which eliminated the uncertainty of NE cleavage at P5 existing in rabbit AT.

The eukaryotic expression, through secretion of proteins from transfected COS-1 cells, of both rabbit and human AT resulted in the appearance in conditioned media of proteins which reacted with affinity-purified monospecific anti-AT antibodies of a gel-based mobility of approximately 60 kDa. In addition, these COS-derived proteins inhibited thrombin in both progressive and heparin-catalyzed manners. Put together, these observations appeared to confirm that rabbit and human AT were successfully expressed in functional forms in this expression system.

4.1.1 Effects of Reactive Centre Loop Mutagenesis in AT on Thrombin-Inhibitory Activity

Wild-type rabbit and human AT that had been produced in the rabbit reticulocyte lysate cell-free expression system qualitatively behaved similarly to plasma-derived AT

in that the recombinant molecules formed inhibitory complexes with thrombin, based on SDS-PAGE and autoradiographic analysis. This was confirmed in the current study by SDS-PAGE analysis, but has also been demonstrated by others (Austin *et al.*, 1990; Austin *et al.*, 1991a; Austin *et al.*, 1991b). The effects of P4 mutagenesis in rabbit AT produced several results. Screening of a family of six mutant AT proteins expressed in the rabbit reticulocyte lysate cell-free system revealed that charged residues reduced thrombin-inhibitory activity approximately 2-fold, while polar uncharged, aromatic or hydrophobic substitutions had little effect. Although the P4 residue is not conserved amongst the serpins, there are no known serpins with a charged residue at this position (Stein and Carrell, 1995). These results are consistent with results obtained in the mutagenesis of the P2 region of the reactive centre loop. In that study, charged amino acid substitutions also resulted in an AT molecule with impaired thrombin-inhibitory activity (Sheffield and Blajchman, 1994a; Sheffield and Blajchman, 1994b; Chuang *et al.*, 1999). These results also suggested that a reduction in function of AT would be anticipated with a polar amino acid substitution at P4, if what holds true at P2 can be generalized. Indeed, this appears to occur with the substitution of serine at this position. Interestingly, substitution of serine for wild-type isoleucine at P4 in the current study abolished the ability of rabbit AT to form stable complexes with thrombin.

The expression of rabbit AT variants in a cell-free system served to provide an initial screening method to evaluate the effects of P4 mutagenesis on the ability of the altered molecules to inhibit thrombin and resist NE inactivation. Subsequently, the serine and tryptophan P4 mutants were recreated in pSG5-(rab)WT in order to analyze the

effects of mutagenesis on more physiologically relevant rabbit AT proteins. Like their cell-free-derived counterparts, COS-derived rabbit AT P4 variants displayed similar characteristics. While the serine P4 mutant lost all thrombin-inhibitory activity, the tryptophan P4 mutant inhibited thrombin at levels of approximately 72% of that observed with the wild-type recombinant molecule. A similar result was seen when the P4 tryptophan mutation was made in human AT, as that molecule maintained essentially the same thrombin-inhibitory activity as COS-derived wild-type human AT. In addition, the creation of a double tryptophan mutant at P4 and P5 in human AT was well-tolerated as evidenced by its ability to inhibit thrombin at approximately 78% compared to the wild-type recombinant serpin.

The observation that double tryptophan substitutions at P4 and P5 are well-tolerated by AT suggests that this region of the reactive centre loop is relatively unconstrained in the presence of heparin with respect to its secondary structure. This is consistent with crystallographic structures of human AT complexed with pentasaccharide (Elliot *et al.*, 1996) and of the related serpin α_1 -proteinase inhibitor (Carrell *et al.*, 1997). In these structures, the reactive centre loop is exposed from the body of the molecule in the presence of heparin, and this facilitates the interaction between AT and thrombin. Additionally, studies by several groups have shown that the rate of reactive centre loop insertion into β -sheet A within the body of AT determines inhibitor or substrate status after the covalent interaction with thrombin has occurred (Huntington *et al.*, 1996; Mellet and Bieth, 2000; Lawrence *et al.*, 2000). These studies have demonstrated the importance of the P14 residue in anchoring the proximal portion of the reactive centre

loop within the underlying β -sheet of the inhibitory serpin in order to facilitate full insertion of the reactive centre loop after its interaction with its target protease. The retention of the thrombin-inhibitory activity by the P4/P5 double tryptophan mutant AT suggests that the presence of two bulky residues at these positions does not adversely affect the rate of reactive centre loop insertion. This observation is possibly not surprising, given that substitution of a glutamic acid residue at either P4, P6, P8, P10, or P12 of the serpin, PAI-1 does not significantly affect its function (Tucker *et al.*, 1995) although they did not conduct the study on odd numbered residues within the reactive centre loop. Interference of the P4 or P5 residues with P14 insertion is not expected, based upon the crystal structures of AT showing that the P14 residue does not come into close contact with either P4 or P5 (Carrell *et al.*, 1994).

