ENGINEERING OF SERPINS FOR VASCULAR APPLICATION

Jill Camille Rau

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine.

Chapel Hill
2008

Approved by:

Frank C. Church, Ph. D.

Herbert C. Whinna, M. D., Ph. D.

Alisa S. Wolberg, Ph. D.

Maureane R. Hoffman, M. D., Ph. D.

David R. Clemmons, M.D.

ABSTRACT

Jill Camille Rau: *The Engineering of Serpins for Vascular Application*

(Under the direction of Frank C. Church, Ph.D.)

"The Engineering of Serpins for Vascular Applications" contributes to the understanding of serine protease inhibitors (serpins) in cardiovascular disease, the relationship between their structures and activities, and the manipulations of those structures for the benefit of medicine.

Chapter 1 presents a comprehensive overview of serpins' structure-activity relationships, pathophysiology and their roles in thrombosis, hemostasis and fibrinolysis. Chapter 2 establishes the co-localization of (pro)thrombin with heparin cofactor II (HCII) in the core of atherosclerotic plaques and demonstrates a positive correlation between the presence of HCII, (pro)thrombin or antithrombin (AT) and lesion severity. Additionally, these results indicate that atheromas may act as a non-specific watershed for plasma proteins and should serve as a warning against assumptions that (co-)localization of protein in atheromas implies a pathophysiologic role in atherosclerosis.

Chapter 3 utilizes a gain-of-function approach to examine glycosaminoglycan (GAG)-binding in serpins. An α1-protease inhibitor (α1PI) mutant, $\alpha1PI_{Pitt\text{-}GAG}$, containing five basic residues homologous to the HCII D-helix was shown to have HCII-like GAG-accelerated thrombin inhibition. Results confirm the benefit of using a gain-of-function approach to the study of serpins. The finding that GAG-binding and associated functionality

can be added to a non-GAG binding serpin has exciting implications for the engineering of serpins for biomedical purposes.

In Chapter 4, a chimeric serpin, HATpin, was engineered with intent to create a potent anti-thrombotic, anti-inflammatory, anti-atherogenic protein. HATpin was designed to comprise 1) the reactive center loop of AT for specificity of thrombin and factor Xa over activated protein C; 2) the N-terminal acidic domain of HCII to utilize thrombin exosite 1 for specific thrombin inhibition; 3) the GAG-binding region of HCII to target it to areas of vascular injury; 4) on an α1PI backbone. Although HATpin was successfully created, its inhibitory profile failed to meet expectations. The results from Chapter 4 illustrate our need for a better understanding of serpin structural domain interactions and challenge the concept that serpin domains are strictly modular building blocks that can be exchanged without a contextual effect.

Finally, Chapter 5 outlines additional future studies that can be pursued based on questions that arose during the completion of this dissertation.

ACKNOWLEDGEMENTS

This endeavor took almost five years to achieve. I started, even before my graduate school training, as a technician in Frank Church's lab four years prior with virtually no understanding of basic laboratory science. In the nine years since I began learning in this field, I've come a very long way. This hasn't been on my own. There have been many, many people and groups who have supported me, encouraged me, taught me, challenged me, kept me sane, paid for me and contributed in some way, big and small to the completion of this dissertation. I would like to recognize those people and put down in black and white that they have contributed to my growth and to this dissertation. I owe them much and am very appreciative of their help.

First, this dissertation project could not be undertaken without funding. I appreciate those institutions who have believed in me and my science. The Holderness Foundation provided me funds for a summer research fellowship during the summer after my first year of medical school. The Integrative Vascular Biology Training Program funded by the National Institutes of Health, National Heart Lung and Blood Institute (NIH-NHLB 5T32HL069768-04) and directed by Nobuyo Maeda, Ph. D. took me on as a trainee for my first several years of graduate school. The Triangle Community Foundation selected me to receive the 2006-2007 Gertrude B. Elion Mentored Medical Student Award which provided me funds for educational purposes that I have put to wonderful use and has greatly enhanced my

education and the completion of this dissertation. The American Heart Association then took a chance on me and provided me with a Pre-Doctoral Fellowship in 2007 (AHA 0715191U) . Finally, the National Institutes of Health, Institute on Aging have granted me a Ruth L. Kirschstein National Research Service Award (NIH-NIA 1F30AG029053 - 01A2) to pay for both the completion of my Ph.D. and my M.D. degrees. Additionally, I am grateful to The Department of Pathology and Laboratory Medicine and to Frank Church for providing additional funds for lapses in financial support and for funding the laboratory research I performed.

Second, I would like to acknowledge and thank my committee members for their diligence, support, and mentoring. David Clemmons, M.D. initially came on board because I was going to devote some of my dissertation to a topic related to his own research. That did not pan out, however, Dr. Clemmons cheerfully remained on my committee to support me and my research. Maureane Hoffman, M.D., Ph.D. is an amazing woman and a wonderful role model for me. She's acted as a friend, mentor and scientific consultant. She provides calm and insightful advice and always makes the time to work and meet with me. Alisa Wolberg, Ph. D. is an inspiration. Not much older than myself, Alisa is running her own laboratory. She is also always willing to go the extra mile to meet with me to review my work. She is very thoughtful and level headed and has kept my Ph.D. on track. Herb Whinna, M.D., Ph.D. is combination friend, mentor and boss. I've worked with Herb for 9 years and enjoyed doing so. As a committee member Herb is superb. He cuts to the chase, understands the science very well and gives the bottom line. As a friend and a person, I have the utmost respect for Herb. He has helped to shape my laboratory and Ph.D. experience into

an enjoyable one and I appreciate his passion and cynicism both. My final committee member, Frank Church will be discussed later.

Next, I would like to acknowledge all those individuals (of which there are many) who provided a scientific contribution to my dissertation and or training. Peter Gettins, Ph. D. of the Department of Biochemistry and Molecular Genetics at the University of Illinois at Chicago provided me with the cDNA for both a1PI and $a1PI_{Pittsburgh}$. Steve Olson, Ph.D. of the Center for Molecular Biology of Oral Diseases, College of Dentistry at the University of Illinois at Chicago helped me understand, optimize and interpret the extrinsic fluorescence assays. James A. Huntington, Ph.D. and the members of his laboratory, Denis O'Keeffe, Daniel Johnson, Jonathan Langdown, Wendy Carter, Ph.D. , Stefan Luis, Aiwo Zhou, Li Wei, Ph. D. and Professor Robin Carrell all welcomed me with open arms into their lab for a 4 month long project in the Cambridge Institute for Medical Research, U.K.. There, in addition to having a wonderful time, I learned a considerable amount about serpins and science in general. I am extremely grateful to have had the opportunity to work with them.

I am thankful the following individuals in the UNC School of Public Health: to Bob Schoonhoven from the Center for Environmental Health and Suceptibility for teaching me to perform immunohistochemistry; and the members of the Biometric Consulting Laboratory, Carolyn Deans, Brian Armstrong and Kelly Hunnigan under the guidance of Gary Koch, Ph. D. for performing my statistical analysis.

In the UNC School of Medicine, I would like to thank John Sondek, Ph.D. and his laboratory in the Department of Biochemistry and Biophysics for letting me use their fluorometer; James Faber, Ph.D. in the Department of Cell and Molecular Physiology for acting as a collaborator on one of my grants what was funded. I would like to thank David

Siderovski, Ph. D. and Lee Graves, Ph. D. and their laboratories in the Department of Physics for lending me pretty much anything I asked for, including their advice and guidance.

In the Department of Pathology and Laboratory, my home department, there are many individuals who contributed to my research and training and deserve recognition. First, the graduate students, they are fun and welcoming and always helpful when in need of scientific help, a shoulder to cry on or a beer. Next, Lawrence Kiss, M. D. ranked some of my immunohistochemistry slide sets. David T. Thomas, M.D., served on my committee before moving to New York and guided me through much of the pathology in my dissertation. Donna Thompson trained me to section paraffin embedded tissue and let me use her facilities. Similarly, Lisa Cremeans taught me to formalyn-fix and paraffin embed and also to snap freeze fresh tissue. Howard Reisner, Ph. D. and Gayle McGhee trained me to use the Aperio Scanning Scope so that I would have easily used and attractive images of my slide sets.

I am thankful to all the individuals associated with the MD/PhD Training Program here at UNC. They have been incredibly supportive during this very long endeavor. The students in the program all experience similar experiences and they are invaluable assets and friends. Eugene Orringer, M.D., Liz Garman and David Siderovski, Ph. D. have created a maintained a wonderful program an environment for which I cannot be more grateful. Finally, Patricia Byrns, M.D. while not officially part of the MD/PhD training program, but whom I was introduced through it, has been incredibly encouraging and was very helpful in the submission of one of my grant applicatioins.

The Church Lab group extends beyond just the Church lab members themselves and there are extended members whom I am in frequent contact who have been helpful in more

ways than can possibly be explained in this small space, but I am particularly grateful to Nigel Key, M.D. for his guidance and particularly his help with my grant applications. I would like to thank Nigel Mackman for his contagious enthusiasm for science and thoughtful insight into my research and his post-doctoral fellow, Jianguo Wang, Ph.D. (a.k.a. Kevin) for his help with my fluorescent immunoblots.

Jen-Yea (Peter) Chang, Ph. D. is an amazing resource who is always willing to help. He almost single-handedly forced my molecular biological creation of HATpin to work. I am certain I would be much, much less knowledgeable without the benefit of Peter's tutelage. Likewise, Dougald (Mac) Monroe III, Ph. D. -- I can't say enough about how wonderful Mac has been throughout the years; supportive, intuitive, with insight and perspective, a veritable encyclopedia of a man with incredible capacity for teaching. Mac helped me understand all the math and kinetics that I used in this dissertation and has made me a better scientist.

The immediate members of the Church Lab throughout my years are numerous and all have had an impact on me in one way or another. I thank them for teaching me, making me laugh, supporting me, humoring me and all the other things that goes along with being a labmate. The post-doctoral fellows I'd like to recognize are Jennifer Gibbons, Ph.D., Yolanda Fortenberry, Ph.D., and Sophie Rehault, Ph.D. The graduate students are Jennifer Carter, Mark Grammling, Troy Mceachron, Jessica Cardenas, Lea Beaulieau, Ph.D., Brandi Hilder, Ph.D., Jennifer Mitchell, Ph.D., Alexis Henry, M.Sc., and Diane Palmieri, Ph.D. Thank you all. The technicians, undergraduates, rotating graduate and medical students are numerous and I appreciate the interactions I've had with all of them, however there are too many to name. However, I'd like to thank, in particular, Romer Orada , Amy Stuckey, Ginger Bond, Melissa Cooper, Rachel Whinna, Rebekah Bourland , Lea Hawkins, Emir

Sandhu, Shawna Kelly, Ryan Bilas , John Adams, M.D., Nell Pollard, and Josh Davis. Additionally, the staff members associated with the Church lab are invaluable, especially at grant time, but every day as well. So I'd like to thank both Floyd Whitney and Cheryl Jeanneret for their support.

It is noticeable how many people have supported me and contributed to my education and dissertation. This is largely because Frank Church, Ph.D., my boss and friend fosters such a collaborative and family environment. Frank has supported me in every decision I've made. He doesn't do so blindly, but he does always support me, in every way that he can. I can't begin to describe my appreciation for everything he has done for me. To list just a few, he gave me a job, helped get me into medical school, helped me secure funding, helped get me into the M.D. /Ph.D program, got me a position in Cambridge so that I could live with my husband. The list could go on and on and on. Essentially, Frank has encouraged, taught, mentored and enabled me to carry out my Ph.D. work my way, so that I became the best scientist that I could be. Thank you so much for this wonderful experience, for your help, and for your friendship.

Finally, I'd like to thank all my friends and family for their continued love and support through this whole process. I couldn't have done it without them, and I can't say enough to thank them. In particular, Jennifer Gilner, Ph. D., Anna Ballew and Alice Ma, M.D. have been my best friends through this work. They're always available and have made finishing this much less painful then it could have been. They also fill my life with joy which is much more important. Tom Rau, Pete Rau and Mom (Paula Rau) are my base. I can do everything I do, because I know they love and support me. Life is what it is, (wonderful) because they are my family. And Richard Owen, the love of my life. He has been submitted to every trial

and tribulation, every up and down that I have experienced during my research experience and he's continued to love me and support me. He fills my life and my world with love, companionship, adventure, excitement, and peace. For these four people, there are not words enough to express my gratitude and love.

To all of you I've mentioned and the many more who have touched my life, thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

° C	degrees Celcius
5 x	five times magnification
A; Ala	alanine
A_{280}	light absorbance at 280 nm
AnII	annexin ii
APC	activated protein c
AT	antithrombin
C; Cys	cysteine
Ca++	calcium (ion)
cDNA	complementary dna
CHD	coronary artery disease
-CTRL	negative control
CVD	cardiovascular disease
DEGR	dansyl-glu-gly-arg chloromethyl ketone
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E; Glu	glutamate
EDTA	ethylenediaminetetraacetic acid
EPCR	endothelial cell protein receptor
et al.	and others
F; Phe	phenylalanine

fII	prothrombin (Factor II)
fIIa; IIa	thrombin (Factor IIa (activated))
fIX	Factor IX
fIXa	Factor IXa *(activated)*
FPLC	fast protein liquid chromotography
fV	Factor V
fVa	Factor Va *(activated)*
fVII	Factor VII
fVIIa	Factor VIIa *(activated)*
fVIII	Factor VIII
fVIIIa	Factor VIIIa *(activated)*
fV_{Leiden}	Factor V Leiden
fX	Factor X
fXa	Factor Xa *(activated)*
fXI	Factor XI
fXIa	Factor XIa *(activated)*
fXIII	Factor XIII
fXIIIa	Factor XIIIa *(activated)*
g	gravitational force
G; Gly	glycine
GAG(s)	glycosaminoglycan(s)
H; His	histadine
HCII	heparin cofactor ii

HIT	heparin induced thrombocytopenia
I; Ile	isoleucine
ICC	intra-class correlation coefficient
IgG	gamma immunoglobulin
IIa ; fIIa	thrombin
K; Lys	lysine
k_2	second order rate of inhibition
K_d	dissociation constant
kDa	kilodaltons
L; Leu	leucine
LAD	left anterior descending coronary artery
LDL	low density lipoprotein
LMWH	low molecular weight heparin
M	molar
M; Met	methionine
mg	milligram
mL	milliliter
mM	millimolar
M_r	molecular weight
N; Asn	asparaginine
NaCl	sodium chloride
nM	nanomolar
nm	nanometer

NO	nitric oxide
P; Pro	proline
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator-2
PAR	protease activated receptor
PBS	phosphate buffered saline
PBStw	phosphate buffered saline with Tween 20
PC	protein c
PCI	protein c inhibitor
PDAY	Pathobiological Determinants of Atherosclerosis in Youth Study
PEG	polyethylene glycol
Penta	pentasaccharide
PL	phospholipid
PMSF	phenylmethanesulfonyl fluoride
PN-1	protease nexin-1
PPACK	d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone
Q; Gln	glutamine
R; Arg	arginine
r^2	correlation coefficient
RSL ; RCL	reactive site loop; reactive center loop (interchangeable)
s	seconds
S; Ser	serine
SDS	sodium dodecyl sulfate

SDS-PAGE	sds-polyacrylamide gel electrophoresis
Serpin	serine protease inhibitor
SMB	somatomedin b
SNAC-heparin	sodium n-(8-[2-hydroxybenzoyl] amino) caprylate bound heparin
T; Thr	threonine
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLCK	tosyl-l-lysine chloromethyl ketone
TM	thrombomodulin
TNS	toluidino-2-naphthalenesulfonic acid
tPA	tissue plasminogen activator
TPCK	tosyl-l-phenylalanine chloromethyl ketone
UFH	unfractionated heparin
uPA	urokinase plasminogen activator
uPAR	upa receptor
VN	vitronectin
ZPI	protein z-dependent protease inhibitor
α1PI	α1- protease inhibitor
α2AP	α2-plasmin
μg	micrograms
μL	microliters

μM	micromolar
μm	micrometer

INTRODUCTION

Vascular Pathophysiology and Serpin Engineering

Implications of Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death in the United States[1]. It was responsible for over 800,000 deaths, more than 33% of deaths in the U.S in 2007[2]. Worldwide, CVD is responsible for more than seven million deaths each year[3]. It has been estimated that at an average of one in three Americans have one or more types of cardiovascular disease (over 80 million), only 47% of whom are 60 years old or more. The economic burden in the United States alone is expansive, estimated at over $400 billion dollars for 2008 in direct and indirect costs[1]. The personal burdens are incalculable.

Cardiovascular Disease Components

CVD is an aggregate of interrelated pathological conditions affecting the cardiovascular system. The Center for Disease Control has categorized the most commonly occurring conditions of CVD as coronary heart disease (CHD also known as coronary artery disease), stroke, heart failure, high blood pressure, and diseases of the arteries/peripheral artery disease. Eighty-six percent of all deaths from CVD are due to these causes[2]. CHD accounts for over 50% of deaths from CVD. It is caused primarily by atherosclerosis of the coronary arteries. Similarly, peripheral vascular disease can also be attributed predominantly to atherosclerosis, but in the peripheral arteries, commonly those of the legs and pelvis. The

most commonly occurring type of stroke, ischemic stroke, accounting for over 80% percent of strokes, is frequently caused by atherosclerosis of the carotid or cerebral arteries[4]. Heart failure and high blood pressure have numerous underlying causes, but atherosclerosis often is a key contributor. Thus, understanding and combating atherosclerosis is critical to reduction of the devastation of CVD.

Atherosclerosis Overview

Atherosclerosis is a chronic inflammatory process affecting the innermost layer of the arterial wall, the intima. While there are many contributing causes to the development of atherosclerosis, prevailing theory favors a combination of high plasma concentrations of low-density lipoprotein (LDL) concentration and endothelial cell dysfunction as the principle initiators of atherosclerosis[5,6]. A very basic description of the pathogenesis of atherosclerosis includes the initiation of a cascade of inflammatory events by increased LDL levels that begins with endothelial cell activation[7] in areas susceptible due to hemodynamic stress[8-10]. This induces the expression of surface receptors and adhesion molecules and which recruit monocytes and other inflammatory cells and facilitate their entry into the vascular intima[11]. Simultaneously, endothelial permeability to LDL is increased resulting in intimal accumulation of LDL[7]. Bound to the extracellular matrix, LDL can be enzymatically modified or oxidized. The resultant LDL species, are considerably more pro-inflammatory than native LDL[12]; they release phospholipids which can activate platelets and induce smooth muscle cells and endothelial cells to release additional cytokines[13]. These cytokines have a multitude of pro-inflammatory effects including the differentiation of monocytes to

macrophages[14]. Mature macrophages ingest the LDL creating foam cells, macrophages packed with lipid droplets. This is the first histopathological identifier of an early atheroma[15]. The foam cells and other recruited cells continue to express inflammatory signals, stimulating smooth muscle cell proliferation and migration. A fibrous cap of matrix proteins, rich in collagen I and interspersed with smooth muscle cells, may form over the accumulation of foam cells which often necrose and form the lipid-rich necrotic core of a more advanced atheromatous plaque[16]. The intima, thickened with increased cellularity and matrix deposition, may impinge on the vascular lumen. Consequently, this stenosis can cause ischemia of the region fed by blood flow through this artery, resulting in heart attack, stroke and claudication. However, this atherosclerosis is a relatively slow chronic process. Collateral blood flow is often able to compensate for such restrictions. Therefore heart attacks and stroke are not frequently the result of arterial stenosis from atherosclerosis. More commonly, at least 70% of the time, they are due to arterial blockage by a thrombus formed from plaque rupture or erosion of the endothelium[17].