The finding that a serine substitution at P4 significantly impairs AT thrombin-inhibitory activity implies that either there are alterations in behavior of the reactive centre loop, or that there is generalized denaturation of AT. The latter hypothesis is based upon the possibility that the polar side group of the P4 serine residue makes an inappropriate intramolecular interaction that results in denaturation of the serpin. Although this hypothesis cannot be ruled out, the apparent lack of a secretion defect, based on similar ELISA-determined protein secretion levels as other functional AT P4 variants, argues against a folding defect. Alterations of reactive centre loop behavior appear to be a more feasible hypothesis. The P1 arginine of AT forms a salt bridge with Glu225 in strand 3 of β -sheet C (Carrell *et al.*, 1994), while the P5 glutamic acid of α_1 -proteinase inhibitor bridges to Arg196 at the apex of strand 3 of β -sheet A (Elliot *et al.*,

1996). It is possible that the presence of a serine residue at P4 causes similar but inappropriate bonding to underlying elements, thus locking the reactive centre loop in an unproductive conformation that is not alleviated by heparin. The apparent lack of cleavage within the P4 serine variant by NE also supports this hypothesis. Alternatively, it is possible that insertion of the reactive centre loop, subsequent to the formation of a covalent bond with thrombin, is impeded by the presence of the polar amino acid at P4. If the rate of insertion of the reactive centre loop is sufficiently impaired, it is possible that the covalent bond between thrombin and AT could be broken before the serpin attains an inhibitory conformation that prevents dissociation between AT and thrombin.

4.1.2 Effects of Reactive Centre Loop Mutagenesis in AT on Susceptibility to Elastase Cleavage

A novel approach was established in this study to analyze the NE-mediated cleavage of cell-free-derived rabbit AT P4 variants. Recombinant AT was incubated with a large molar excess of NE, in order to drive the reaction in a rapid manner. Aliquots of timed reactions were electrophoresed, under non-reducing conditions, by SDS-PAGE followed by fluorography and autoradiography of dried gels. The resultant bands were visualized such that the cleaved AT moiety migrated with decreased electrophoretic mobility compared to intact AT. This phenomenon was possible given the disulfide bond that exists between cysteine residues 247 and 430 (Petersen *et al.*, 1979; Sun and Chang, 1989), that allows the NE-cleaved C-terminus of AT to act as an “anchor” and slow the migration of the remaining cleaved protein through the polyacrylamide gel. Analysis of NE-cleaved AT under non-reducing conditions

increased the resolution between cleaved and intact AT, compared to similar reactions electrophoresed under reducing conditions.

Analyses of six P4 variants of rabbit AT expressed in the cell-free system revealed that none of the amino acid substitutions had significant effects on the NE reaction with AT, except for the glycine mutant, which appeared to be more susceptible to cleavage by the protease. Because the NE-mediated effects on cell-free-derived rabbit AT were observed using large excesses of NE in the absence of heparin, it was not known if the observed electrophoretic change in the serpin corresponded to a reduction in thrombin-inhibitory function, or if the NE cleavage patterns were dependent on the artificial protein expression system that was used as a preliminary step in protein analyses.

Subsequently, wild-type isoleucine, serine and tryptophan P4 variants were expressed in COS-1 cells in order to analyze the effects of NE reaction with physiologically relevant AT proteins. Because the effects of NE on human, but not rabbit AT had been reported previously (Owen and Carrell, 1985; Jordan *et al.*, 1987), the reaction between plasma-derived rabbit AT and NE was characterized prior to the analysis of COS-derived proteins. Reaction of plasma-derived rabbit AT with NE resulted in a loss in ability to inhibit thrombin, as shown by the lack of TAT complex formation subsequent to NE pretreatment of AT. This result was consistent with that observed in the same reaction employing plasma-derived human AT. In addition, an increase in electrophoretic mobility of rabbit AT was observed after incubation with NE. The resolution of this shift in mobility was enhanced by the enzymatic removal of

carbohydrates from the plasma-derived serpin. These experiments made possible the testing of the rabbit P4 AT tryptophan variant for NE reactivity. This variant demonstrated similar susceptibility to NE inactivation as the wild-type recombinant AT. Had there been only one site of NE cleavage, this result would have been surprising, given that NE preferentially hydrolyzes substrates containing a small hydrophobic amino acid at the P1 site, as shown by its cleavage of tripeptide thiobenzyl ester substrates. Substrates with P1 phenylalanine residues are not cleaved, and crystal structures of NE complexed to peptide or ovomucoid inhibitors suggest that the S1 site within the substrate-binding domain of NE is insufficiently large to accommodate bulky residues like phenylalanine or tryptophan (Bode *et al.*, 1989; Bode *et al.*, 1986). It therefore seemed likely that residual proteolysis of the P4 tryptophan rabbit AT variant was due to cleavage at another site or sites.

The human AT P4 tryptophan variant had similar properties to its mutant rabbit AT counterpart, with respect to both its thrombin-inhibitory activity and its susceptibility to inactivation by NE. Mutation of P5 to tryptophan to form a double tryptophan mutant, however, failed to prevent NE inactivation. This result suggests two possibilities; firstly, that NE cleaves at a secondary site when its primary sites are blocked by mutagenesis; or, alternatively, that the reactive centre loop is a specialized structure in which NE is capable of cleaving at residues that it could not recognize in other conformations or in short peptides. Insufficient quantities of the double tryptophan mutant were available to permit amino acid sequencing, but immunoblot visualization showed no differences in mobility between wild-type and mutant NE-cleaved AT. Given that differences of

approximately 2 kDa are usually resolvable with this technique, cleavage at another site within the reactive centre loop is certainly possible, especially considering that other small, non-polar residues exist within the reactive centre loop. Cleavage outside of the reactive centre loop seems unlikely because in the absence of heparin, AT is essentially impervious to the effects of NE (Jordan *et al.*, 1987).

4.2 Recombinant Expression of Heparin Cofactor II

Although the study of reactive centre loop mutagenesis of AT provided some useful information on the effects of mutagenesis on thrombin-inhibitory and NE-inactivation activities, production of greater quantities of proteins was desired to allow for more informative physicochemical characterization of serpins with reactive centre loop amino acid substitutions. Transiently-transfected COS-1 cells provided appropriately secreted proteins, but in insufficient quantities to conduct a more complete protein analysis. In addition, results observed with the analyses of AT reactive centre loop mutants suggested that the presence of two adjacent NE cleavage sites might complicate the analysis of the effects of amino acid substitutions on the primary NE cleavage sites. Moreover, it has been demonstrated that AT is unusual in that expression in both BHK and Chinese Hamster Ovary (CHO) mammalian cell systems yields a heterogeneous product composed of sub-populations of AT with different degrees of functionality (Björk *et al.*, 1992; Fan *et al.*, 1993). Based on these reports and observations, it seemed to be logical to repeat the reactive centre loop mutagenesis study on recombinant human HCII.

Human HCII is another thrombin-inhibitory serpin that circulates in plasma and like AT, is susceptible to NE inactivation via cleavage within the reactive centre loop (Pratt *et al.*, 1990). However, in contrast to AT, only one NE cleavage site is present within the reactive centre loop at valine residue 439 (P6), a difference which potentially provided a better molecule in which to study the effects of site-directed mutagenesis on the susceptibility to NE inactivation by cleavage at its primary site of hydrolysis. In addition to the NE cleavage site within the reactive centre loop of HCII, an additional site of cleavage exists C-terminal to the residue 66 isoleucine. In contrast to the NE cleavage site in the reactive centre loop, cleavage by NE at Ile-66 does not inactivate HCII, but decreases its second order rate constant of thrombin inhibition by approximately 100-fold (Pratt *et al.*, 1990). Reports by others have shown that cleavage of this N-terminal portion of HCII removes an acidic hirudin-like domain that is involved in the high affinity binding of HCII to thrombin (Pratt *et al.*, 1990; VanDeerlin and Tollefsen, 1991; Bauman and Church; 1999). Removal of this binding domain does not inactivate HCII but reduces its ability to bind avidly to thrombin.

The cDNA encoding human HCII was inserted into the pCDNA3.1 eukaryotic expression vector in order to produce glycosylated, optimally folded HCII in stable BHK cell lines. Production of permanent cell lines, through selection by resistance to the antibiotic G418, would theoretically yield unlimited recombinant HCII proteins through repeated growth of G418-resistant cells and harvesting of conditioned media. However, it was determined that, while several HCII P6 variants were secreted from transfected

BHK cells, the level of protein secretion was far too low to allow the accumulation of sufficient HCII protein to conduct an extensive analysis of recombinant protein behavior.

In contrast to AT, HCII has been successfully produced in bacterial expression systems that yield large amounts of functional protein (Blinder *et al.*, 1988). This information led to the subsequent expression of HCII variant molecules in bacteria, which were used to conduct the rest of the studies in this thesis. Initially, HCII was expressed in BL-21 *E. coli* cells as a constituent of a glutathione-S-transferase (GST)-HCII fusion protein. These two constituents of the fusion protein were separated by a factor Xa cleavage site, which permitted the removal of the GST protein moiety from the N-terminus of HCII once the fusion protein had been isolated from bacterial lysates. The presence of a GST component in the fusion protein facilitated its purification by immobilized glutathione affinity chromatography, and it also facilitated efficient production of HCII through its genetic association with GST, a slime mold protein that, fortuitously, is very efficiently produced by bacteria (Smith and Johnson, 1988; Kaelin *et al.*, 1991). Analysis of SDS-PAGE gels and immunoblots employing anti-HCII antibodies demonstrated that the expression and purification of the GST-HCII fusion protein was possible. However, analysis of bacterial lysates that had been passed over glutathione-Sepharose columns demonstrated that the fusion protein constituted only a minor fraction of the total purified protein. The vast majority of the purified proteins consisted of either GST or of other GST-HCII degradation products that were immunoreactive with anti-HCII antibodies. Incubation of the GST-HCII fusion protein with factor Xa, however, demonstrated that technically, the GST fusion protein system

worked, given the appearance of a 60 kDa band that was immunoreactive with anti-HCII antibodies. However, the low yield of GST-HCII fusion protein did not warrant the continued use of this expression system, given that a significant amount of protein was necessary for the remaining studies.