Atherosclerotic Plaque Rupture

A thin fibrous plaque and an abundance of activated inflammatory cells mark the plaques most vulnerable to rupture[18]. The persistent stimulation of accumulated inflammatory mast cells, T-lymphocytes and macrophages results in the secretion and activation of matrix metalloproteases, which degrade the integrity of the collagen rich matrix of the fibrous cap and induces apoptosis of smooth muscle and endothelial cells[19]. Together, this weakens the stability at the surface of atheromatous plaque[20].

Progressive endothelial cell dysfunction reduces the ability of the endothelium to properly produce nitric oxide (NO)[19]. NO has many properties including, vasodilation, and inhibition of smooth muscle cell proliferation, platelet aggregation and leukocyte adhesion[8]. In normally functioning endothelial cells, NO is released by activation of specific receptors and by shear stress. In atherosclerotic conditions, which include a dysfunctional endothelium, intimal thickening (potentially protruding into the intima) and a thick fibrous cap with decreased flexibility, shear stress is increased in the region of the atheroma. However, NO is not released in response. Thus, vasodilation does not occur, the vascular lumen remains constricted, and inflammatory processes remain unmediated by NO. Additionally, increased shear stress itself can promote local inflammation[9].

With increased shear stress at the luminal surface and inflammatory cells eroding the fibrous cap from the inside, the interface between the blood and the intima is diminished and destabilized. When a plaque does rupture, tissue factor that is located on macrophages, lipid rich microparticles and smooth muscle cells within the atherosclerotic core is exposed to the blood[18]. This initiates a series of enzymatic reactions which promote blood clotting. (See Chapter I for a more detailed description of coagulation.) Ultimately, thrombin, the most crucial protease in coagulation is activated and a thrombus is formed. Thrombi formed from exposure of the lipid-rich core of advanced atheromas are up to six times greater in size than those formed from exposure to non-atherosclerotic vessel intima[21]; thus, increasing the likelihood of vascular occlusion and clinical events.

Atherosclerotic Plaque Erosion

In some cases of sudden death from coronary thrombosis, plaque rupture or exposure of the lipid rich atherosclerotic core to blood has not occurred. Rather, superficial erosion of the endothelium results in occlusive thrombus formation. The frequency of myocardial infarction and death due to atherosclerotic plaque erosion versus plaque rupture is controversial with percentages reported from 44%[17] to only 9%[22-24]. Regardless, tissue factor, normally shielded from the blood by the endothelium, is exposed to coagulation factors, thrombin is generated and an occlusive thrombus forms.

Non-Atherosclerotic Thrombosis: Venous Thrombosis and Atrial Fibrillation

Thrombus formation, or thrombosis, in non-atherosclerotic vessels is also a critical health concern. Venous thrombosis affects over 900,000 people in the United States each year and causes over 300,000 deaths[25,26] Additionally, atrial fibrillation which has a morbidity of over 2.2 million people in the United States carries the significant risk of thrombosis and thromboembolism, and itself is a leading risk factor for ischemic stroke[27]. In both venous thrombosis and atrial fibrillation, the most significant health threat is from emboli separated from thrombi that travel to the lungs (from deep vein thrombosis) or the brain (from the atrium) resulting in the severe consequences of pulmonary embolism and ischemic stroke.

The pathophysiologic mechanisms driving venous thrombosis are less understood than arterial thrombosis. The established perspective on the causes of venous thrombosis has centered on Virchow's triad[28,29] which ascribes thromogenesis to a combination of hemodynamic stasis or altered blood flow, intimal injury or endothelial dysfunction, and

hypercoagulability. Thrombosis in atrial fibrillation is generally attributed to the same causes with particular emphasis on "stasis"[30]. Fibrillations of the heart alter the natural flow of blood through the atrium leading to intimal injury and alteration in protein expression of the endothelium which upsets the hemostatic balance resulting in thrombus formation in the atrium[31]. In venous thrombosis, the inciting element is less apparent; any component of Virchow's triad can lead to the other. In the past decade however, an emphasis on inflammation in venous thrombosis has emerged[25,32,33].

As described above the endothelium is a producer of numerous regulatory molecules that modulate vascular flow, leukocyte adhesion, migration and activation. It is also vulnerable to the cytokines and proteases released by inflammatory cells[19,34]. Under normal conditions endothelium also produces thrombomodulin and tissue factor pathway inhibitor, potent anticoagulant molecules. When distressed the endothelium releases many thrombogenic agents including platelet activating factor, von Willibrand factor, plasminogen activator inhibitor-1 (PAI-1) and factor V[25]. Both locally and systemically, inflammation is associated with an increased risk of thrombosis[35]. This is evidenced by the many conditions of inflammation in which there is an increased risk of thrombosis such as in sepsis and cancer[36-38]. Thrombosis eventually occurs when an imbalance between pro-coagulant and anti-coagulant and also between pro-fibrinolytic and anti-fibrinolytic elements (See Chapter 1 for more details on coagulation and fibrinolysis.) is tipped in favor of those pro-coagulant and antifibrinolytic.

Therefore, pharmocotherapies aimed at the treatment of prevention of venous thrombosis, pulmonary embolism and thromboembolic stroke focus on re-establishing the balance and are either profibrinolytic or antithrombotic. Fibrinolytics are used only acutely

as they associated with severe side effects[39]. Antithrombotics however, are widely used and are the most common treatment for those at risk of venous thrombosis and atrial fibrillation[40,41]. Ultimately they target a reduction in thrombin generation.

Thrombin in vascular inflammation

In addition to its prominent role in cardiovascular disease as the essential protease in thrombosis, thrombin has many pro-inflammatory functions and plays a central role in vascular lesion formation[42,43]. It regulates inflammatory processes including leukocyte adhesion molecule expression on endothelium, platelet activation, leukocyte chemotaxis and endothelial production of pro-thrombotic factors. Thrombin is also a potent growth factor, initiating endothelial, fibroblast and smooth muscle cell proliferation and upregulating other cytokines and growth factors[35,44]. Because of its role in both atherosclerotic development and thrombus formation, the study of thrombin and its regulators is pertinent to the understanding of cardiovascular disease.

Thrombin and its inhibitors in CVD

Thrombin is a serine protease. Its cellular effects occur largely through its cleavage of the G-coupled protein receptors, protease activated receptors -1 and -4 (PAR-1 and PAR-4). In order for thrombin to cleave PAR-1 or PAR-4, it must be in its native form with the active site intact. Similarly, in order to promote thrombosis, thrombin must be proteolytically active, maintaining its structural integrity.

Serine protease inhibitors (serpins) are a family of proteins classified by their homologous structure. The majority of the family members inhibit serine proteases. Several

serpins are capable of inhibiting thrombin: antithrombin (AT), heparin cofactor II (HCII), protease nexin-1 (PN-1), plasminogen activator inhibitor (PAI-1), and protein C inhibitor (PCI). All of these serpins utilize a similar mechanism to inhibit thrombin (described in more detail in Chapter 1) in which thrombin cleaves the reactive site of the serpin forming a covalent bond between the molecules and releasing the serpin from its metastable state. This translocates thrombin to the opposite pole of the serpin, inducing a strain on the protease causing its inactivation. AT and HCII are particularly relevant to the inhibition of thrombin in cardiovascular disease. AT is the predominant thrombin inhibitor in coagulation[45], and HCII has been shown to be protective against atherosclerosis[46-50].

Serpin Engineering for Vascular Applications

This dissertation is focused on understanding and utilizing serpins, HCII in particular, for the purpose of combating cardiovascular disease. Chapter 1 provides an overview of serpins in hemostasis, thrombosis and fibrinolysis[51]. It describes their biological functions, mechanisms of action, key structural components, and pathophysiological consequences of their dysfunction. Chapter 2 focuses on the role of HCII in atherosclerosis, examining its presence in atherosclerotic plaques. Chapter 3 investigates a unique gain-of-function approach to serpin engineering, utilizing the prototypical serpin α1-protease inhibitor, α1PI, as a scaffold for the addition of HCII-like structural components. This study shows that HCII-like activity can be conferred upon α1PI through the mutation of specific homologous residues. Chapter 4 describes the design, creation and properties of a novel serpin created through domain engineering. The novel serpin named HATpin was designed to incorporate beneficial elements of both AT and HCII on α1PI in an attempt to create a superior thrombin

inhibitor to battle cardiovascular disease. Chapter 5 discusses the major findings within the dissertation and describes additional directions that would be interesting for investigation based on this work.

Overall, this dissertation represents the culmination of five years of work and learning. It illustrates the facets of serpin structure and activity and contributes to the understanding of serpins and their engineering for the amelioration of the burdens of cardiovascular disease.

CHAPTER 1

Serpins in Thrombosis, Hemostasis and Fibrinolysis

Jill C. Rau[‡], Lea M. Beaulieu[‡], James A. Huntington and Frank C. Church

[‡]These authors contributed equally to the review. (2007) ***J. Thromb. Haemost.*** 5: Suppl. 1, 102-115.

SUMMARY

Hemostasis and fibrinolysis, the biological processes that maintain proper blood flow, are the consequence of a complex series of cascading enzymatic reactions. Serine proteases involved in these processes are regulated by feedback loops, local cofactor molecules, and serine protease inhibitors (serpins). The delicate balance between proteolytic and inhibitory reactions in hemostasis and fibrinolysis, described by the Coagulation, Protein C and Fibrinolytic Pathways, can be disrupted resulting in the pathological conditions of thrombosis or abnormal bleeding. Medicine capitalizes on the importance of serpins, using therapeutics to manipulate the serpin-protease reactions for the treatment and prevention of thrombosis and hemorrhage. Therefore, investigation of serpins, their cofactors, and their structure-function relationships is imperative for the development of state-of-the-art pharmaceuticals for the selective fine-tuning of hemostasis and fibrinolysis. This review describes key serpins important in the regulation of these pathways: antithrombin, heparin cofactor II, protein Z-dependent protease inhibitor, α_1-protease inhibitor, protein C inhibitor, α_2-antiplasmin and

plasminogen activator inhibitor-1. We focus on the biological function, the important structural elements, their known non-hemostatic roles, the pathologies related to deficiencies or dysfunction, and the therapeutic roles of specific serpins.

INTRODUCTION

Blood flow is maintained by the proper balance of hemostasis and fibrinolysis, an interdependent network of physiologic processes and succession of proteolytic reactions. Hemostasis, the physiologic cessation of bleeding, involves the interaction of vasoconstriction, platelet aggregation and coagulation. The end result of coagulation is the deposition of cross-linked fibrin polymers to form blood clots. Both the Protein C and the Fibrinolytic Pathways are activated by the Coagulation Pathway and serve to restrict excessive clot formation or thrombosis. The enzymatic reactions that propel these pathways are dominated by serine proteases and are subject to control by serpins and their local cofactors. Dysfunction, deficiencies or over-expression of serpins can cause either abnormal bleeding or thrombosis. Investigations into the structure and related activities of serpins, their target proteases and cofactors has provided valuable information regarding both serpin-related disease states and potential mechanisms by which medicine can manipulate serpin-protease interactions for the treatment and prevention of thrombosis and bleeding.

HEMOSTASIS

Coagulation Pathway

The factors of the Coagulation Pathway generally circulate in an inactive state until they are activated through proteolysis by an upstream factor. While the end goal of

coagulation is fibrin polymerization, the most crucial feature of the Coagulation Pathway is the generation of thrombin (Figure 1.1). Thrombin is responsible for cleaving fibrinogen to fibrin, activating fXIII to fXIIIa (which cross-links fibrin), activating platelets, and positively feeding back into the cycle by activating upstream factors[52].

Thrombin generation is initiated when damage to a vessel wall exposes the blood to tissue factor (TF) in the subendothelium[53]. TF is also expressed by activated platelets and leukocytes[54]. Therefore, coagulation can also be initiated by inflammation. TF forms a complex with fVIIa and activates fX. Together, fVa and fXa form the prothrombinase complex which then cleaves a small amount of prothrombin (fII) to thrombin (fIIa). This small amount of thrombin activates platelets, fV, fVIII and fXI, feeding back into the cycle to increase thrombin formation. Factor IXa, previously activated by either TF-VIIa or by fXIa on the platelet surface, and fVIIIa in the presence of calcium, complex on the platelet surface to form the platelet tenase complex. Platelet tenase activates more fX, which with fVa, generates a "thrombin burst" (Figure 1.1). It is this burst of thrombin rather than the initial thrombin activation that is crucial for the formation of a stable hemostatic plug[53].

In addition to its role in hemostasis, thrombin regulates many pro-inflammatory processes including leukocyte adhesion molecule expression on the endothelium, platelet activation, leukocyte chemotaxis and endothelial cell production of pro-thrombotic factors[55]. Thrombin is also a potent growth factor, initiating endothelial, fibroblast and smooth muscle cell proliferation and up-regulating other cytokines and growth factors[42]. These activities have been attributed to proteolytic cleavage of insulin-like growth factor binding proteins[56] and protease activated receptors -1, -3, and -4 (PAR-1, -3, -4)[57] on cell surfaces, and account for thrombin's central role in atherosclerotic lesion formation[43].

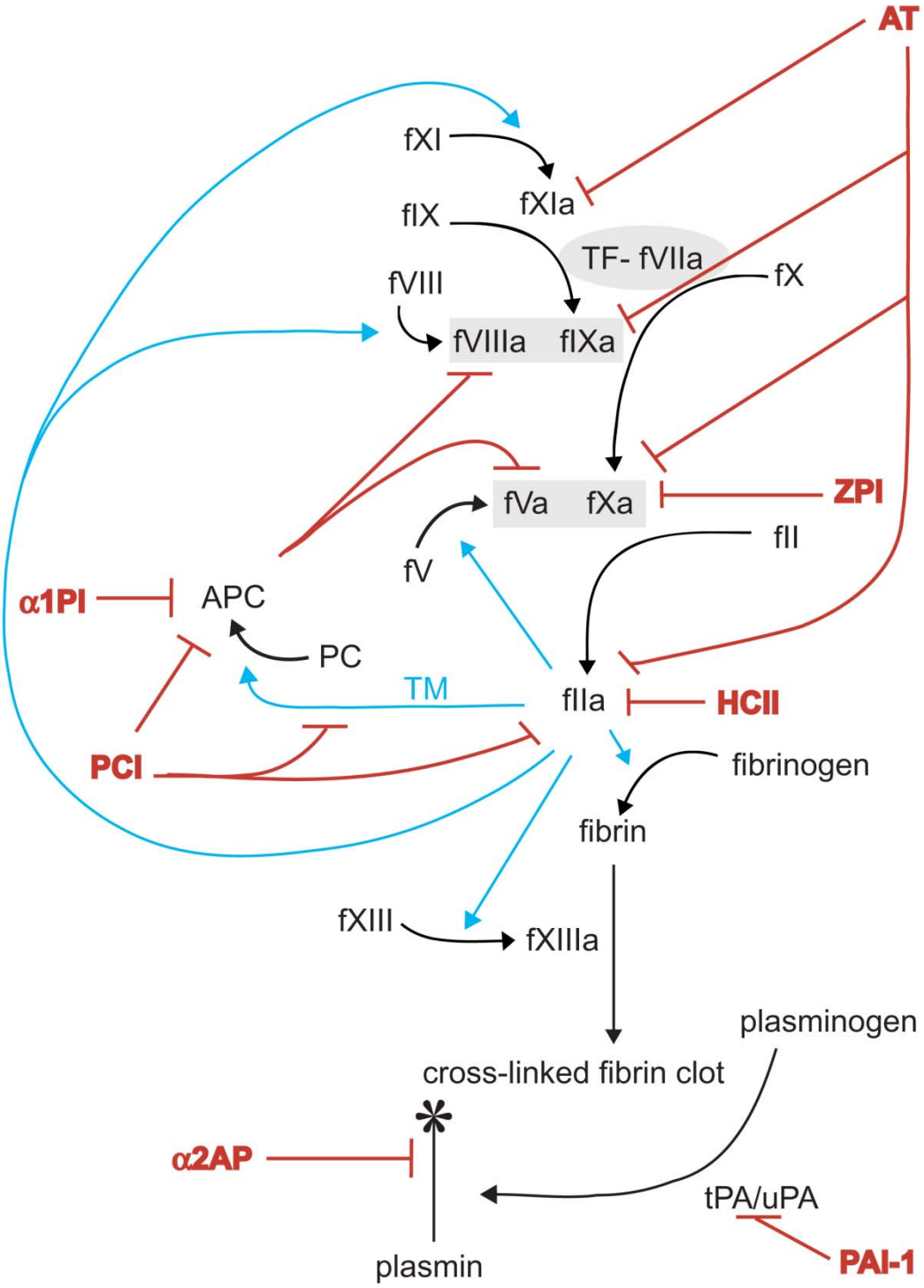


Figure 1.1. *Serpin Regulation of Coagulation, Protein C and Fibrinolytic Pathways.* Serpins and inhibitory functions are shown in red, thrombin activity is shown in cyan. Prothrombinase and tenase complexes are shown in grey boxes. Coagulation is initiated by the exposure of tissue factor to fVIIa shown in grey oval. The symbol ✻ indicates degradation. Necessary cofactors, Ca^{++}, phospholipids, proteins S and Z, vitronectin and GAGs are not shown to maintain the simplicity of the schematic.

Coagulation is regulated predominantly by antithrombin (AT)[45], tissue factor pathway inhibitor (TFPI)[58], the Protein C Pathway[59] and to a lesser extent heparin cofactor II (HCII)[48] and protein Z-dependent protease inhibitor (ZPI)[60]. Protein C inhibitor (PCI) and plasminogen activator inhibitor (PAI-1) may also contribute by inhibiting thrombin[61,62]. TFPI is not a member of the serpin family and so will not be discussed in this paper.

Protein C Pathway

The Protein C Pathway works in hemostasis to control thrombin formation in the area surrounding the clot[63]. The zymogen protein C (PC) is localized to the endothelium by endothelial cell protein C receptor (EPCR)[64]. Thrombin, generated via the Coagulation Pathway, is localized to the endothelium by binding to the integral membrane protein, thrombomodulin (TM). TM occupies exosite I on thrombin which is needed for fibrinogen binding and cleavage, thus reducing thrombin's pro-coagulant activities[65]. However, TM bound thrombin is able to cleave PC to activated protein C (APC), a serine protease, on the endothelial cell surface[66]. In the presence of protein S, APC inactivates fVa and fVIIIa[67] (Figure 1.1). This limits further thrombin generation on the clot periphery where the endothelium is not damaged[35].