Expression of N-terminally truncated and full-length HCII was subsequently accomplished by employing a bacterial expression system that facilitated the prokaryotic expression of HCII variant proteins in an arabinose-inducible manner using the pBAD(B) expression vector . In this system, arabinose regulates the expression of recombinant proteins encoded on the pBAD(B) plasmid. Arabinose binds to, and activates the AraC transcription factor that then binds to the pBAD promoter and facilitates protein expression (Lee *et al.*, 1987). Analysis of total proteins from bacterial lysates of cells harbouring the appropriate pBAD expression constructs by SDS-PAGE and immunoblotting demonstrated that both histidine-tagged full-length and H₆-Δ66HCII could be induced, as evidenced by the presence of a 60 and 50 kDa band, respectively, that reacted with anti-human HCII and anti-histidine antibodies. Purification of both HCII proteins by successive nickel-chelate and ion exchange chromatographic steps yielded relatively pure HCII preparations, although the full-length HCII preparation typically contained more contaminants than its N-terminally truncated counterpart. This difference probably arose from the combined effects of a greater susceptibility to proteolysis of the full-length HCII than the truncated form, and the less avid binding of the deleted HCII to positively charged Q-Sepharose than the full-length protein.

4.2.1 Effects of Reactive Centre Loop Mutagenesis in Recombinant HCII on Thrombin-Inhibitory Activity

Although the GST- $\Delta 66$ HCII fusion protein produced low yields after bacterial expression and purification by glutathione-Sepharose, sufficient amounts of protein were obtained to permit gel-based assays that would determine if the fusion protein could form inhibitory complexes with thrombin. After electrophoresing aliquots of heparin-catalyzed reactions of a five-fold molar excess of GST- $\Delta 66$ HCII with thrombin, a thrombin-dependent band of approximately 110 kDa was visible after Coomassie-staining the SDS-PAGE gels. Most inhibitory serpins described to date consist of proteins that are approximately 400 residues, or 45 kDa in size (not including carbohydrate mass) (Gettins *et al.*, 1996). This observation that GST- $\Delta 66$ HCII formed complexes with thrombin demonstrates the thrombin-inhibitory activity of the largest functional serpin described to date. This finding could potentially be exploited in the future design of chimeric serpin molecules. Unfortunately, insufficient amounts of purified fusion proteins were obtained, which consequently did not permit a more extensive kinetic characterization of the anti-proteinase activity of this interesting molecule.

Initially, SDS-PAGE gel-based analyses were conducted in order to determine if the bacterially-derived HCII molecules could form visible inhibitory complexes with thrombin by Coomassie staining. In preliminary experiments with wild-type H₆-full-length and H₆- $\Delta 66$ HCII, HCII-thrombin complexes were observed by 2 minutes after incubation with heparin. A time course of thrombin inhibition demonstrated that the

serpin-enzyme complexes were detectable and were stable over at least 20 minutes. Subsequent analyses of P6 mutants in the full-length and H₆-Δ66HCII were visualized after incubation with thrombin and heparin for 5 minutes. In the H₆-Δ66HCII background, the Phe, Ser, and Trp P6 variants formed visible complexes with thrombin, while the Cys, Glu, Arg and Leu variants, instead of forming inhibitory complexes with thrombin, were cleaved by the protease, as evidenced by the presence of a faster migrating protein product, compared to intact H₆-Δ66HCII, corresponding to the expected size of H₆-Δ66HCII cleaved at the reactive site.

Next, extensive kinetic characterizations of H₆-Δ66HCII P6 variants were completed by determining the progressive and heparin-catalyzed second order rate constants of thrombin inhibition, as well as the inhibition stoichiometries for all of the recombinant P6 mutants. As a first step, second order rate constants for wild-type H₆-Δ66HCII were determined, which were in agreement with published ranges for similar N-terminally truncated HCII molecules that lacked the first acidic domain (Pratt *et al.*, 1990; VanDeerlin and Tollefsen, 1991; Bauman and Church, 1999). For instance, the progressive second order rate constant for H₆-Δ66HCII was $1.6 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$, which was close to the published range of $1.0 - 1.3 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ for other similarly truncated HCII molecules. Moreover, the second order rate constant determined for H₆-Δ66HCII in the presence of heparin was $7.8 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, which corresponded well to other similarly truncated HCII proteins with reported heparin-catalyzed second order rate constants ranging from 1.5 to $6.4 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$.

In contrast to the other, previously described truncated HCII molecules lacking the first acidic domain, H₆-Δ66HCII contained an N-terminal hexahistidiny tag that was used as a purification and detection tool. While Bauman and Church (1999) reported that the presence of a hexahistidiny tag at the C-terminus of HCII increased its heparin-catalyzed thrombin-inhibitory activity by over two-fold, the N-terminal hexahistidine tag did not affect the activity of H₆-Δ66HCII. Bauman and Church (1999) reported that C-terminally tagged HCII had a higher affinity for heparin, but not dermatan sulfate binding, and they proposed that the C-terminal hexahistidiny tag interacted in an intramolecular manner with internal residues of HCII, perhaps by altering the heparin-binding site within the serpin. This proposal is also consistent with our observations, as it suggests that the position of the His tag, rather than merely its presence in HCII, accounted for the supranormal activity. The N-terminus is clearly more amenable to modification, as previously demonstrated by Blinder *et al.* (1988), who deleted residues 1 to 18 of HCII without effect on the function of the inhibitor.