The Protein C Pathway is also associated with non-hemostatic functions. APC has been shown to be an anti-inflammatory protein[68,69] and modulates gene expression[70]. It also enhances vascular permeability by signaling through both PAR-1 and sphingosine 1-phosphate receptor-1[71]. Using focal ischemic stroke animal models, APC treatment restored blood flow, reduced infarct volume and inflammation[72]. These neuroprotective effects of APC were shown to be mediated through EPCR, PAR-1[73], and PAR-3[74]. In the PROWESS

Study, patients diagnosed with severe sepsis were treated with recombinant human APC, resulting in a mortality reduction of 19.4%[75].

The proteolytic activity of APC is regulated predominantly by protein C inhibitor (PCI)[45]. Additionally, plasminogen activator inhibitor-1 (PAI-1)[76] and α_1-protease inhibitor (α_1PI)[77] have been shown to inhibit APC, although their role in the hemostasis is not well understood.

FIBRINOLYSIS --

Fibrinolytic Pathway

Fibrinolysis is the physiologic breakdown of fibrin to limit and resolve blood clots[78]. Fibrin is degraded primarily by the serine protease, plasmin, which circulates as a zymogen, plasminogen. In an auto-regulatory manner, fibrin serves as both the cofactor for the activation of plasminogen and the substrate for plasmin (Figure 1.1). In the presence of fibrin, tissue plasminogen activator (tPA) cleaves plasminogen to plasmin which proteolyzes the fibrin. Because it is a necessary cofactor for the reaction, the degradation of fibrin limits further activation of plasminogen[79-81]. The serine protease, tPA, is synthesized and released by endothelial cells[78]. In addition to binding fibrin, tPA binds Annexin II (AnII) and other receptors on endothelial cell and platelet surfaces[82]. Thus, plasmin generation and fibrinolysis are restricted to the site of thrombus formation.

In addition to its role in fibrinolysis, plasmin has other physiologic functions as evidenced by its ability to degrade components of the extracellular matrix[83] and activate matrix metalloproteases 2 and 9[84,85]. Plasminogen can also be converted to plasmin by the serine protease, urokinase plasminogen activator (uPA)[83]. Urokinase-catalyzed events are

localized on the cell surface through the uPA receptor (uPAR). Complex formation and subsequent reactions are thought to be more important during pericellular proteolysis, cell adhesion and migration than it is for vascular fibrinolysis[78]. These additional functions contribute to the role of the Fibrinolytic Pathway in cancer[83,86,87].

Fibrinolysis is controlled predominantly by α_2-antiplasmin (α_2AP)[88], PAI-1[79,89] and thrombin activatable fibrinolysis inhibitor (TAFI)[90]. PCI can inhibit tPA and uPA[91,92], but its role in fibrinolysis is unclear. TAFI is not a member of the serpin family and so will not be discussed in this paper.

SERPIN OVERVIEW

Serpins

Serpins are a superfamily of proteins classified into 16 clades (A-P). The systematic name of each serpin is, SERPINXy where X is the clade and y is the number within the clade[93]. Serpins have been identified in the genomes of organisms representing all of the branches of life (Bacteria, Archaea, Eukarya and Viruses), and the genome of humans contains ~36 serpins[94]. While serpins are named for their ability to inhibit *serine* proteases (of the chymotrypsin family) (Table 1.1), some are capable of cross-class inhibition of proteases from the subtilisin, papain and caspase families. In addition, some serpins utterly lack protease inhibitory activity and serve other roles, such as hormone transporters, molecular chaperones or catalysts for DNA condensation. Serpins are typically composed of ~400 amino acids, but can have large N-, C-terminal or internal insertion loops[93]. Serpins can also be post-translationally modified by glycosylation, sulfation, phosphorylation and oxidation to alter their function. In spite of a low overall primary sequence identity for the

Table 1.1. *Second Order Rate Constants of Protease Inhibition by Serpins in the Presence and Absence of Cofactors.*[a]

SERPIN	***SYSTEMATIC NAME***	***TARGET PROTEASE***	***COFACTOR***	***SECOND ORDER RATE k_2 (M^{-1} s^{-1})***	***CITATION***
AT	*SERPINC1*	*thrombin*	--	7.5×10^3, 1×10^4	95,96
			UFH	2×10^7, 4.7×10^7	95,97
			LMWH	5.3×10^6	97
			pentasaccharide	2×10^4	96
		fXa	--	2.5×10^3, 6×10^3	98
			UFH	5×10^6, 6.6×10^6	96,97
			LMWH	1.3×10^6	97
			pentasaccharide	7.5×10^5	96
		fIXa	--	1.3×10^2, 5×10^2	96,99
			UFH	8×10^6, 1.75×10^6	96,99
			LMWH	3.7×10^5	99
			pentasaccharide	3×10^4	96
HCII	*SERPIND1*	*thrombin*	--	6×10^2	95
			UFH	5×10^6	95
			LMWH	$\sim 5 \times 10^6$	100
			dermatan sulfate	1×10^7	95
			hexasaccharide	2×10^4	101
ZPI	*SERPINA10*	*fXa*	-- , Ca^{++}, PL	2.3×10^3	102
			protein Z, Ca^{++}, PL	6.1×10^5	102
		fIXa	--	2×10^5	103
			UFH	4×10^5	103
PCI	*SERPINA5*	*thrombin*	--	1.7×10^4	104
			UFH	$\sim 2 \times 10^5$	104
			thrombomodulin	2.4×10^6	104
		APC	--	3×10^2	105
			UFH	5×10^4	105
			UFH, Ca^{++}	2.9×10^5	106
		tPA (2-chain)	--	8×10^2	91
			UFH	3×10^4	91
α_1*PI*	*SERPINA1*	*thrombin*	--	4.8×10^1	107
		APC	--	4×10^1	108
$\alpha_1PI_{Pittsburgh}$		*thrombin*		4.8×10^5	109
		APC		7×10^4	109
α_2*AP*	*SERPINF2*	*plasmin*		2×10^7	88,110
PAI-1	*SERPINE1*	*thrombin*	--	7.9×10^2	62
			UFH	1.6×10^5	111
			vitronectin	1.9×10^5	111
		APC	--	5.7×10^2	76
			vitronectin	1.8×10^5	76
		tPA(1- , 2-chain)	--	4×10^7, 1.5×10^8	112

[a]The rates constants indicated here are from selected references and may vary slightly under different experimental conditions. UHF = Unfractionated heparin, LMWH = low molecular weight heparin, PL = phospholipids.

family, serpins share a highly conserved three-dimensional fold comprised of a bundle of 9 α-helices (A-I) and a β-sandwich composed of three β-sheets (A-C) (Figure 1.2A). It is useful to view a serpin in the 'classic orientation' to illustrate the important structural features (Figure 1.2A, left panel). In this view the main β-sheet A is facing and the reactive site loop (RSL) is on top. The RSL is typically composed of 20 amino acids running from P17 at the N-terminus (at the C-terminal end of strand 5A) to P3′ at the C-terminal end (using the nomenclature of Schechter and Berger, where residues are numbered from the scissile P1-P1′ bond). In the normal native state of a serpin, β-sheet A is composed of five strands and the RSL (bridging the C-terminus of strand 5A to the N-terminus of strand 1C) is exposed. This state is, however, not the most stable. An astounding increase in thermodynamic stability (best estimate -32 kcal/mol)[113] can be achieved through the incorporation of the RSL into β-sheet A, triggered either through extension of strand 1C (to form the so-called 'latent' state), or through proteolytic nicking anywhere near the scissile bond (the cleaved state). The metastability of the native serpin is critical for its unusual mechanism of protease inhibition[114]).

The Serpin Mechanism of Protease Inhibition

The serpin mechanism of protease inhibition has been worked out over the last 20 years through a series of biochemical, fluorescence and structural studies. A minimalist kinetic scheme is composed of two steps: the formation of the encounter complex (also known as the Michaelis complex) where the sequence of the RSL is recognized by the protease as a substrate; and, the formation of a final covalent complex where the protease is trapped in an inactive state (Figure 1.2B). The rates of formation and dissociation of the

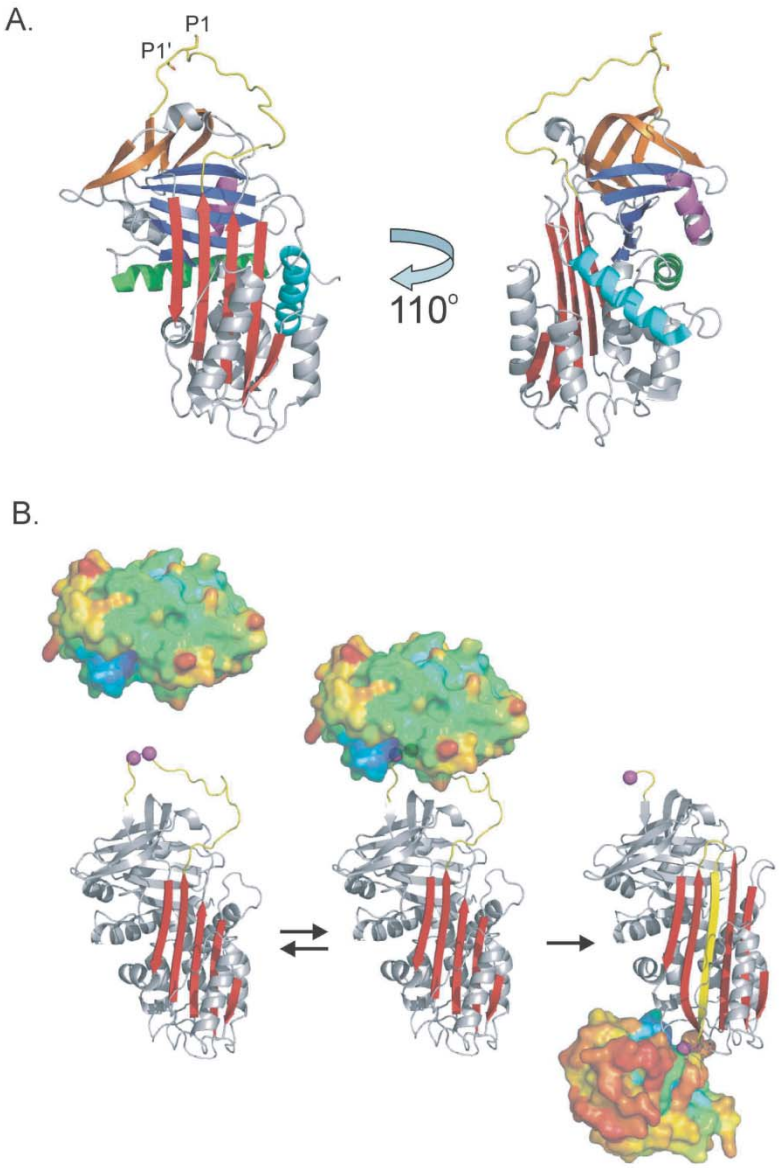


Figure 1.2. *Serpin Structure and Mechanism of Protease Inhibition.* A. The shared serpin fold is illustrated by the structure of the prototypical native serpin α_1PI. The 'classic' orientation shown on the left places the RSL (yellow) on top and the main β-sheet A (red) to the front. Sheets B and C are blue and orange, respectively, and helices A, D and H are colored green, cyan and magenta. The accessibility of the RSL is illustrated by rotating the molecule by 110° to the left along the long axis. It shows how the P1-P1′ (rods) scissile bond is exposed for proteolytic attack. Also clearer in this orientation are helices D and H which are the heparin binding helices. B. The serpin mechanism of protease inhibition is minimally expressed as a two step process. In the first step native serpin (ribbon with the P1 and P1′ residues as magenta balls, below) interacts reversibly with a protease (surface representation, colored according to temperature factors from blue to red) to form the Michaelis complex (middle). After formation of the acyl-enzyme intermediate the protease is flung to the opposite pole of the serpin and its catalytic architecture is destroyed, and consequently there is a loss of ordered structure (notice the smaller size and increase in temperature factors).

reversible Michaelis complex, along with colocalization in tissues, determines the specificity of the serpin-protease interaction[115,116]. While the obligate RSL-active site contacts contribute significantly to the formation of the Michaelis complexes, exosite interactions may also be involved. As with actual substrates of serine proteases, this step is followed by the nucleophilic attack of the peptide bond between the P1-P1′ residues by the catalytic Ser195 of the protease. This ultimately results in the formation of a covalent ester bond between the P1 residue and Ser195 of the protease (acyl-enzyme intermediate), and then separation of the P′ residues from the active site of the protease. At this stage the serpin rapidly adopts its lowest energy conformation through the incorporation of the N-terminal portion of the RSL into β-sheet A. The tethered protease is thus flung from the top to the bottom of the serpin (~70Å), and the resulting pulling force exerted on the catalytic loop results in a conformational distortion of the protease[117]. The acyl-enzyme intermediate is thus trapped, with deacylation prevented largely due to the destruction of the oxyanion hole. Two structures of final complexes have been solved by X-ray crystallography[118,119], with one showing an additional distortion of ~37% of the protease structure[119]. This mechanism is particularly well suited to tightly regulated processes such as hemostasis and fibrinolysis because inhibition is irreversible, and the conformational changes in the serpin and the protease alter cofactor interactions. An example of the physiologic relevance of the conformational change in the protease component of the complex is the complete destruction of thrombin's exosite I in complex with serpins[120]. Thus, when PCI inhibits thrombin bound to thrombomodulin the interaction with thrombomodulin is broken, allowing the serpin-protease complex to diffuse away so that another thrombin molecule can bind[104].

Cofactor Interactions

Because serpin specificity is determined largely by the rate of formation of the Michaelis complex, cofactors which bind to serpins (and sometimes the protease) can radically alter specificity[115]. Table 1.1 presents serpin second order rates of protease inhibition in the presence and absence of relevant cofactors. The best understood cofactor for serpins is the glycosaminoglycan (GAG), heparin. It binds to and activates most of the serpins involved in hemostasis and thrombosis[121]. Acceleration of protease inhibition is generally conferred through a template effect where the protease and the serpin bind to the same heparin chain. The hypothesis is that this co-occupation will limit the diffusional freedom from three to one dimension to increase the likelihood (rate) of encounter. In addition, heparin also provides a bridge between the serpin and the protease to help stabilize the Michaelis complex. However, heparin and other GAGs are also capable in some cases of altering the conformation of the serpin to permit more rapid complexation with proteases. The best-characterized examples are AT and HCII, whose activation by heparin is the basis of its therapeutic anti-coagulant effect. In the next sections we describe each of the serpins involved in hemostasis and fibrinolysis, their targets, the role of cofactors and available structural data.

SERPINS IN HEMOSTASIS AND FIBRINOLYSIS

Antithrombin – SERPINC1

Antithrombin (AT) is a 58 kDa, 432 amino acid glycoprotein[122], synthesized in the liver, circulating at approximately 150 μg/mL with a half-life of ~3 days[123]. It is the most important physiologic inhibitor of the coagulation pathway[124]. As its name implies,

antithrombin inhibits thrombin. Additionally, AT is capable of inhibiting all of the other proteolytic coagulation factors (e.g. factors IXa, Xa, and XIa). The predominance of its anti-coagulant activity, however is focused on the regulation of fXa, fIXa and thrombin. Measurement of thrombin-AT (TAT) complex is used as a marker of hemostatic activation and helps diagnose thrombotic events[125]. Thrombin bound to fibrin, clot-bound thrombin, is protected from inhibition by AT[126]. This may explain the occurrence of rethrombosis after fibrinolytic therapy as clot-bound thrombin is released from the dissolving hemostatic plug[127].

The anti-coagulant activity of AT is dependent on its cofactor, heparin. Consisting of variably sulfated repeating dissacharide units, heparin can have a molecular weight ranging from 3 to 40 kDa[128-130]. A unique pentasaccharide sequence in heparin is responsible for the high affinity binding to AT[131]. *In vivo*, forms of heparin relevant to AT include heparan sulfate found on the endothelium, and heparin released from endothelium-associated mast cell granules. The interaction of AT with heparan sulfate on the endothelium and subendothelium localizes AT activity to the vessel wall and maintains its normal, non-thrombogenic nature[123]. AT is expressed as both an α-form and a β-form. α-AT represents 90% of AT and is glycosylated at all four positions. While comprising only 10% of AT, β-AT, which is not glycosylated at one position (N135), has a higher affinity for heparin and is thought to exert an overall larger anti-coagulant effect[132].