Determinations of second order rate constants with the H₆-Δ66HCII P6 variants mirrored the results obtained in the gel-based assays, in that the P6 variants that formed visible HCII-thrombin complexes had the highest rate constants in both the progressive and heparin-catalyzed reactions with thrombin. The Phe mutant retained the closest to wild-type levels of thrombin-inhibitory activity, while the Ser and Trp variants also had significant rate constants of thrombin inhibition. As observed in the study of P4 and P4/P5 mutagenesis in AT, the presence of bulky residues within this region of the reactive centre loop had little effect on the ability of HCII to inhibit thrombin. Moreover,

the presence of charged residues (Glu, Arg) at P6 significantly reduced the activity of HCII, similarly to the results obtained with AT, further supporting the requirement that charged residues not exist within this region of the reactive centre loop for maintenance of anti-proteinase activity. In contrast, the Ser P6 variant had significant thrombin-inhibitory activity in HCII while the same substitution at P4 in rabbit AT resulted in a protein with severely abrogated function. The results of P6 mutagenesis in H₆-Δ66HCII were the direct result of amino acid substitutions, as shown by the fact that the Glu and Ser P6 variants, which lost and retained thrombin-inhibitory activity, respectively, both had similar heparin-binding profiles and similar heparin binding profiles as that observed with wild-type H₆-Δ66HCII. This suggests that the differences in function of the P6 variants were a direct result of the amino acid substitution within the reactive centre loop and not due to acquired differences in the ability to bind heparin.

This apparent discrepancy in results between the substitution of serine at P4 and P6 in AT and HCII, respectively, can possibly be explained by the orientation that the serine residue side chain maintains in the reactive centre loop. In the crystal structure of an AT dimer complexed with heparin pentasaccharide, the P4 isoleucine and P6 valine side chains of the active half of the dimer face into the solvent (Figure 36A), while in cleaved α₁-proteinase inhibitor, the P4 isoleucine and P6 leucine residue side chains are oriented within the body of the molecule, where they make non-covalent interactions with β-sheet A (Loebermann *et al.*, 1984; Skinner *et al.*, 1997) (Figure 36B). The substitution of a polar serine residue at P4 or P6 may alter the orientation of the reactive centre loop after cleavage and insertion into the underlying β-sheet. In the case of AT,

A



B



the orientation may be such that the insertion of the reactive centre loop is unstable, possibly due to electrostatic interactions between the hydroxyl group on the serine residue and the residues making up the underlying environment in the body of AT. Unfortunately, there is currently no known crystal structure of HCII. It is possible that the P6 residue in the reactive centre loop of HCII faces the solvent when inserted into β -sheet A, where a serine substitution could have minimal contact with underlying amino acids and subsequently would have normal reactive centre loop insertion stability. This hypothesis could provide an explanation for the divergent results of serine substitutions within the reactive centre loops of AT and HCII. Incidentally, the P6 residues in cleaved forms of both AT and α_1 -proteinase inhibitor both face the solvent (Delarue *et al.*, 1990; Loebermann *et al.*, 1984), which provides further support of this hypothesis. However, the observation that the P6 Ser variant had elevated inhibition stoichiometries in both the progressive and heparin-catalyzed reactions with thrombin suggest that this mutation does contribute some instability in the HCII-thrombin complex, presumably by a less thermodynamically favourable association between the inserted reactive centre loop and β -sheet A.

Wild-type H₆- Δ 66HCII had SI values of 1.4 and 1.8 in the absence and presence of heparin, respectively, indicating that the wild-type molecule behaved almost exclusively as an inhibitor of thrombin. A slight increase in the inhibition stoichiometry is observed when heparin is present in the reaction; however, this heparin-dependent observation has been previously reported (Olson, 1985; Björk and Fish, 1982). The higher SI indicates that, in the presence of heparin, some thrombin molecules escape

inactivation by avoiding being trapped in an inhibitory complex with H₆-Δ66HCII. Instead of becoming trapped, thrombin cleaves the serpin and inactivates it. Some non-inhibitory interaction of thrombin and H₆-Δ66HCII occurred when heparin is present because small amounts of cleaved H₆-Δ66HCII were present in the gel-based thrombin-inhibition assays, which were conducted in the presence of heparin.

Given that several of the P6 variants in H₆-Δ66HCII formed visible complexes with thrombin in gel-based assays, and that they had elevated SI values indicate that these molecules form, to varying degrees, unstable complexes with thrombin. Some other point mutations within the reactive centre loops of both HCII and α₁-proteinase inhibitor have shown similar tendencies to form less stable complexes with their target proteases. For example, the substitution of arginine for wild-type leucine at P1 in HCII creates a thrombin-inhibitory serpin that forms unstable complexes in the presence of heparin (Ciaccia, 1997). In α₁-proteinase inhibitor, mutagenesis at P5 that resulted in substitution of a positively charged residue in the place of a wild-type negatively charged residue caused the serpin to inhibit elastase, its target protease, at 50% efficiency compared to wild-type, and form less stable complexes (Chaillan-Huntington and Patston, 1998). The apparent discrepancies between the gel-based analyses and SI determinations can be explained by the differences in reaction conditions that were used in each set of experiments. The gel-based analyses of H₆-Δ66HCII-thrombin complex formation were conducted essentially under initial reaction conditions, whereas the SI values were determined once the reactions had gone to completion. Therefore, direct comparisons between gel-based and SI observations cannot be made. The kinetic analyses

convincingly show, however, that the elevated SI values and low rate constant values for the Cys, Glu, Leu, and Arg P6 mutants demonstrate the tendencies of these molecules to act as substrates in the reaction with thrombin. These observations are consistent with the gel-based observations of exclusive substrate activities of these P6 mutants.

Similar amino acid substitutions at the P6 residue in full-length, N-terminally hexahistidinyI-tagged HCII gave similar results with respect to the ability to inhibit thrombin. The Phe, Ser, and Trp variants all formed complexes with thrombin in gel-based assays and had second order rate constants most similar to wild-type bacterially derived H₆-HCII. The amino acid substitutions in the full-length H₆-HCII background appeared to result in molecules which formed less stable complexes with thrombin compared to the same residue substitutions in H₆-Δ66HCII. This was evident in both the gel-based thrombin inhibition assays, as well as in the determination of inhibition stoichiometries. Wild-type H₆-HCII had an SI value of 1.1 in the absence and presence of heparin. However, it is not readily evident why the SI did not increase, as expected, when heparin was added to the reaction, especially when the gel-based assays provided evidence that some wild-type molecules have substrate activity. This was evident by the presence of cleaved H₆-HCII in the gel-based assays.

The SI values determined for all of the P6 H₆-HCII demonstrated that all P6 variants had varying degrees of substrate activities. The Trp variant had the most similar SI to wild-type H₆-HCII in the absence of heparin but its susceptibility to cleavage by thrombin in the substrate reaction appeared to be extensive when heparin was present. This observation is consistent with the gel-based observation that minimal H₆-HCII-

thrombin complex formed with the Trp P6 variant, despite the difference in reaction conditions between the gel-based assays and SI determinations. It appears that all P6 substitutions were tolerated less well in the H₆-HCII background compared to those in the H₆-Δ66HCII molecule. The reasons for this observation are not known; however, it is possible that the presence of the N-terminal hexahistidinyI tag and the first acidic domain, which are both present in the full-length serpin whereas only the histidine tag is present in H₆-Δ66HCII, complicates the appropriate folding of the protein. Purification of H₆-HCII results in the isolation of two pools of protein, which are immunoreactive with anti-HCII antibodies (V. Bhakta and Dr. W.P. Sheffield, unpublished observations). The first pool, which elutes from an ion exchange column at low salt concentration is functional, whereas another protein fraction, which elutes at higher salt concentrations does not inhibit thrombin. This is not observed with H₆-Δ66HCII, suggesting a possible assembly defect in the full-length His-tagged, bacterially-derived serpin.

4.2.2 Effects of Reactive Centre Loop Mutagenesis in HCII on Susceptibility to Elastase Cleavage

Prior to analyzing the effects of reactive centre loop mutagenesis on the susceptibility to NE cleavage in HCII, the effects of NE incubation with plasma-derived HCII were evaluated, and compared to the results reported by Pratt *et al.* (1990). The presence of a 60 kDa band in the reaction between plasma-derived HCII and NE in the absence and presence of heparin was observed, as expected. In the reaction lacking heparin, this band appears at earlier time points than that observed in the reaction containing heparin. Also, at any given time point, the intensity of this 60 kDa band is

greater in the reaction conducted in the absence of heparin. These observations are consistent with reported preferential cleavage of the N-terminal NE cleavage site at Ile-66. While cleavage at this site occurs when heparin is present, it occurs less frequently than when heparin is absent from the reaction.

Analysis of plasma-derived HCII after NE cleavage in the presence of heparin did not yield an expected 65 kDa band, which would have corresponded to cleavage of HCII within the reactive centre loop. It was initially thought that heterogeneous glycosylation of plasma-derived HCII might obscure the cleavage pattern of the protein, given that the expected cleavage product would migrate with similar electrophoretic mobility as the intact molecule on SDS-PAGE gels. However, enzymatic removal of N-linked glycans from HCII after incubation with NE did not resolve the appearance of a C-terminally cleaved HCII band. Subsequently, chromogenic substrate analyses of residual thrombin activities were used to demonstrate that cleavage, and inactivation of HCII occurred after incubation with NE in the presence of heparin. As reported by Pratt *et al.* (1990) and as mentioned previously, the heparin-independent reaction between HCII and NE decreases its ability to inhibit thrombin, based on second order rate constant determination, by 100-fold. Conversely, when HCII is incubated with NE and heparin, the serpin is inactivated. It was shown that, although a heparin-dependent NE cleavage product of HCII could not be visualized, HCII that had been incubated with NE and heparin was significantly less functional than HCII that had been treated with NE without heparin being present in the reaction. This provided support for NE cleavage within the reactive centre loop of HCII.