Heparin utilizes two distinct mechanisms for accelerating protease inhibition by AT. AT undergoes a well characterized conformational change upon heparin binding, which expels the N-terminus of the RSL from β-sheet A (Figure 1.3A). This 'liberation' of the RSL is sufficient to confer the majority of the acceleration of fIXa and fXa inhibition, but

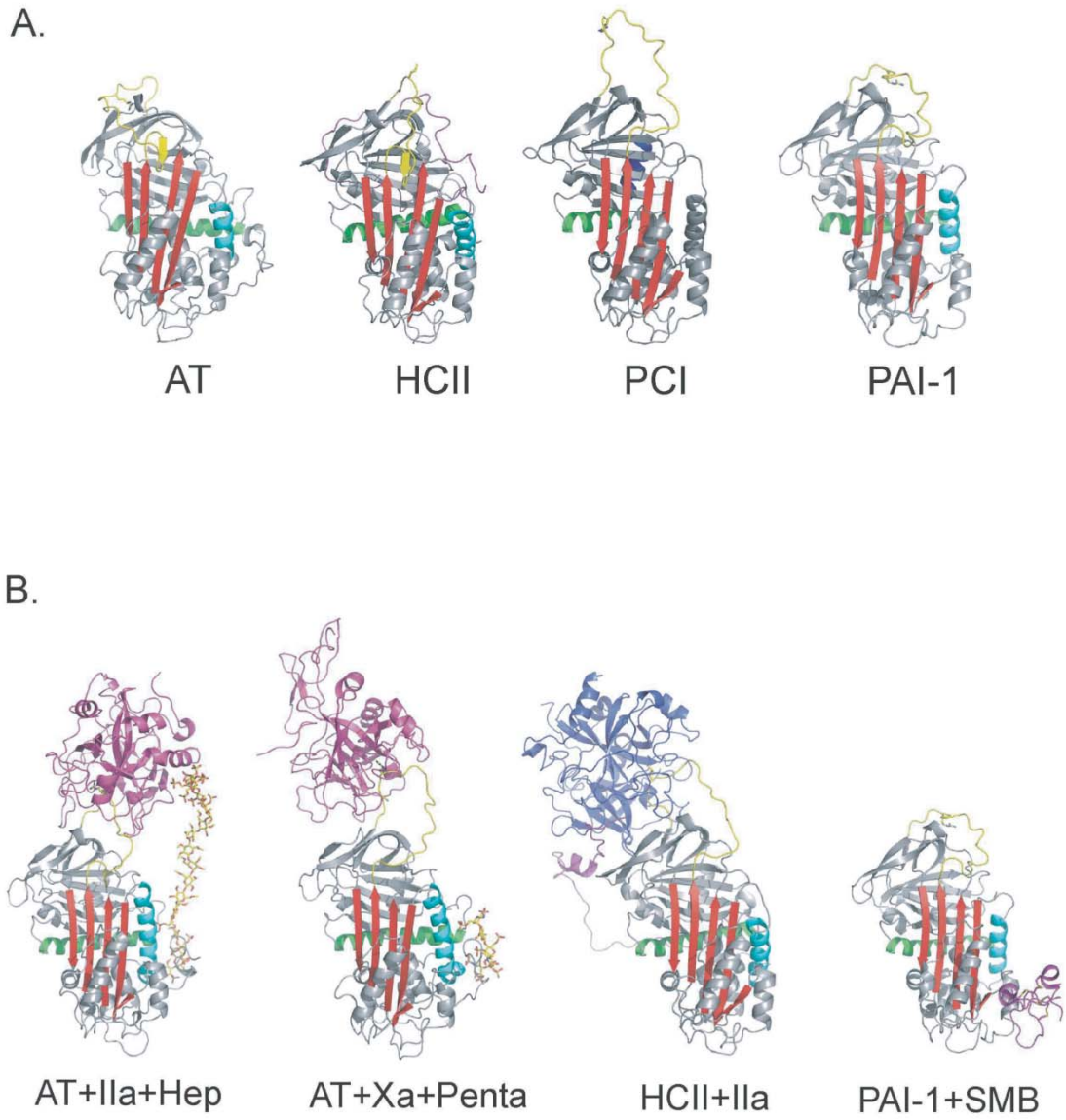


Figure 1.3. *Native and Complexed Serpin Structures.* A. The native structures of important hemostatic and fibrinolytic serpins are shown as ribbon diagrams, colored essentially as in Fig. 2. The monomeric structure of AT is shown in the left panel, and is similar to that of HCII with the partial insertion of the N-terminal portion of the RSL. A modeled position for the N-terminal tail of HCII is shown in magenta although its true position is not known. For AT, HCII and PAI-1 the heparin binding helix (helix D) is shown in cyan, but for PCI heparin binds to helix H (blue). The increased size and flexibility of the RSL of PCI is also evident from this depiction. B. Some important serpin complexes are shown. Using S195A proteases it was possible to obtain the structures of the AT Michaelis complexes with thrombin (magenta) and fXa (magenta) with their activating synthetic heparins (SR123781 and fondaparinux, rods). Similarly, the HCII-thrombin (blue) complex was also solved. The somatomedin domain of VN (magenta) binds to s1A and helix E to prevent the latent transition through expansion of sheet A.

thrombin inhibition is not appreciably affected. Recently, the structures of the AT-heparin-protease Michaelis complexes have been solved[118,133,134] revealing the interactions behind the allosteric and template mechanisms (Figure 1.3B).

In addition to its anti-coagulant activity, AT has been shown to have anti-inflammatory and anti-angiogenic functions. These properties are independent of AT's inhibitory activity. AT regulates inflammation by signalling through heparan sulfate on endothelial and leukocyte cell surfaces[135]. Latent and cleaved AT exert anti-angiogenic effects[136] by binding cell surface heparan sulfate. This blocks fibroblast growth factor-2 and vascular endothelial cell growth factor from forming pro-angiogenic ternary signalling complexes with their protein receptors and the heparan sulfate co-receptors[137].

Antithrombin in Disease - Inherited and acquired AT deficiency predisposes individuals to different degrees of thrombotic disease. The severity of thromophilia can be exacerbated by other risk factors for thrombosis. Inherited AT is classified as type I or type II. Type I deficiencies, which generally confer a higher thrombotic risk, are caused by genetic mutations that impair the synthesis and secretion of AT. Type II deficiencies are caused by genetic mutations that functionally impaired AT. Variations in the degree of thrombophilia in inherited AT deficiencies can be attributed to homozygosity versus heterozygosity and where the mutation lies in the AT structure[138]. An up-to-date database of AT mutations can be found online at http://www1.imperial.ac.uk/medicine/about/divisions/is/haemo/coag/antithrombin[139]. Some research suggests that certain mutations predispose AT to convert to its latent form, which preferentially dimerizes with native β-AT. Dimerization reduces the presence of highly active AT monomers, thus increasing thrombogenicity[140]. Concern has been raised that

therapeutic preparations of AT-concentrates (contain >10% latent AT) might have thrombotic effects. However, a recent study demonstrated that the addition of latent AT alone does not decrease the activity of AT in plasma[141]. Other mutated forms of AT do not show impaired activity or decreased AT levels in the standard hospital laboratory assays despite associated thrombophilia. In particular, the antithrombin Cambridge II (A384S) variant was found to be undetected by some protocols, but estimated to be the most frequent cause of antithrombin deficiency in Caucasian populations[142]. These results suggest a need for alternative methods for detection of AT deficiencies[143].

Antithrombin related treatements for coagulation disorders-- Therapeutic unfractionated heparin (UFH), derived from porcine mucosa, is one of the most commonly used anti-coagulant agents administered for treatment and prophylaxis of thrombotic events. Additionally, UFH is used to coat blood collection tubes and surgical devices to prevent clotting on their surfaces. UFH's primary mechanism of action is to accelerate AT's inhibition of thrombin, fXa and fIXa. UFH also accelerates thrombin inhibition by other circulating serpins. It has a very short half-life and optimal dosing of heparin is notoriously difficult to achieve, therefore requiring frequent monitoring[144]. Still in trials, an orally available form of heparin, sodium N-(8-[2-hydroxybenzoyl] amino) caprylate bound heparin or SNAC-heparin, has dose-dependent antithrombotic effects, and has an efficacy comparable to low-molecular weight heparin in reducing venous thrombosis in patients undergoing hip replacement surgery[145]. Contraintuitively, heparin can cause a dangerous thrombotic condition called heparin induced thrombocytopenia (HIT). In this autoimmune reaction antibodies develop against platelets[146]. Currently in development stages, synthetic

oligosaccharide heparin mimetics show thrombin and fXa inhibition comparable to UFH without inducing HIT and with far fewer side effects[147].

Low molecular weight heparin (LMWH) is a fractionated preparation of heparin between 1 and 10 kDa with an enriched population of high affinity pentassacharide sequences. Because of the smaller average size, LMWH acts predominantly by inducing conformational change in AT, the mechanism which activates fXa. It has a longer half-life than UFH and does not need coagulation monitoring. LMWH also has significantly reduced risk of HIT. Multiple LMWH variants are available or in clinical trials[148].

Fondiparinux and idraparinux are synthetic pentassacharide sequences derived from heparin, which activate AT to specifically inhibit fXa. Because of this the single target, the side effect of over-anticoagulation, bleeding, is reduced. Both have longer half-lives than LMWH. Additionally, neither preparation causes HIT[148,149].

Thrombotic events due to antithrombin deficiency are treated with AT purified from human plasma. A covalent AT-heparin complex is currently under preliminary investigations for possible use as a novel anticoagulant because unlike AT or heparin alone, it is able to inhibit clot-bound thrombin[150].

Heparin Cofactor II – SERPIND1

Heparin cofactor II (HCII) is a 66.5 kDa 480 amino acid glycoprotein, synthesized in the liver, circulating at ~80 μg/mL with a half-life of 2-3 days. HCII inhibits thrombin in the presence of many polyanionic molecules including the GAGs heparin and dermatan sulfate[48]. A unique hexasaccharide sequence within dermatan sulfate has been determined to be responsible for its high affinity binding to HCII[151]. Dermatan sulfate does not accelerate any

other serpin activity. HCII has a unique N-terminal extension of ~80 residues that contains two acidic regions, critical for its GAG-associated anti-thrombin activity[152]. HCII inhibits thrombin and clot-bound thrombin, but not other coagulation proteases[48]. Evidence suggests that HCII contributes 20-30% to thrombin inhibition in coagulation. Neither humans nor mice deficient in HCII exhibit thrombophilia under normal conditions[153]. However, HCII homozygous deficient mice form occlusive thrombi faster than wild-type mice after photochemical vascular endothelial cell injury to the carotid artery[154]. Recent data suggests that the primary physiologic function of HCII is to inhibit thrombin's non-hemostatic roles such as in the development of atherosclerosis. Elevated levels of HCII are shown to protect against atherosclerosis and restenosis[50,155,156].

The structure of native HCII was solved in 2002 and revealed a surprising resemblance to native antithrombin[152], with the N-terminus of the RSL inserted into β-sheet A (Fig. 3). As HCII also shares a similar heparin binding site along helix D[45,157], it was proposed that HCII underwent a similar conformational change upon heparin binding[152]. Recently, it was shown that the smallest heparin length capable of tight binding to HCII was 14 monosaccharide unit chains, and that the majority of the acceleration effect was due to this allosteric change in HCII conformation[100]. Disappointingly, however, the native structure could not resolve the position of the N-terminal extension. Several mutagenesis studies concluded that the acidic tail is binding to the basic heparin binding site in native HCII[157], but it is still unclear how the tail interacts with the body of HCII in the native state. From the structure of HCII bound to S195A thrombin[152] it was clear how the tail confers specificity to thrombin. The tail binds to exosite I of thrombin in a manner similar to the hirudin, primarily through hydrophobic contacts. The tail was found sandwiched between thrombin and the

body of HCII, essentially providing a shared exosite (Fig. 3B). A complex allosteric mechanism has been proposed based on these structures, supporting biochemical studies and analogy to AT[116,121].

HCII related treatements for coagulation disorders **-** As alternatives to heparin-based treatments, dermatan sulfate derivatives and other polyanionic molecules that act to accelerate HCII's antithrombotic activity are being investigated. They are of particular interest for use in HIT, AT deficiency and for the inhibition of clot-bound thrombin. Two types of fractionated dermatan sulfate enriched for the hexasaccharide sequence (Intimatan™ and Desmin™) have been tested in humans[158,159]. Intimatan is beginning Phase I trials. Other HCII agonists in laboratory investigations include over-sulfated dermatan sulfate[160], fucosylated chondroitin sulfate[161] and fucoidan[162].

Protein Z-dependent Protease Inhibitor– SERPINA10

Protein Z-dependent protease inhibitor (ZPI) is a 72 kDa, 444 amino acid glycoprotein, synthesized in the liver, circulating at ~1.5 μg/mL. In the presence of protein Z, phospholipids and calcium, ZPI rapidly inhibits fXa. In the absence of cofactors, ZPI also inhibits factor XIa, which can be accelerated two-fold by heparin. It is thought that the major physiologic function of ZPI is to attenuate the coagulation response prior to the formation of the prothrombinase complex[103]. In humans, mutations in ZPI are associated with increased risk of venous thrombosis[163-165]. Additionally, reductions in protein Z plasma concentrations result in an aggravated thromboembolic risk in humans and mice with fV_{Leiden}[166].

Protein C Inhibitor – SERPINA5

Protein C Inhibitor (PCI) is a 57 kDa, 387 amino acid glycoprotein[167], synthesized in the liver, circulating at ~5 μg/mL. It is also found in other bodily fluids including urine, saliva, amniotic fluid, milk, tears and seminal fluid[168]. PCI is a heparin-binding serpin that inhibits many proteases including APC[169], IIa, IIa bound to TM[104] tPA and uPA[170]. PCI may have contrary anti-coagulant and pro-coagulant functions depending on the target protease and the presence of specific cofactors. In the presence of heparin, PCI is anti-coagulant, inhibiting the proteolytic cleavage of fibrinogen by thrombin. However, in the presence of TM, PCI is pro-coagulant, inhibiting the activation of PC by thrombin[45].

The structures of RSL-cleaved PCI[167] and of native PCI (PDB # 2HI9 and # 2OL2) (Fig. 3A) have now been solved, revealing a typical serpin structure with some notable differences. The RSL of PCI is unusually long and flexible, accounting for its broad protease specificity, and its heparin-binding site is found along the highly basic helix H. The effect of heparin on PCI activity can either be to accelerate protease inhibition (e.g. APC)[171] or to abrogate it (tissue kallikrein)[172]. The position of the heparin-binding site close to the protease docking site may help explain this property.

Protein C Inhibitor in Disease - PCI is not synthesized by the liver in mice, and thus PCI is unlikely to play a role in hemostasis or fibrinolysis in the mouse[173]. Transgenic mice that over-express human PCI (hPCI) provide evidence of PCI's ability to inhibit thrombin, APC and tPA. These mice do not exhibit symptoms of pulmonary hypertension induced by monocrotaline. TAT complexes are reduced compared to their wild-type counterparts suggesting that PCI competes with AT to inhibit thrombin. Additionally, a decrease in free tPA and subsequent reduction in fibrinolysis is seen. Finally, when APC was administered

for endotoxemia, hPCI-expressing transgenic mice demonstrated a reduction in the anti-coagulant and anti-inflammatory effects of the treatment[174]. Male homozygous PCI-knockout mice were infertile due to abnormal spermatogenesis caused by loss of the Sertoli cell barrier[175] due unregulated proteolytic activity.

In humans, APC-PCI complex is indicative of atherosclerosis and aortic aneurysms[176], an early indicator of myocardial infarction[177] and predicts poor patient outcome after aortic surgery[178]. Additionally APC-PCI complex is increased (4-fold) in patients with fV_{Leiden} who have suffered a previous venous thrombosis[179]. PCI alone has been shown to be elevated in survivors of acute coronary events[180].

α_1-Protease Inhibitor – SERPINA1

α_1-protease inhibitor (α_1PI) – historically known as α_1-antitrypsin – is a 51 kDa, 394 amino acid glycoprotein, synthesized in the liver, circulating at ~1.3 mg/mL with a half-life of 4.5 days (structure shown in Figure 1.2). Its physiologic target is neutrophil elastase[181], however, it has also been shown to inhibit APC in a heparin-independent manner[77]. In pediatric ischemic stroke patients, α_1PI levels were significantly increased independent of other pro-thrombotic factors. Authors suggest this pathology is due to APC inhibition[182].

α_1PI is not thought to contribute significantly to coagulation. However, a variant of the protein ($\alpha_1PI_{Pittsburgh}$) with a reactive site mutation (M358R) can cause a fatal bleeding disorder[183]. The Met → Arg polymorphism creates a potent inhibitor of several coagulation serine proteases, especially thrombin and APC, that is not dependent on heparin or other cofactors[184]. Therapies utilizing recombinant $\alpha_1PI_{Pittsburgh}$ were considered but abandoned due to side effects of promiscuous protease inhibition[185,186].

α_2-antiplasmin – SERPINF2

α_2-antiplasmin (α_2AP) is a 63 kDa, 452 amino acid glycoprotein, synthesized in the liver, circulating at ~70 µg/ml with a half-life of 2.6 days[88,187,188]. α_2AP is the primary physiological inhibitor of plasmin, but has also been reported to inhibit other enzymes such as trypsin, elastase, and APC. Homozygous deficiency of α_2AP results in uncontrolled fibrinolysis and subsequent severe hemorrhagic tendencies[187,189]. While α_2AP has all of the key structural features of the serpin family, it uniquely has both N- and C-terminal extensions of 42 and 55 residues, respectively[88,190]. In thrombus formation, the N-terminal region of α_2AP is cross-linked to fibrin by fXIIIa, and the C-terminal Lys binds to the Lys-binding site of plasmin. The rate of fibrinolysis is proportional to cross-linked α_2AP.

Plasminogen Activator-1 – SERPINE1

Plasminogen activator-1 (PAI-1) is a 50 kDa, 379 amino acid glycoprotein, synthesized in endothelial cells, platelets and other mesenchymal cells surrounding the vasculature[191-193]. This serpin is relatively unstable with a half-life of 1-2 hours in circulation[194]. However, PAI-1 is found bound to the extracellular matrix protein, vitronectin (VN)[195,196]. The PAI-1-VN complex has an enhanced half-life of 4-6 hours[194]. PAI-1 regulates both tPA and uPA and is considered the main physiological inhibitor of plasminogen activation[78,79,89,197]. As platelets are activated following vessel injury, they release PAI-1 to protect the developing thrombus from premature fibrinolysis. Later in the coagulation process, tPA and plasminogen/plasmin are bound to fibrin within the thrombus, which protects tPA from inhibition by PAI-1, resulting in plasmin generation and fibrinolysis[78,79,89,197].

Several structures of PAI-1 have been solved , but, due to its rapid conversion to the latent form, all structures of native PAI-1 are of a stabilized quadruple mutant[198-200]. Although the structure shows a native state similar to α_1PI, not AT and HCII, some mutagenesis studies suggest an equilibrium for wild-type PAI-1 where the native state is in equilibrium between α_1PI-like and AT-like states[201]. The structure of the stabilized mutant bound to the somatomedin domain of VN revealed the mechanism of stabilization of the native state through a blocking of the expansion of β-sheet A[196] (Fig. 3B).

PAI-1 can also inhibit APC[76] and thrombin[62,111] in the presence of VN and/or heparin. It is not known to what extent these activities contribute to coagulation. Previously, it has been shown that APC cleaves PAI-1, inactivating the serpin[202,203]. Recently, it has been shown that PAI-1 inhibits APC and the rate of inhibition increases in the presence of vitronectin ~300-fold[76].

Plasminogen Activator-1 in Disease - Studies show that PAI-1 levels are sensitive to many different pathophysiological factors and increased synthesis of PAI-1 contributes to numerous cardiovascular disease states. In metabolic syndrome, both glucose and insulin increase PAI-1 synthesis in vascular endothelial and smooth muscle cells[204]. Controlling hyperglycemia in type 2 diabetes results in a decrease in PAI-1 levels. One of the clinical benefits of "statins" may be due to their decrease of PAI-1 expression and simultaneous increase of tPA expression, altering the balance of the Fibrinolytic Pathway[205,206]. Circadian clock proteins, including CLOCK, BMAL, and CRY, regulate PAI-1 gene expression which may explain the increased risk of adverse cardiovascular events in the morning[207]. Inhibition of nitric oxide synthase induces PAI-1 expression, which contributes to the development of perivascular fibrosis[208]. Increased PAI-1 levels are associated with coronary artery disease

and myocardial infarction. However, studies examining the association of cardiovascular disease with a polymorphism within the PAI-1 promoter region (4G/5G) which increases the expression PAI-1 are controversial[209]. Stents with rapamycin and paclitaxel are used in interventional cardiology due to the antiproliferative effects of these drugs[210,211]. These stents have been shown to be associated with an increased risk of thrombosis and it is speculated that this may be due to an up-regulation of PAI-1 by rapamycin and paclitaxel[212]. There is also strong evidence for a role of PAI-1 in cancer metastasis independent of its protease inhibitory activity[87,213,214].