The interaction between NE and the GST- $\Delta 66$ HCII fusion protein was analyzed by visualizing Coomassie-stained products of the reaction that had been electrophoresed by SDS-PAGE. The reaction containing NE did not show any visible bands around the molecular weight of intact GST- $\Delta 66$ HCII, which suggested that the fusion protein was subjected to extensive proteolytic degradation. The interaction between NE and GST has not been characterized, so the influence of these two proteins on the cleavage pattern of the fusion protein could not be anticipated.

Cleavage of H₆- $\Delta 66$ HCII by NE in the absence and presence of heparin appeared to yield the expected protein products. In the absence of heparin, no NE cleavage was expected or observed, given that the Ile-66 cleavage site was absent in this protein. At later time points, however, cleavage appears to occur, but the major cleavage product corresponded to the expected size of H₆- $\Delta 66$ HCII that had been cleaved in the reactive centre loop. The observation that anti-histidine antibodies were immunoreactive with this NE cleavage product further supported this hypothesis. In the presence of heparin, cleavage within the reactive centre loop occurred quickly in the reaction with NE. At later time points, however, it appeared that H₆- $\Delta 66$ HCII was susceptible to additional NE proteolysis. Although the site of this proteolysis is not currently known, the protein was not reactive with anti-histidine antibodies, which suggests that the previously undescribed proteolytic site exists at the N-terminus of the bacterially-derived protein. Interestingly, similar incubation of NE with plasma-derived HCII also yielded a similar protein band, but it is not known if the extra site of NE cleavage is the same in both HCII molecules.

A quantitative analysis of the effects of NE pretreatment on the H₆-Δ66HCII P6 variants that retained thrombin-inhibitory activities demonstrated that the substitutions of Phe, Ser and Trp all conferred similar degrees of resistance to NE cleavage in the presence of heparin. As mentioned previously, this result is not surprising given that NE has a preference for small, non-polar residues at its primary site of cleavage. All of the P6 variants were, however, still susceptible to some degree of NE inactivation. It is unknown if NE reacted with the P6 variants at the same site of NE cleavage at a slower rate, or if NE cleaved at an adjacent site with a more favourable small, alkyl side group present. This possibility seems to be the most feasible answer given that the reactive centre loop of HCII contains five small, non-polar amino acids in the region between P13 and P5'.

H₆-HCII was also incubated with NE in the absence and presence of heparin and the resultant protein products were visualized by Coomassie-stained SDS-PAGE gels. In the absence of heparin, preferential cleavage of Ile-66 is expected. The appearance of a 55 kDa band, which would have corresponded to approximately the expected size of H₆-HCII that had been cleaved by NE near the N-terminus was not observed to a very large degree. Other cleavage products were produced, but they reacted with anti-histidine antibodies and this indicated that the cleavage that had occurred was not the result of N-terminal cleavage. In addition, another bacterially-derived HCII molecule, H₆-HCIIP1' did not appear to react with NE in the absence of heparin, as shown by the lack of NE cleavage products from the input protein. The observations of the reaction of NE with these two bacterially-derived HCII molecules suggest that production of these proteins in

an expression system that does not glycosylate may contribute to the observed differences in interacting with NE, compared to the plasma-derived protein. The observation that all seven P6 variants of full-length H₆-HCII did not form inhibitory complexes with chymotrypsin, another target protease of this serpin, further supports the notion that bacterially-derived HCII may not assume the physiological conformation as that seen in the plasma-derived molecule. It is possible, however, that the presence of the N-terminal histidine tag may interfere with the inhibitory ability of HCII towards chymotrypsin, but this hypothesis was not tested.

Heparin-dependent cleavage of H₆-HCII by NE, however, appeared to occur in an expected manner. Cleavage of full-length H₆-HCII occurred by 2 minutes and the observation that the major NE cleavage product reacted with anti-histidine antibodies demonstrated that cleavage had occurred at the C-terminus of H₆-HCII.

Repetition of the quantitative analysis of the effects of NE pretreatment on the thrombin-inhibitory activity of the functional H₆-HCII P6 variants yielded similar results as those obtained in the H₆-Δ66HCII background. All three of the Phe, Ser, and Trp P6 variants demonstrated similar levels of resistance to NE inactivation compared to the wild-type full-length molecule. These results were expected, given that H₆-HCII differed from H₆-Δ66HCII only at its N-terminus, which presumably would not have affected the reactive centre loop of the molecule.

With both H₆-Δ66HCII and H₆-HCII having demonstrated the ability to be less susceptible to NE cleavage after amino acid substitution at P6, the possibility of using “hardened serpins” to combat sepsis can be postulated. The use of NE-resistant

thrombin-inhibitory serpins could theoretically reduce the consumptive coagulopathy that is observed in this disease (Fourrier *et al.*, 1992). NE-resistant AT and HCII could potentially remedy the decrease in regulation of thrombin activity that contributes to disseminated intravascular coagulation that is sometimes observed in sepsis, although it is important to note that sepsis is a multi-factorial disorder with several abnormal processes occurring in coagulation and fibrinolysis simultaneously (Fourrier *et al.*, 1992). Greater control of thrombin activity through the use of recombinant serpins would not necessarily remedy all of the metabolic abnormalities that are observed in the disease, but might contribute to a decrease in mortality that is very often a consequence of sepsis.