Effectors of Plasminogen Activator Inhibitor-1 Activity and Synthesis - Physiological levels of PAI-1 provide crucial regulation of fibrinolysis, yet excess levels contribute to disease. Numerous factors have been found that up-regulate PAI-1 expression and secretion, including inflammatory cytokines, angiotensin II, aldosterone, transforming growth factor-β, and very-low density lipoproteins[89,215,216]. Monoclonal antibodies have been prepared against PAI-1, which express inhibitory activity by (i) preventing the formation of the encounter complex between PAI-1 and tPA/uPA, (ii) increasing PAI's susceptibility to cleavage by target proteases, and (iii) promoting the tendency of PAI-1 to become latent and inactive[217]. Sequence-specific catalytic DNA enzyme, short-interfering RNA structures, and antisense technology have all been used to down-regulate PAI-1 levels[218]. Negatively charged organochemical compounds have been found to bind to a hydrophobic site on PAI-1 and induce polymerization and inactivation. Finally, several small molecules (XR5118, ZK4044, and PAI-039) have been developed to inhibit PAI-1 activity by either reduction of accessibility to the RSL, by promoting a latent-like state or by favoring a substrate-like conformation[219-227].

CLOSING STATEMENT

In this State of the Art manuscript, we have described our current knowledge of serpins that regulate hemostasis and fibrinolysis. Utilizing the "suicide substrate" mechanism unique to serpins AT, HCII, ZPI, α_1PI, PCI, α_2AP and PAI-1 provide rapid and specific inhibition of the activated serine proteases in the Coagulation, Protein C and Fibrinolytic Pathways. These pathways are not single independent systems, but they represent a dynamic balance between pro-coagulant, anti-coagulant, pro-fibrinolytic and anti-fibrinolytic states with serpins playing multiple and sometimes conflicting roles. While we have learned a considerable amount about their physiologic control, their structure, related activities and regulation by local cofactors, much is left to be understood. Of note, there is deciphering the primary physiologic roles of HCII and PCI, resolving the crystal structures of ZPI and α_2AP, and learning more about the non-hemostatic functions of AT and PAI-1. Continued research with the less-studied serpins such as ZPI and α_2AP will undoubtedly provide useful information about control of hemostasis and fibrinolysis and of serpins in general. The serpins described in this paper have a multitude of functions, which under some circumstances contribute to disease, but which often can be maniupulated for the benefit of medicine. Therefore, it is of paramount importance that we continue the investigations of serpins in thrombosis, hemostasis and fibrinolysis.

ACKNOWLEDGEMENTS

We gratefully acknowledge all the serpin scientists and their contributions over the last three decades, and we apologize to our respected colleagues whose important publications we were not able to cite or discuss due to space limitations. The FC Church

laboratory is supported in part by the National Institutes of Health (HL-32656) and in part by the Susan G. Komen Breast Cancer Foundation (BCTR0503475 and BCTR45206). Current stipend support for JC Rau is through the UNC-CH Integrative Vascular Biology Program NIH grant (T32 HL69768). Current stipend support for LM Beaulieu is supported in part through an NRSA-NIH predoctoral fellowship (F31 NS054590) and was previously supported through the UNC-CH Integrative Vascular Biology Program NIH grant (T32 HL69768) and the Susan G. Komen Breast Cancer Foundation (BCTR0503475). The JA Huntington laboratory is supported by the Medical Research Council (UK), the British Heart Foundation, and the National Institutes of Health (HL-068629).

CHAPTER 2

Heparin Cofactor II in Atherosclerotic Lesions

SUMMARY

Heparin cofactor II is a serine protease inhibitor that has been shown to be a predictor of decreased atherosclerosis in the elderly and protective against atherosclerosis in mice. HCII inhibits thrombin *in vitro* and HCII-thrombin complexes have been detected in human plasma. Moreover, the mechanism of protection against atherosclerosis in mice was determined to be the inhibition of thrombin. Despite this evidence, the presence of HCII in human atherosclerotic tissue has not been reported. In this study, using tissue obtained from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study and the McLendon Clinical Laboratory, Division of Autopsy Services at the University of North Carolina Hospitals, we explore the local relationship between HCII and thrombin in atherosclerosis. We found that HCII and (pro)thrombin are co-localized in the lipid-rich necrotic core of atheromas. A significant positive correlation between each protein and the severity of the atherosclerotic lesion was also present. Complexed HCII and thrombin were not detected. These results suggest that HCII is in a position to inhibit thrombin in atherosclerotic lesions where thrombin can exert a proatherogenic inflammatory response. However, these results should be tempered by the additional results from this and other

studies that indicate the presence of many circulating plasma proteins in the same region of the atheroma.

INTRODUCTION

Heparin cofactor II (HCII) is a unique member of the serine protease inhibitor (serpin) family. Although it is present in human plasma at levels over 1μM, its physiologic role is not yet fully understood. In vitro, HCII is known to inhibit only thrombin, chymotrypsin and cathepsin G[228,229]. Although one study has reported that HCII inhibits chymotrypsin *in vivo* during acute pancreatitis[230], the majority of studies into HCII's function have focused on its inhibition of thrombin.

HCII, antithrombin (AT) and several other serpins that inhibit thrombin, require cofactors to reach physiologically relevant rates of inhibition. HCII and AT utilize glycosaminoglycans (GAGs), unbranched sulfated polysaccharide chains composed of repeating disaccharide units, for thrombin inhibition. While the activity of both HCII and AT can be activated by the GAGs heparin and heparan sulfate, HCII is distinctive amongst the serpins in its ability to utilize the GAG dermatan sulfate as a cofactor[45,152,231-236]. *In vitro,* GAGs can accelerate AT and HCII to similar rates of thrombin inhibition (second order rates of inhibition over 1 $x10^7$ M^{-1} s^{-1})[51].

Despite the ability of HCII to inhibit thrombin to rates comparable to that of antithrombin (AT) *in vitro,* and the detection of HCII-thrombin complexes in human plasma[237] evidence suggest that under normal conditions HCII does not appear to play a major role in the regulation of thrombosis in hemostasis. Individuals with HCII deficiency do not appear to have increased risk of venous thrombosis compared to those with normal

HCII levels[153]. There has been only one report of individuals with homozygous HCII deficiency, a pair of sisters who showed only 10-15% of normal plasma HCII activity and only 2-5% antigen[238]. It was determined that their genetic mutation caused a E428K mutation in HCII which almost virtually abolished its hepatic secretion[239]. One of these sisters did experience thrombotic events; however, it was determined that she had a comorbid AT deficiency[238].

Recently, it has been suggested that HCII acts as an adjunct to AT in hemostasis regulation in AT deficiency. Tanaka et al.[240] demonstrated that Intimatan, a pharmaceutical form of dermatan sulfate, could be used effectively for anticoagulation when patients had deficient antithrombin levels indicating that HCII could be a sufficient backstop for hemostasis. Evidence also suggests that HCII may play a role in hemostasis during pregnancy, most likely in maintaining placental hemostasis[241]. Heparin cofactor II and HCII-thrombin complex levels are elevated, during pregnancy[242,243]. The placenta is abundant in dermatan sulfate proteoglycans[241] and traces of dermatan sulfate can be found in maternal and fetal blood[244]. Additionally, HCII levels are reduced ~50% of normal in women with preeclampsia[245].

One conclusion that could be drawn from this incomplete story is that complete HCII deficiency is embryonically lethal, that at least some HCII is required to maintain placental function; thus, individuals with complete HCII deficiency do not exist. Another possibility is that HCII deficiency does not produce a clinical phenotype within a realm that is typically examined within the context of HCII plasma levels. In 2000 He et al.[246] reported the creation of homozygous HCII knockout mice. These mice were born with predicted Mendelian frequencies and had normal hemostasis when unchallenged. However, they

reported that these mice showed decreased time to vascular occlusion after arterial injury than their wildtype counterparts suggesting that HCII may play a role in response to vascular injury[154]. More recently, another group created homozygous HCII null mice. They reported that complete HCII deficiency is embryonically lethal[49]. This has renewed the debate regarding the role of HCII in placental function in pregnancy and in pathophysiology in general.

Since the finding that HCII may play a role in response to vascular injury, there has been a flurry of research investigating the role of HCII in atherosclerosis[48]. Carotid atherosclerosis measured by ultrasound was shown to be decreased in individuals with high levels of HCII[50]. Furthermore, in this study, HCII was a better predictor of decreased atherosclerosis than high-density lipoprotein which is generally regarded as offering cardioprotection. Decreased levels of HCII activity were correlated with endothelial dysfunction and were predictive of cardiovascular events in one study[247]. However, another study showed no correlation between HCII and subsequent cardiovascular events[248]. Two studies which examined restenosis after arterial stent placement, a process dominated by smooth muscle cell proliferation and migration that are also factors in atherogenesis, indicated that elevated blood concentrations of HCII were associated with decreased incidence of restenosis[155,156]. Finally, in experiments using either homozygous HCII null mice or heterozygous HCII knockout mice crossed with the apolipoprotein E null mice, HCII was shown to protect against atherosclerosis. Furthermore, it was shown that HCII was acting to inhibit thrombin in and vascular remodeling after injury and in atherosclerosis[47,49]. Although HCII is thought to regulate thrombin in atherosclerosis, the presence of HCII within atherosclerotic lesions has not been reported. In this study we immunologically

probed normal artery and atherosclerotic lesions for the presence of HCII, (pro)thrombin and other plasma proteins. We proceeded with the hypothesis that we would find decreased levels of HCII and increased levels of thrombin in atherosclerotic plaques, and the reverse in normal arterial wall, reasoning that less HCII would result in decreased thrombin inhibition and therefore more severe atheromas. Furthermore, because dermatan sulfate, but not heparin was shown to be ineffective in reducing atherosclerosis in laboratory mice, we hypothesized that AT would be absent in the lesions[47].

MATERIALS AND METHODS

Histological Samples

Samples of human left anterior descending (LAD) coronary artery were collected, formalin-fixed, paraffin-embedded, cut and mounted on glass slides by the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study[249-253]. All subjects in this study were persons from the ages of 15-34 who died of external causes (accidents, homicides, suicides) from fifteen cooperating centers managed by the Department of Pathology at Louisiana State University Health Science Center. Serial sections from 33 cases with varying degrees of atherosclerosis were used in this study. For simplicity of coding, the cases were renumbered 1-33. Upon examination, five cases (13, 15, 20, 30, 32) were found to lack enough tissue for evaluation, leaving 28 cases for analysis.

Sections of formalin-fixed, paraffin-embedded liver were obtained from the University of North Carolina Surgical Pathology cut into 4 μm sections and mounted on glass slides. These served as positive controls slides for immunohistological staining as almost all of the proteins being probed are of hepatic origin. The exception to this is maspin

which served as a negative control and is not synthesized by the liver. However, a liver section with a maspin-positive tumor was found to serve as a positive control.

Slide Preparation and Mounting

Before staining, paraffin was dissolved and slides were rehydrated by immersion for three minutes each in successive baths of the following solutions: 100% xylene, 100% xylene, 100% xylene, 100% ethanol, 100% ethanol, 95% ethanol, 95% ethanol, 70% ethanol, 30% ethanol, water. All water, unless otherwise specified, was distilled and deionized. After staining, all slides were dehydrated by immersion for three minutes each in successive baths of the following solutions: 50% ethanol, 50% ethanol, 100% ethanol, 100% ethanol, 100% xylene, 100% xylene. This was modified slightly for hematoxylin and eosin staining so that instead of 50% ethanol, slides were soaked in 95% ethanol and the time in each bath was extended to five minutes. After dehydration all slides were coverslipped using 1 drop of Permount Mounting Media (Fisher Scientific) and air dried overnight.

Hematoxylin and Eosin Staining

For staining with hematoxylin and eosin, rehydrated slides were immersed first for eight minutes in filtered Mayer's hematoxylin (Dako), rinsed in tap water until no more dye was evident and then soaked in tap water for 10 minutes. Slides were then rinsed in distilled and deionized water before being dipped ten times in 95% ethanol. Slides were then submersed for 45 seconds in Eosin Y (Sigma) followed by 95% ethanol for five more minutes.

Antigen Retreival

Heat-induced antigen retrieval was employed for probing the AT antigen only. Six slides at a time were placed into plastic Copeland jars with a large hold drilled in the lid filled with Tissue Unmasking Fluid (Invitrogen). These jars were placed into 400 mL glass beaker in 180 mL of distilled, deionized water and heated on highest power in a Samsung Classic Collection Microcooking microwave for 2.5 minutes. The water was then exchanged for 180 mL of fresh water and the beaker containing the Copeland jar with slides was heated again on highest power for 2.5 minutes to bring the internal temperature to 95° C. The water was exchanged again for 180 mL of tap water and everything was permitted to cool for 20 minutes.

Immunohistochemical Staining

Slides were laid flat on a rack and covered with 460 μL per slide of each successive solution. To remove solution, slides were placed at an angle and then rinsed with $PBStw_{1\%}$, Dulbecco's phosphate buffered saline (PBS) (Gibco) containing 1% Tween 20 (Sigma). Before and between incubation with each solution, slides were washed two times by being laid flat and covered in $PBStw_{1\%}$ for three minutes. First, endogenous peroxidase activity was blocked with either HRP-Block (Dako) for five minutes or with 3% hydrogen peroxide for ten minutes. Slides were then incubated for one hour in the dark with primary antibody diluted in PBS with either 1% ovalbumin or 1% bovine serum albumin. Table 2.1 describes the antibodies used, their dilutions and incubation times. Next, slides were incubated in the dark with peroxidase conjugated secondary antibody appropriately matched to primary antibody either HRP-conjugated donkey anti-goat IgG (Serotec) or HRP labeled polymer

(Dako). Secondary antibodies were diluted in the same solution as their primary antibodies. Slides were then covered with nine drops per slide of diaminobenzidine solution (DAB) (DAKO) and let sit for eight minutes. DAB staining was enhanced using a solution of 2.5% cobalt chloride, 2.5% nickel ammonium sulfate for eight minutes and rinsed in water. Slides were counterstained for five minutes with Mayer's Hematoxylin (Dako or Sigma), rinsed in tap water until no more stain was detected in the water and then immersed in tap water for five minutes before dehydrating and coverslipping.

Table 2.1. *Description of Antibody, Dilutions and Incubation Times Used in Immunohistological Staining.*

1° Antibody	Clonal	Raised in	1° Ab Dilution	1° Ab time (min)	2° Ab Dilution	2° Ab time (min)
Anti-human HCII IgG --affinity purified (Affinity Biologicals)	poly	goat	1:1000	60	1:100	30
Anti-human antithrombin IgG (Diasorin)	poly	goat	1:500	60	1:200	20
Anti-human (pro)thrombin* IgG (Dako)	poly	rabbit	1:2000	60	None	10
Anti-human α1-protease inhibitor IgG (Fitzgerald Industries)	poly	goat	1:10,000	60	1:100	10
Anti-human albumin IgG --affinity purified (Antibodies Inc)	poly	goat	1:1000	60	1:1000	10
Anti-human maspin IgG (Pharmingen)	poly	goat	1:100	60	1:100	30
Goat IgG		goat	1:1000	60	1:100	30

* Note: Antibodies that distinguish between human prothrombin and human thrombin are not commercially available. Therefore, under circumstances such as tissue staining when molecular weight is not detectable, the two are indistinguishable by antigen staining.

Lesion Classification

One serial section from each case was stained with hematoxylin and eosin. Using this slide set, the severity of each atherosclerotic lesion was classified separately by three trained individuals. Plaque severity was rated from I to VI according to the American Heart Association classification system (Table 2.2)[15,16].

Table 2.2. *American Heart Association Classification of Atherosclerotic Lesions*

Classification	Description
I	(initial) Intimal thickening and isolated macrophage foam cells
II	(fatty streak) Mainly intracellular lipid accumulation
III	(intermediate) Type II changes and small extracellular lipid pools
IV	(atheroma) Type II changes and core of extracellular lipid
V	(fibroatheroma) Lipid core and fibrotic layer, or multiple lipid cores and fibrotic layers, or mainly calcific or mainly fibrotic
VI	(complicated) Surface defect, hematoma-hemorrhage, thrombus

Staining Intensity

The intensity of staining for each probed antigen was also independently ranked on a scale of 0 to 3 with 0 indicating no staining, 1 indicating weak staining, 2 indicating intermediate staining and 3 indicating strong staining. Note, the reviewers vary by protein, but each slide set was rated by 3 reviewers. Images were created using the Aperio ScanScope and Aperio ImageScope software version 9.0. Slides were scanned using factory settings for immunohistochemistry.

Statistical Analysis

Statistical analysis was carried out by Carolyn Deans of the University of North Carolina Biometric Consulting Laboratory. Mean scores for lesion severity and staining intensity was computed for each slide. Spearman correlation coefficients, with their corresponding p-values, were computed to estimate the correlation between the mean scores for each of the probed proteins and severity of the atherosclerotic lesions. Using the mean score for each protein helped to decrease the variance of scores, thereby increasing the chance of detecting a significant correlation when it did exist. To support the use of the mean scores, intra-rater reliability was assessed using intra-class correlations. Since five comparisons between lesion severity and staining intensity were made, it was necessary to make an adjustment for multiple comparisons. The Bonferroni-Holm method was used, comparing p-values for all five correlations, initially comparing the smallest p-value to 0.05/5=0.01. If significant, then the second-smallest p-value was compared to 0.05/4=0.125. If significant, the third-smallest p-value was compared to 0.05/3=0.1667, and so on for each of the five p-values, stopping when a non-significant result was observed.

Aorta Collection and Protein Extraction

Samples of aorta were gathered by the University of North Carolina Hospital McLendon Clinical Laboratory, Division of Autopsy Services. Use of these tissues for the experiments described was subject to review and was preapproved by the appropriate individuals. Three samples each of normal or atherosclerotic aorta as determined by gross examination were collected from autopsy patients and numbered 1 through 6. Even-numbered samples contained non-diseased tissue and odd-numbered samples contained

atherosclerotic lesions. After washing rinsing in $PBStw_{0.1\%}$, tissues were submerged in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek USA), frozen in liquid nitrogen and stored at -80° C. Vessels were thawed quickly and washed 3 times in $PBStw_{0.1\%}$. The adventitia was carefully dissected and discarded to help ensure the removal of contamination from trapped blood and fluids. A 5 mm^2 piece was cut from each sample for homogenation. Each 5 mm^2 section was placed in 200 μL of modified RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Tergitol-type NP-40, 0.25% sodium deoxycholate, 1% TritonX-100 pH 7.4, containing 1 Complete Protease Inhibitor tablet (Roche) per 50mL) on a glass plate and diced with a fresh razor blade and scalpel. This was transfer to a polypropylene tube on ice containing 300 μL more of modified RIPA buffer. Each slurry was sonicated using a Sonicator Dismembrator (Fisher Scientific) in five one-second bursts with 2 minute rest on ice, five times with an output power of between 10 and 14 watts. 250 μL more of modified RIPA buffer was added to each homogenation which was then left on ice overnight. Protein suspensions were centrifuged at ~20,000 x g for 20 minutes at 4° C. Supernatant was removed, aliquoted and stored at -80° C for future use.

Immunoblot Analysis of Normal and Atherosclerotic Vessel

Protein extracted from normal and atherosclerotic aorta, HCII, AT, prothrombin (all purified from human plasma as previously described[254,255], α-thrombin (Haematologic Technologies) and serpin-thrombin complex were mixed with sample buffer containing SDS and heated for 10 minutes at 95° C. Serpin-thrombin complexes were made by incubating a 1 to 1 molar ratio of serpin and thrombin with 25 μg/mL unfractionated heparin (Diosynth), for 5 minutes at room temperature and stopping the reaction with 10 μM final concentration each

of the protease inhibitors, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR), tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK) (all from Calbiochem) and phenylmethanesulfonyl fluoride (PMSF) (Sigma). Denatured solutions were then electrophoresed through either 10% SDS-polyacrylamide gels or through 4-15% gradient SDS-polyacrylamide PhastGel (General Electric Healthcare). Protein was transferred to Immobilon-B transfer membrane, 45 μm pore-size (Millipore) and blocked with 5% milk in PBS. Membranes were then incubated with two antibodies: either goat anti-human HCII (affinity purified, 1:1000, Affinity Biologicals) and rabbit anti-human prothrombin (1:1000, Dako) or goat anti-human AT (1:1000, Diosorin) and human prothrombin (1:1000, Dako) for 1 hour at 37° C in 5% milk in $PBStw_{0.1\%}$. After washing 3 times for 10 minutes in $PBStw_{0.1\%}$, membranes were incubated in the dark for 1 hour at room temperature with Alexa Fluor 680 donkey anti-goat (Invitrogen) and IRDye 800CW donkey anti-rabbit (Rockland Immunochemicals) fluorescent secondary antibodies. Membranes were washed two times for ten minutes with $PBStw_{0.1\%}$ and one time for ten minutes with PBS. Antigen was then detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

Ratings of Lesion Severity and Antigen Staining

Atherosclerotic lesions were categorized from 1 to 5. No category 6 lesions were observed. Staining of each of the circulating proteins was observed with intensity range of 0 to 3. Mean scores for each slide are listed in Chapter 2, Supplemental Material , Table 2.S1. Assessment of inter-rater reliability by inter-class correlation coefficients (ICCs) results in

ICCs above 0.5 (Table 2.3), indicating that there is reliability among raters. This supports the use of the mean scores to assess correlations between plaque severity and staining intensity.

Table 2.3. *Intra-class Correlation Coefficients (ICC) to Address Intra-Rater Reliability*

Variable	ICC
Severity	0.824
HCII	0.705
(Pro)thrombin	0.719
AT	0.603
α1PI	0.725
Albumin	0.845

All of the plasma proteins probed for were found in atheromatous lesions. Mapsin was not detected in vessel. Antigen staining was detected primarily in the lipid rich, necrotic core of the atheromas. Images of each slide have been compiled in Supplemental Materials Figures 2.S1-2.S7. Figure 2.1 shows an example of serial sections of one sample, #5, immunoprobed for each circulating protein. This atheroma was rated with an average lesion severity of 4.67. Mean staining intensity for HCII = 3.00, AT = 1.67, (pro)thrombin = 3.00, α1PI = 3.00, albumin = 3.00 and goat IgG = 0.

It is evident from Figure 2.1 that there is protein from all of the circulating proteins found in this atherosclerotic plaque. From first examination, AT appears to have less staining than the other proteins. However, due to discrepancies in the quality of antibodies

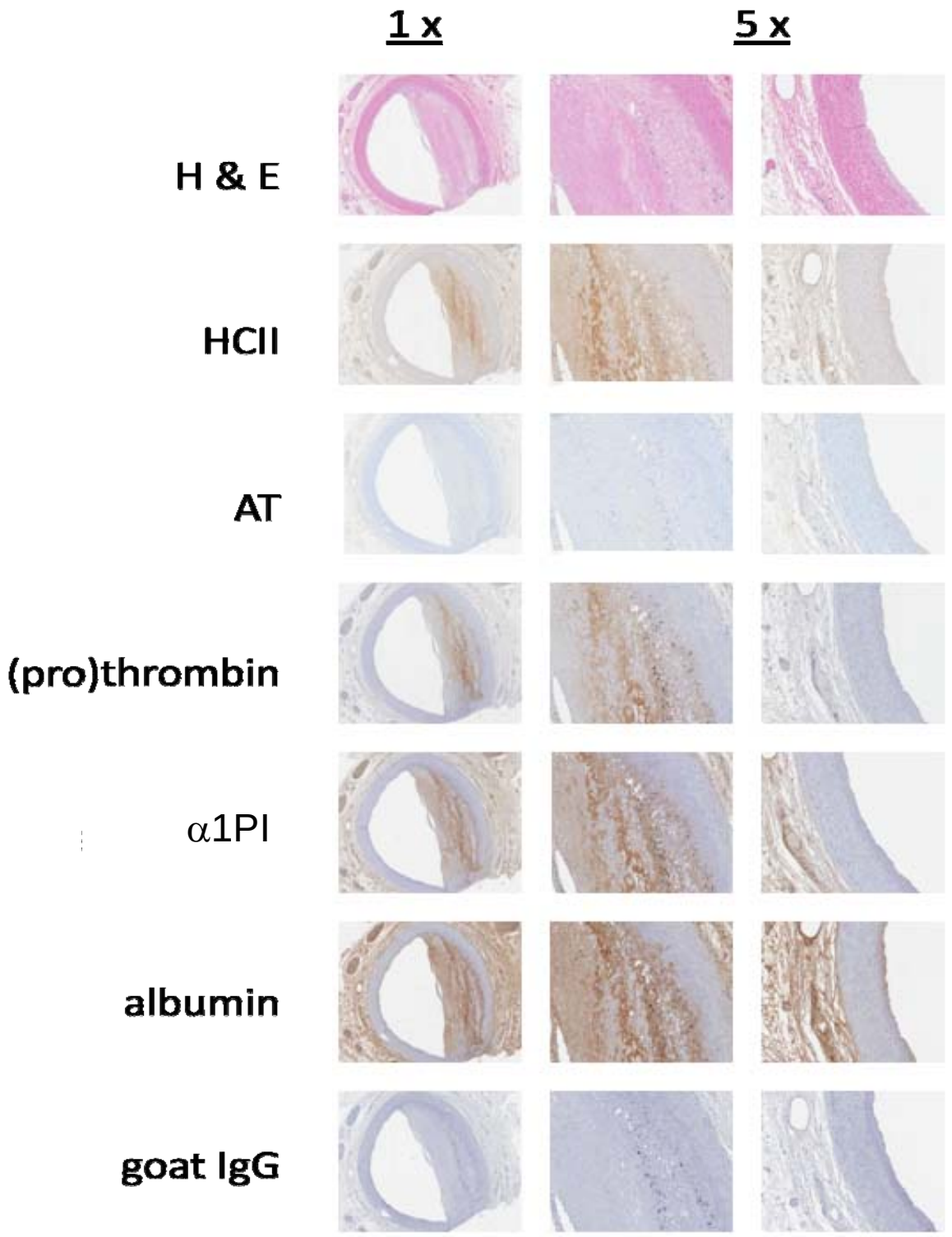


Figure 2.1. *H&E and immunological staining of left, anterior descending coronary artery #5 for various plasma proteins and goat IgG negative control.* The left-most column shows a 1x view of the vessel, the middle column shows a 5x view of the atheromatous lipid core and the right-most column shows staining in region of the least atherosclerosis in this vessel.

and the requirement to use heat-induction for antigen retrieval on this slide set, it is impossible to compare protein concentration across protein types in these stained vessels.

It is interesting to note the paucity of staining in the less atherosclerotic tissue from this vessel (seen in the right-most column in Figure 2.1). There is also some detectable HCII, AT and albumin in the endothelial region, but not the other proteins. Furthermore, no antigen staining was detected in the normal region of this or any of the LAD coronary arteries used in this study.

Relationship Between Plaque Severity and Antigen Staining

Statistical analysis using Spearman correlation coefficients comparing lesion severity and staining intensity indicates that there is a significant positive correlation between lesion severity and HCII staining, lesion severity and (pro)thrombin staining, and lesion severity and antithrombin staining (Table 2.4).

Table 2.4. *Spearman Correlation Coefficients to Estimate Correlation of Severity and Antigen Staining.*

	HCII	**(pro)thrombin**	**AT**	**α1PI**	**albumin**
correlation coefficient	0.63378	0.80537	0.48451	0.34403	0.38671
p-value	0.0003	<.0001	0.0090	0.4499	0.1918
N	28	28	28	7	13

Since five comparisons were being made, it was necessary to make an adjustment for multiple comparisons. Analysis using the Bonferroni-Holm method supported the data above, indicating a positive correlation between lesion severity and (pro)thrombin, HCII and AT, but not with α1PI and albumin (Table 2.5).

Table 2.5. *Breakdown of Tests of Significance Using Bonferroni-Holm Method to Adjust for Multiple Comparisons*

Step	**Variable**	**Observed p-value**	**Compare to**	**Significant?**
1	(Pro)thrombin	<0.0001	0.01	YES
2	HCII	0.0003	0.0125	YES
3	AT	0.0090	0.01667	YES
4	Albumin	0.1918	0.025	NO → Stop after this step.

It is useful to note that sample sizes stained for for α1PI (n=7) and albumin (n=13) were smaller than those for severity, HCII, (pro)thrombin, and AT (n = 28 for each). If α1PI and albumin staining are truly positively correlated with severity, then an increased sample size would be necessary to demonstrate this significance.

Protein Extraction and Immunoblot Analysis

Figure 2.2 shows immunoblotting on protein extracted from atherosclerotic and normal aorta along with purified proteins for comparison. Panel A depicts the samples run on the PhastGel system, through a SDS 4-15% polyacrylamide gradient gel and probed for HCII (red) and (pro)thrombin (green). Complexes and overlap between the red and green fluorescence emissions appear yellow. Column 1 is Aorta 1 (atherosclerotic), column 2 is

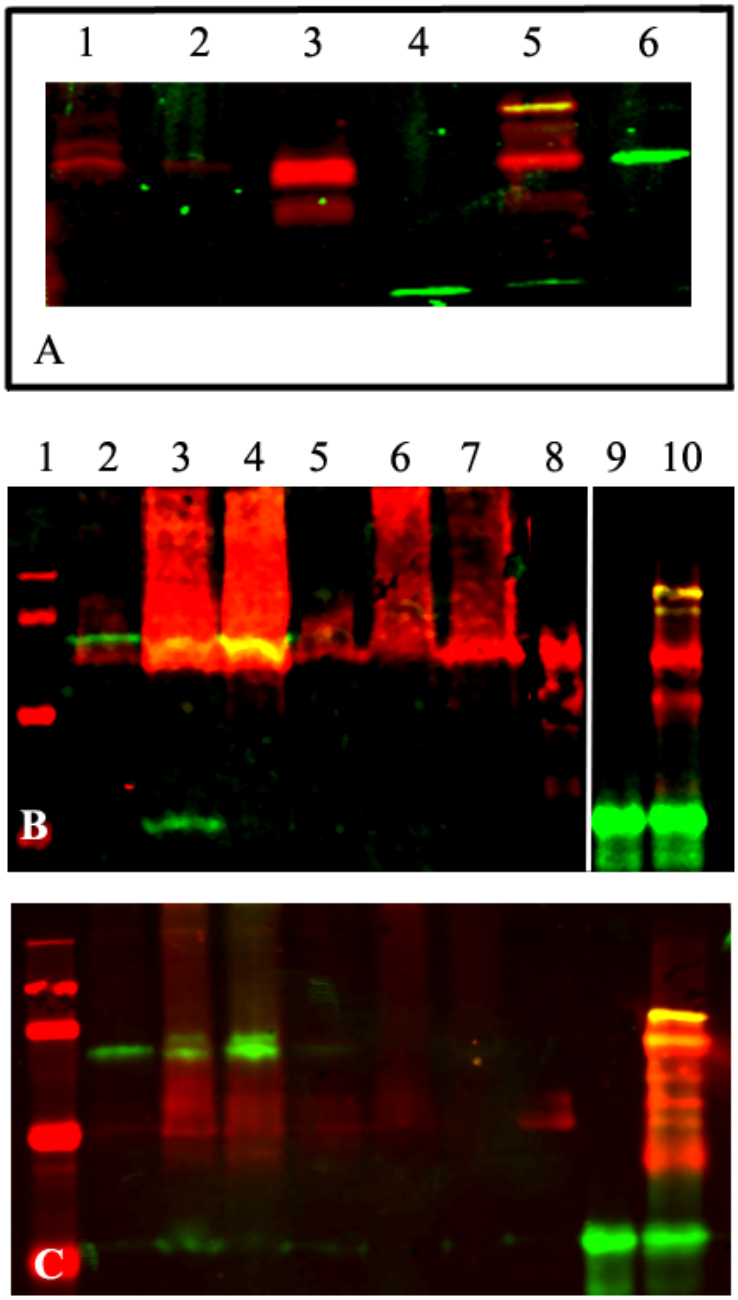


Figure 2.2. *Presence of HCII, AT, thrombin and Prothrombin in homogenized atherosclerotic and normal aorta.* Panels A and B have been probed for HCII (red) and (pro)thrombin (green). Panel C has been probed for AT (red) and (pro)thrombin (green). Complexes and overlap appear yellow. The columns contain the following proteins, A1 = Aorta 1 (atherosclerotic) ; A2 = Aorta (normal) 2; A3 = HCII; A4 = thrombin; A5 = HCII-thrombin-complex; A6 = prothrombin; B1, C1 = molecular weight marker; B2, C2 = Aorta 1(atherosclerotic); B3, C3 = Aorta 2 (normal); B4, C4 = Aorta 3(atherosclerotic); B5, C5 = Aorta 4 (normal); B6, C6 = Aorta 5 (atherosclerotic); B7, C7 = Aorta 6 (normal); B8 = HCII; C8 = AT; B9, C9 = thrombin, C10 = serpin-thrombin complex.

Aorta 2 (normal), column 3 is human HCII, column 4 is human thrombin, column 5 is HCII-thrombin complex, column 6 is prothrombin. The higher molecular weight band in column 3 is intact HCII while the lower molecular weight band is cleaved HCII. Note HCII is detectable in both normal and atherosclerotic tissue. On this blot, no prothrombin or thrombin (complexed or not) is detectable.

Additional studies are shown in Figure 2.2 panels B and C. These experiments utilized 10% SDS-polyacrylamide gels. Column 1 is molecular weight marker, column 2 is Aorta 1 (atherosclerotic), column 3 is Aorta 2 (normal), column 4 is Aorta 3 atherosclerotic), column 5 is Aorta 4 (normal), column 6 is Aorta 5 (atherosclerotic), column 7 is Aorta 6 (normal), column 8 is human serpin (Panel B = HCII, Panel C = AT), column 9 is human thrombin, column 10 is serpin-thrombin complex. Panel B is probed with and probed for HCII (red) and (pro)thrombin (green). Panel C is probed with and probed for AT (red) and (pro)thrombin (green). Complexes and overlap between the red and green fluorescence emissions appear yellow. Note that Panel B has been divided between columns 8 and 9 to indicate that these sections of the gel were adjusted separately for color curves, brightness and contrast so as best to detect distinct bands within each segment. HCII is detected in all the aortic sections as is AT. Additionally, prothrombin can be detected in Aortas 1-5 and thrombin can be detected in Aorta 2. While Panel A would suggest that there may be more HCII in normal aortic vessel than in atherosclerotic vessel, the results shown in Panel B lends some uncertainty to this observation. Finally, no serpin-thrombin complex is detectable in the aortic sections.

DISCUSSION

Somewhat at odds with our hypothesis, we detected more HCII in the more severe atherosclerotic lesions as evidenced by a statistically significant positive Spearman Correlation (Table 2.3). However, as predicted (pro)thrombin staining exhibited a positive correlation between intensity and lesion severity (Table 2.3). Both HCII and (pro)thrombin were detected in the same lipid-rich regions and necrotic cores of the atheromas (Figure 2.1). Based on this data, we developed the working hypothesis that increasing amounts of HCII was present in increasingly severe atheromas because there are greater amounts of thrombin to be inhibited in the more severe plaques. Consistent with this hypothesis is that dermatan sulfate found in atheromas shows decreased ability to accelerate HCII inhibition of thrombin[256]. We reasoned therefore, that to reach the same level of thrombin inhibition, higher levels of HCII must be present.

The studies which show that HCII inhibits thrombin in atherosclerosis, also indicate that AT, the prominent thrombin inhibitor in coagulation, is unable to fulfill this role[47,49]. Therefore, to substantiate the functional specificity of HCII in the atheromas, we probed adjacent sections for the presence of AT, expecting to find little there. To our surprise, AT was detected in the atheromas, in the same location as HCII and (pro)thrombin (Figure 2.1). The AT staining was somewhat less intense in the atheromas (Figure 2.1) despite comparable staining intensity in the liver positive controls to those of HCII and (pro)thrombin (data not shown). However, a positive correlation between staining intensity and plaque severity was still evident (Table 2.4).

To further investigate the specificity of the co-localization of these thrombin-inhibiting serpins and (pro)thrombin, we probed additional adjacent LAD coronary artery sections for a non-thrombin-inhibiting plasma serpin, α1-protease inhibitor; a non-serpin

plasma protein, albumin; and a non-circulating serpin, maspin. Although we can find no evidence establishing a role for α1-protease inhibitor or albumin in the pathophysiology of atherosclerosis, both of these proteins were found localized in the same region as HCII, (pro)thrombin, and AT (Figure 2.1). While correlations between staining intensity and plaque severity were not significant with albumin and α1-protease inhibitor, this could be due to the smaller number of slides probed. It is perhaps more important that these proteins can be found in the atheroma at all.

In an attempt to determine if the (pro)thrombin in atherosclerotic lesions was thrombin or prothrombin and if it complexed with either the HCII or AT, we examined homogenized aorta by immunoblot analysis. Results from these experiments confirmed the presence of HCII, AT and prothrombin in atherosclerotic tissue and also in normal vessel. Thrombin was only detectable in one sample of aorta and there was no indication of thrombin-serpin complexes present. These results do not eliminate the possibility or even probability that thrombin is inhibited by HCII in the atherosclerotic vessel. It is very possible that complexes are cleared more quickly from the vessel, or that they are somehow less detectable to the antibodies used here.