5. CONCLUSIONS AND FUTURE EXPERIMENTS

The aim of this thesis was to examine the limits of amino acid substitutions at the sites of NE cleavage in AT and HCII, which would ideally provide information on the structure-function relationship of this region of two thrombin-inhibitory serpins. The expression, purification and analysis of recombinant AT and HCII proteins demonstrated that, while some plasticity is permissible in the amino acid composition of this region of the reactive centre loop with respect to non-conservative amino acid substitutions and maintenance of thrombin-inhibitory activity, there are some limitations to residue substitutions. It appears that the charge or polarity of the substituted residue is more important than its size with respect to maintaining the ability of these serpins to inhibit thrombin.

In regards to susceptibility to NE inactivation, the significance of the results are not as clear. In AT, where two NE cleavage sites exist adjacent to one another within the reactive centre loop, substitution of the bulkiest amino acid, tryptophan, does not confer any significant NE resistance on the molecule. It is possible that the interaction between NE and its target protein is determined not primarily through the interaction of protease and the primary cleavage site, but that the contribution of the entire substrate binding domain of NE towards the substrate target could be important. Preliminary evidence suggests that in addition to the primary NE cleavage site, adjacent residues to the primary sites of NE cleavage in human AT play an important role in determining the cleavage status of this serpin (Zendejrouh *et al.*, 1999). Those investigators made human AT resistant to NE inactivation by altering, in addition to the primary NE cleavage sites,

several sites around the reported NE cleavage sites in AT. Perhaps the interaction between several sites of the reactive centre loop of AT with the substrate binding domain of NE is required for efficient proteolysis of the serpin.

Mutagenesis of the NE cleavage site within the reactive centre loop of HCII, however, appears to confer more significant resistance to NE inactivation. Perhaps the differential results obtained in the HCII background compared to AT are the result of the existence of only one NE cleavage site within the reactive centre loop, or maybe the results that were obtained were dependent on the expression system that was used to express each serpin. While AT was expressed as an optimally-folded, glycosylated protein, recombinant HCII was unglycosylated, although its apparent functionality would suggest that it was optimally-folded as well.

It is interesting to note that the mutagenesis at a single amino acid can confer significant differences in protein characteristics. One might argue that if AT or HCII was meant to become resistant to NE inactivation that the selective pressures of evolution would have created such a molecule by now. Arguing against this point, however, is the recent discovery by O'Reilly *et al.* (1999) that cleaved AT possesses powerful anti-angiogenic and anti-tumour activity in mouse models, characteristics that are not shared with intact, functional AT. A similar argument can be made for HCII. Pratt *et al.* (1990) have demonstrated that the N-terminal 66 amino acids of HCII that are liberated after reaction with NE, possess chemotactic activity, which is believed to be involved the recruitment of macrophages and lymphocytes to sites of vascular injury. Obviously, AT

and HCII have roles that are independent of coagulation and perhaps their interactions with NE are necessary in the regulation of other, currently unknown processes.

Further experiments on these interesting molecules could be conducted to provide a more complete understanding of AT and HCII:

- 1.) Determination of the NE cleavage sites of AT and HCII P6 variants with reduced susceptibility to NE inactivation should be determined, by amino acid sequencing to answer the question of the importance of the primary NE cleavage site in serpin inactivation. In addition, making the P6 substitutions in glycosylated HCII which would be more similar to the plasma-derived protein might provide some insight into the importance of carbohydrate moieties in the reaction of NE with HCII. This could be accomplished by expressing variant HCII proteins in an insect cell line employing baculovirus vectors, which are commercially available and have previously been shown to be effective in expressing recombinant HCII (Bauman and Church, 1999).
- 2.) While the recognition sites for NE and thrombin within the reactive centre loop of AT and HCII are only 4 and 6 residues, respectively, distant from the site of interaction of these serpins with thrombin that normally result in the formation of inhibitory complexes, NE merely cleaves and inactivates the proteins. Determination of the minimum size of the reactive centre loop to facilitate inhibitory versus substrate reactions by serpins with proteases would provide further information on the structure-function relationship of inhibitory serpins.

Analysis of the effects of deletion mutagenesis (ie. deletion of P2 to P7) within the reactive centre loop could answer questions regarding the minimum size of the reactive centre loop that are necessary to maintain thrombin-inhibitory activity.

- 3.) Determination of the crystal structure of the serpin-enzyme complex is the “holy grail” of serpin biochemistry. Knowing the precise structure that a serpin maintains to confer its inhibitory properties would open the door to the development of novel drugs that could aid in the battle against diseases involving abnormalities in coagulation, fibrinolysis, inflammation, and cancer.**

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FCR/JP/025
04 April 2000

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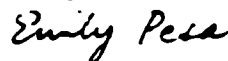
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