The presence in atherosclerosis of all of the circulating proteins probed for in this study, but not the non-circulating serpin, maspin, suggests severe atherosclerotic lesions may act as a watershed for plasma proteins and that the co-localization of proteins in this region may be non-specific. The positive correlation with plaque severity for HCII, (pro)thrombin and AT bolster this theory especially since the endothelium is known to show increased permeability with atherosclerosis[19,44,257]. Many other circulating proteins, in addition to those reported here, have been found in the same region of human atherosclerotic lesions including

the apolipoproteins, apoA-I, apoB and apoE[258,259], C-reactive protein[260], vonWillebrand factor[260], and resistin[261]. Sound explanations for the existence and retention of these proteins in atherosclerotic plaques have been presented, just as there appears to be good reason for the presence of thrombin and HCII. One could even make a case for the retention of albumin in the plaque as it is known to bind fatty acids. However, we are unable to rationalize the function of α1-protease inhibitor in the atheroma. This is not to say that none of the proteins found in atherosclerotic lesions influence its progression. Contrarily, we find it likely that many of these proteins play a role in atherosclerotic regulation or advancement. For example, fibrin has been found in atherosclerotic plaques[262]. While it is possible that it had been deposited by infiltrating macrophages, it is just as likely that all of the necessary coagulation factors leak into the plaque and are activated by the presence of tissue factor bearing cells in the atheroma. The result would be fibrin in the atherosclerotic plaque that does not resemble a typical fibrin clot because platelets and red blood cells would be absent from the milieu. This hypothesis also explains how thrombin could be detected in its active form in atherosclerotic plaques. Thus the increased permeability of atherosclerotic lesions may actually be a thrombogenic factor in and of itself. However, this study emphasizes a caveat that must be regarded with when considering this and other immunohistochemical examinations of atherosclerosis. While presence or co-localization of any circulating protein(s) in atherosclerotic lesion may have functionality, it is important to consider that proteins found in the acellular, lipid-rich region of atheromas, may simply be the result of increased endothelial cell permeability may not contribute to atherogenesis or its regulation at all.

CHAPTER 2 SUPPLEMENTAL MATERIAL

Table 2.S1. *Average Ranking of Plaque Severity and Stain Intensity for each Sample of Atherosclerotic Coronary Artery.*

Sample Number	Plaque Severity	HCII Staining	(Pro)thrombin Staining	Antithrombin Staining	α1PI Staining	Albumin Staining
1	5.00	1.33	1.67	1.00		
2	5.33	2.67	2.67	2.00		1.00
3	3.33	0.00	0.33	0.00		3.00
4	4.67	2.00	0.67	0.00	3.00	3.00
5	4.67	3.00	3.00	1.67	3.00	3.00
6	4.67	1.00	2.00	0.67	3.00	
7	1.33	0.00	0.67	0.00		
8	5.00	0.33	2.00	0.67		2.00
9	4.00	0.00	0.67	0.00		2.00
10	2.00	0.00	0.33	0.00		2.00
11	2.33	0.00	0.33	0.00		
12	2.00	0.33	0.33	0.00		
14	3.33	2.33	1.00	0.33		3.00
16	4.00	1.33	2.00	0.67	3.00	
17	2.33	0.00	0.67	0.33		
18	4.33	1.67	1.00	0.33		
19	3.00	0.33	0.33	0.00		
21	2.00	0.33	0.67	0.33		
22	1.00	0.00	0.00	0.00		
23	3.00	0.00	1.67	1.00		
24	3.33	1.33	1.00	0.67	2.33	
25	1.67	0.67	0.33	0.00		
26	2.33	0.00	0.33	0.33		
27	2.67	0.00	0.33	0.33		
28	1.67	0.67	0.00	0.00		
29	3.00	0.00	0.33	0.00		
31	4.67	1.33	1.67	0.67	1.50	2.00
33	5.00	1.67	2.67	1.33	3.00	

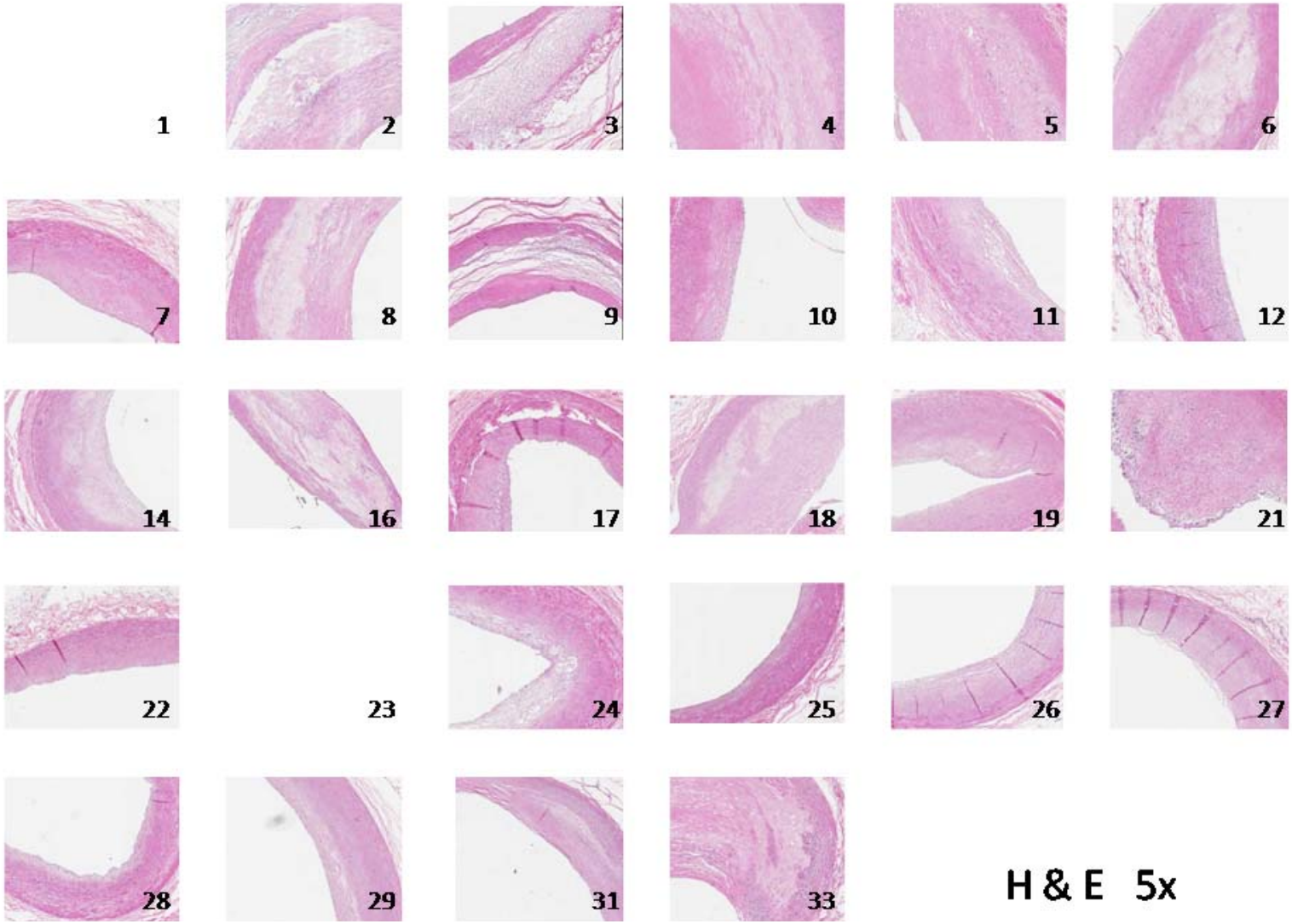


Figure 2.S1. *Images of 5x views of LAD coronary arteries stained with hematoxylin and eosin.* Note, photographic images of samples #1 and #3 were unobtainable using the described method.

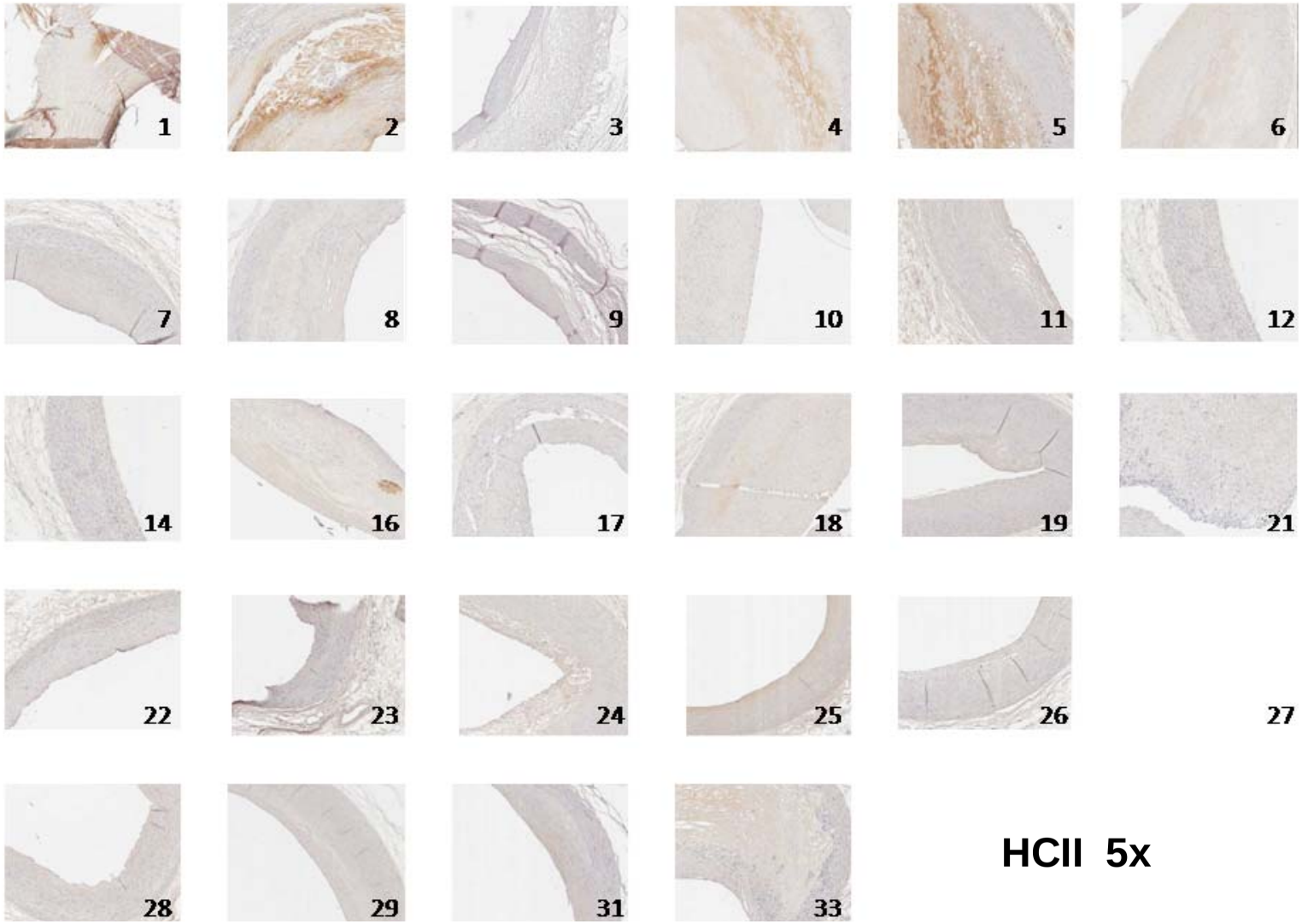


Figure 2.S2. *Images of 5x views of LAD coronary arteries immunoprobed for HCII.* Note, photographic images of sample #27 were unobtainable using the described method.

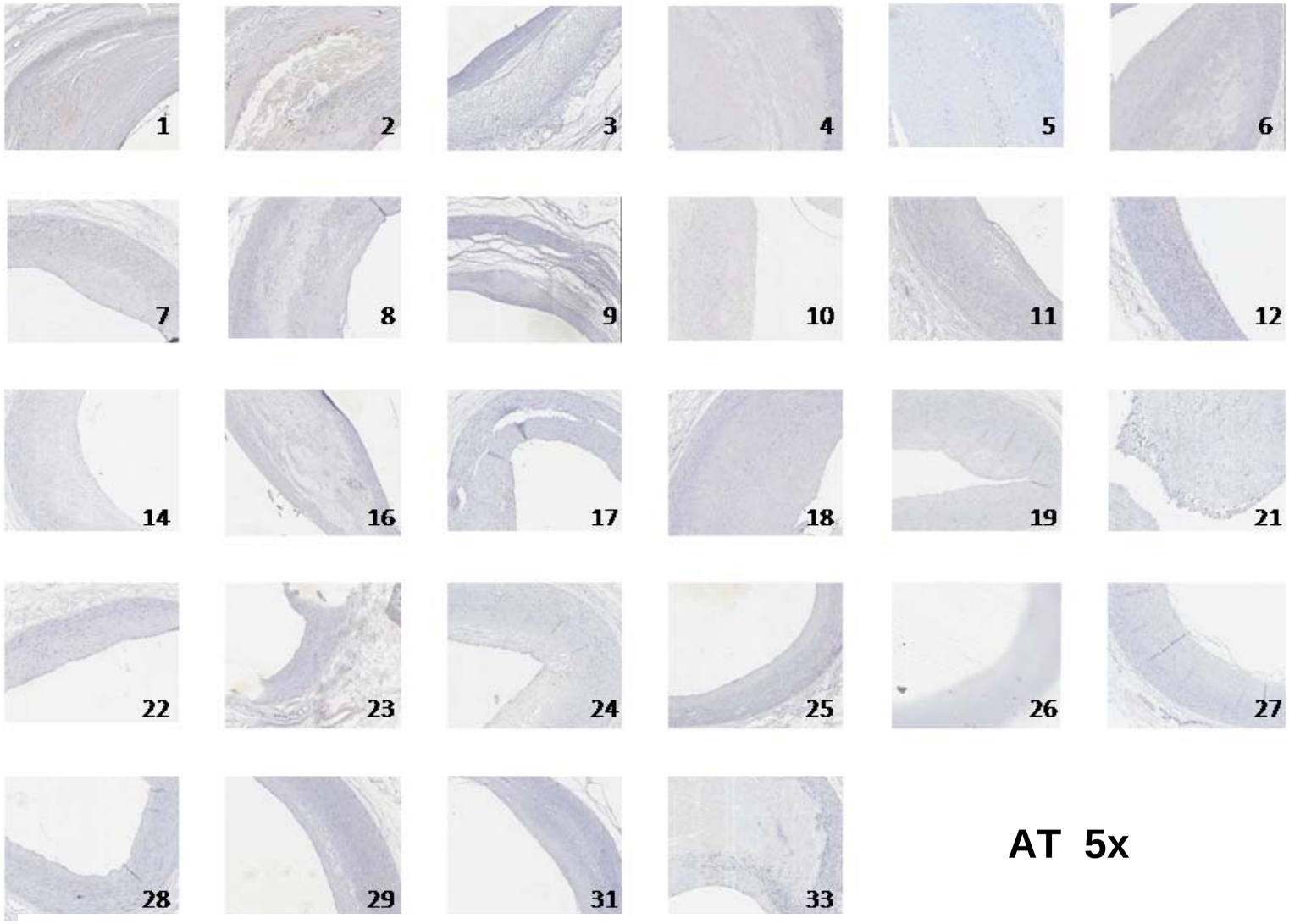


Figure 2.S3. *Images of 5x views of LAD coronary arteries immunoprobed for AT.*

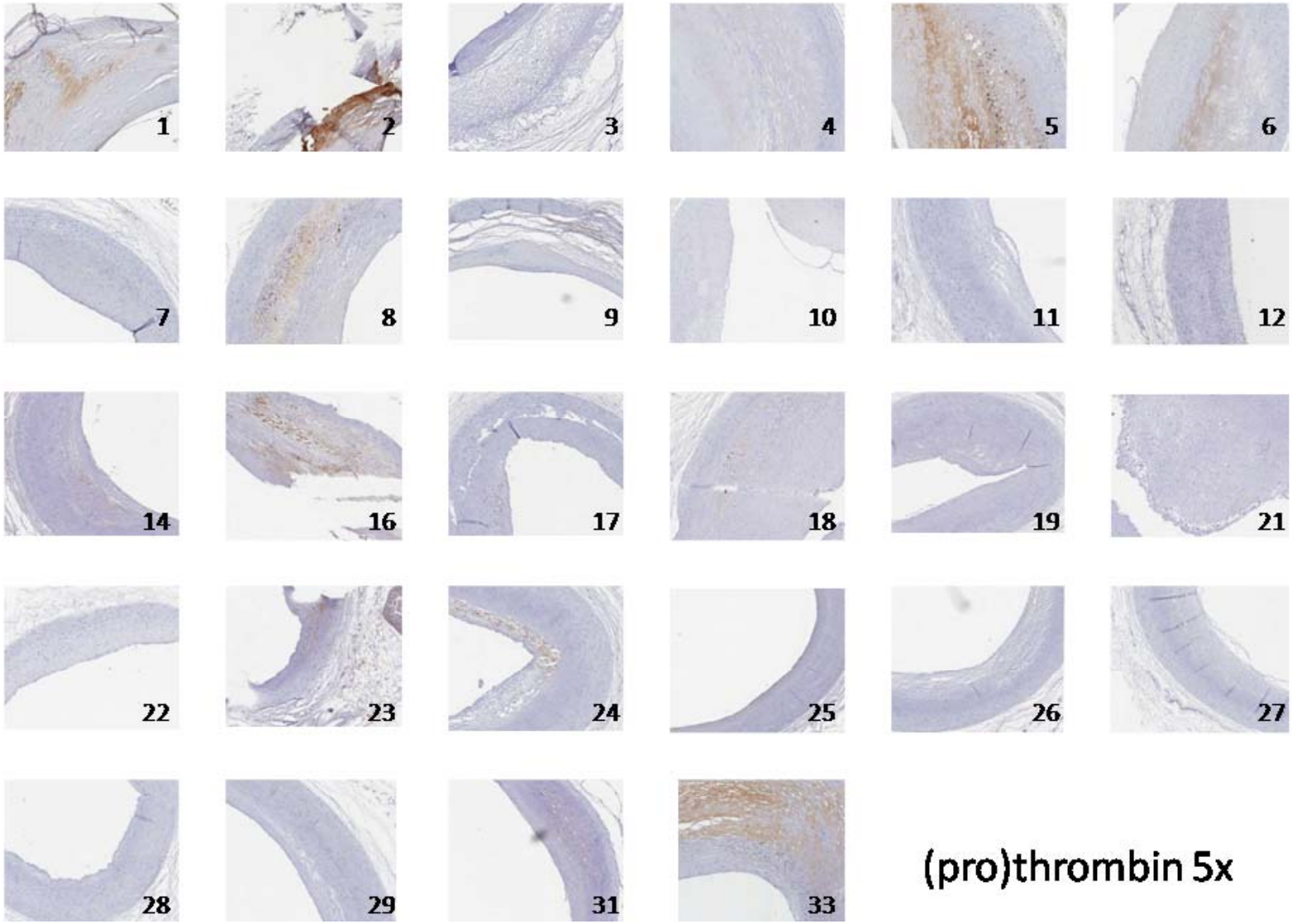


Figure 2.S4. *Images of 5x views of LAD coronary arteries immunoprobed for (pro)thrombin.*

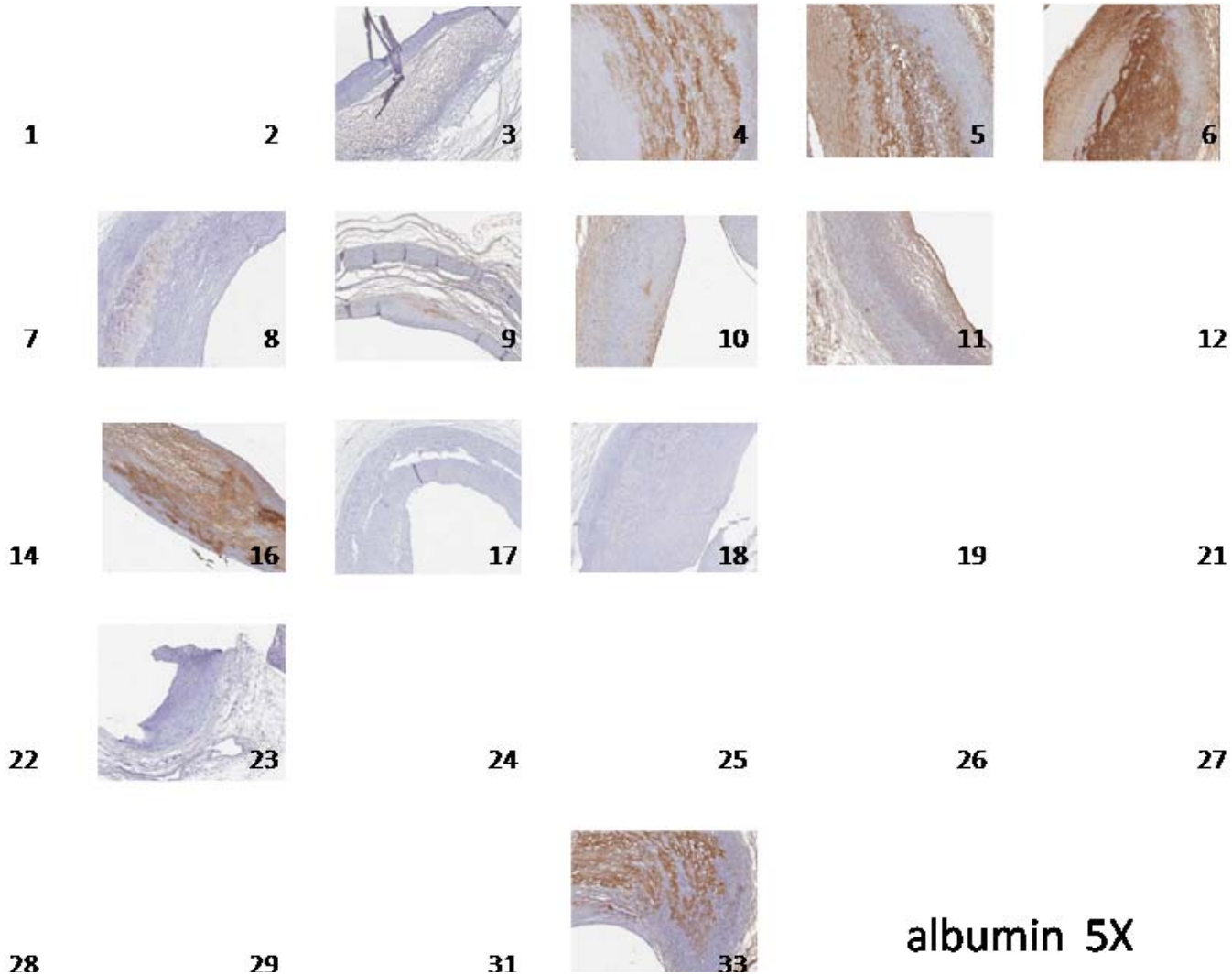


Figure 2.S5. *Images of 5x views of lAD coronary arteries immunoprobed for albumin.*

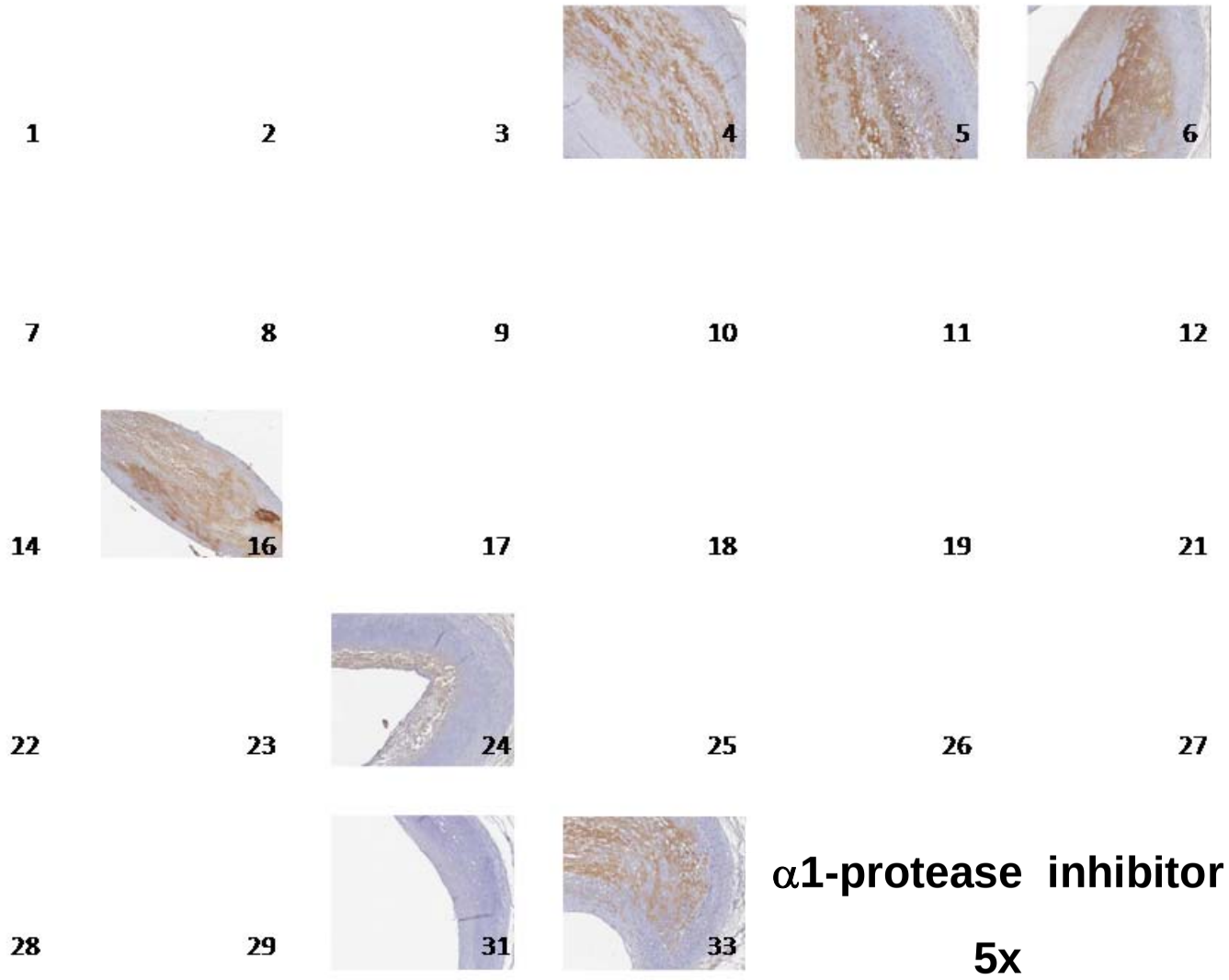


Figure 2.S6. *Images of 5x views of LAD coronary arteries immunoprobed for α1-protease inhibitor.*

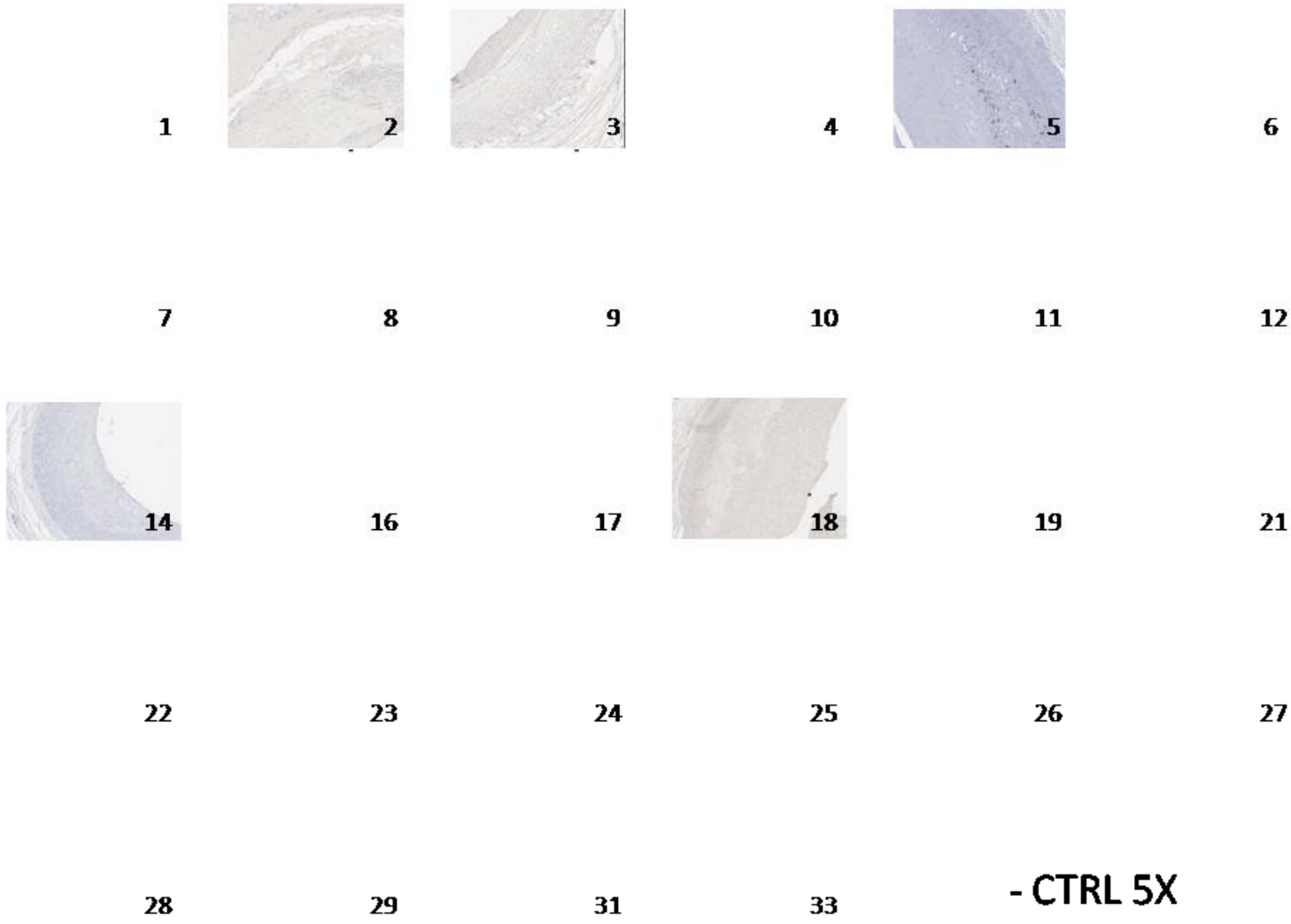


Figure 2.S7. *Images of 5x views of LAD coronary arteries negative controls.* Slides #2, 3 and 18 were probed with anti-maspin primary antibody, while slides #5 and 14 were incubated with goat IgG.

CHAPTER 3

Transferring Glycosaminoglycan-binding Properties of Heparin Cofactor II to α1-Protease Inhibitor$_{Pittsburgh}$

SUMMARY

Glycosaminoglycans (GAGs) are important for the physiologic function of several serine protease inhibitors (serpins). The structural homology of the serpin superfamily is such that it is possible to transfer structural components from one serpin to another and confer the associated specific activity. This provides an excellent opportunity to study structure-activity relationships and allows for the creation of novel proteins for the advancement of science and medicine. Heparin cofactor II (HCII) is a serpin whose physiologic role as a thrombin inhibitor is dependent upon GAGs. Binding to GAGs is centered on its basic D-helix region. The archetypal serpin, α1-protease inhibitor (α1PI), shows no GAG-dependent binding. To confer GAG-binding properties, we mutated five residues in the D-helix of α1PI$_{Pittsburgh}$ (α1PI reactive site variant, M358R) designated α1PI$_{Pitt\text{-}GAG}$, to corresponding positively charged amino acids from the D-helix of HCII: E97K/Q98R/Q105R/P106R/T102R. α1PI$_{Pitt\text{-}GAG}$ gained both heparin and dermatan sulfate binding capacity and enhanced thrombin inhibitory activity. In the presence of GAGs, rates of α-thrombin inhibition were enhanced up to 15 fold, showing maximal inhibition rates, which approached those of HCII-GAG-thrombin. Further studies indicated that the primary

mechanism of GAG-accelerated thrombin inhibition was the creation of a ternary complex, with a small contribution from a conformational change in the serpin. These results indicate (i) the ability to create a novel GAG-binding serpin, (ii) that this gain-of-function approach can further our understanding of serpin structure-activity relationships, and (iii) that the engineering of serpins may prove to be a useful tool in the development of pharmaceuticals.

INTRODUCTION

Serine protease inhibitors (serpins) are a protein superfamily classified based on their structural homology[263,264]. Over 1000 serpins have been identified throughout all domains of life (Eukarya, Bacteria, Archaea and Viruses). Thirty-six serpins are found in the human genome[265]. In human physiology, serpins serve a multitude of functions. The majority of serpins inhibit serine proteases as their name suggests. However, some members of the family inhibit cysteine proteases or caspases, and a few are non-inhibitory proteins that serve as hormone transporters, molecular chaperones, tumor suppressors, or catalysts for DNA condensation. The inhibitory human serpins regulate numerous biologic activities including coagulation, fibrinolysis, inflammation, reproduction and lung elasticity[94].

Serpins inhibit their target proteases with an exceptional mechanism, which is dependent on their unique structure[116]. Members of the family typically comprise around 400 amino acids arranged into nine α-helices (labeled A-I), three β-sheets (labeled A-C) and a reactive center loop (RCL). Upon encounter with its target protease, the serpin's scissile P1-P1′ (by Schechter-Berger nomenclature) bond is cleaved. Without this bond acting as a tether, the serpin shifts its conformation to its most thermodynamically stable state in which the N-terminal portion of the RCL is inserted into β-sheet A. This results in the covalently

bonded protease being translocated to the opposite pole of the serpin, thus disabling its proteolytic functionality. [For review, see[116] and references cited therein.]

While serpins' shared tertiary structure imparts inhibitory activity, specific structural components confer distinct characteristics including protease specificity and cofactor binding[121,264]. "GAG-binding serpins" are a subset of serpins that bind glycosaminoglycans (GAGs). GAGs are negatively charged linear complex carbohydrates found throughout the body both soluble and membrane bound. Heparin, a specific GAG derived from porcine mucosa[235], is widely used for thrombosis prevention[144]. It acts predominantly by accelerating the inhibitory activity of antithrombin (AT) (systematic name: *SERPINC1*) up to 10,000 fold primarily against thrombin, factor Xa and factor IXa[51]. Biochemical and crystallographic studies have determined that heparin accelerates its activity via two mechanisms depending on the target protease. Heparin binding to the positively charged region centered around the D-helix of AT causes a conformational change extending the RCL away from the body of serpin putting it in a more favorable alignment for cleavage by proteases such as factor Xa[266-268]. Heparin can also bind some proteases such as thrombin creating a bridge between serpin and protease forming a ternary complex[134].

Another heparin binding serpin, heparin cofactor II (HCII) (systematic name: *SERPIND1*) specifically inhibits thrombin and is thought to contribute to the regulation of coagulation[51,269,270]. HCII has an N-terminal acidic extension unique among serpins[271,272] and also has an atypical P1 residue, a leucine[272,273]. The inhibitory activity of HCII is accelerated up to 20,000 fold by the presence of the GAGs heparin, heparan sulfate and distinctively, dermatan sulfate[274]. GAGs bind HCII's positively charged D-helix region resulting in activation through a combination of the bridging[275-279] and RCL conformational

mechanisms[100] as well as an additional mechanism unique to HCII, whereby its N-terminal acidic extension is relocated allowing for it to create an additional stabilizing interaction with thrombin[152,157,280].

The understanding of GAG-binding serpins and their mechanisms of action has been of biomedical importance for over thirty years due to our ability to therapeutically manipulate their activity with soluble GAGs such as heparin to control pathological processes such as thrombosis. Traditionally, the approach to investigating GAG-binding has been through a loss-of-function strategy whereby candidate residues or regions are ablated or mutated and subsequent effects are measured. Grasberger et al.[281] illustrate that serpins possess a modular architecture that allows for domain substitution and transfer of activity. Compelled by the concept, we aspired to investigate GAG-binding through a gain-of-function strategy, transferring GAG-binding properties to a non-GAG-binding serpin. Specifically, we confer GAG-binding capacity and GAG-accelerated inhibitory activity to the naturally occurring thrombin-inhibiting Pittsburgh variant (reactive site mutation M358R)[183] of the non-GAG binding, prototypical serpin, α1-protease inhibitor (α1PI)[107] (systematic name: *SERPINA1*). This is achieved by mutating the residues homologous with those on the D-helix of HCII responsible for GAG-binding[152,282].

MATERIALS AND METHODS

Mutagenesis of α1PI$_{Pittsburgh}$

cDNA of α1PI$_{Pittsburgh}$ containing an N-terminal hexahistidine tag for purification, 7 stabilizing mutations (F51L, T59A, T68A, A70G, M374I, S381A, K387R)[283] and an additional mutation (C232S) to prevent dimerization[284] was generously donated by the

Gettins laboratory (Department of Biochemistry and Molecular Genetics, University of Chicago, IL). Primary sequences of the D-helix from $\alpha1PI_{Pittsburgh}$ and HCII were aligned[264] and confirmed three-dimensionally using PyMOL version 1.0 (PDB# 1HP7[285]) and 1JMO[152]). $\alpha1PI_{Pittsburgh}$ residues aligning with the five basic residues in the HCII D-helix were chosen for mutation to the homologous HCII residue (Figure 3.1). The following mutations: E97K/Q98R, Q105R/P106R, E97K/Q98R/Q105R/P106R, and E97K/Q98R/T102R/Q105R/P106R were introduced in the double-stranded plasmid $\alpha1PI_{Pittsburgh}$-pQE30 following the QuikChange site-directed mutagenesis (Strategene) protocol, using these primers:

GTACTTAAGGCCTTTGAGGAGGT (forward, E97K/Q98R)

TGGAGGAGTTTCCGGAAGCCTTCATG (reverse, E97K/Q98R)

GTATGGGAGTTGGCTCTGTCGGTC (forward, Q105R/P106R onto the E97K/Q98R mutant),

CTGGCTGTCTCGCCGGTTGAGGGTATG (reverse, Q105R/P106R onto the E97K/Q98R mutant),

GAGGAGGTATCCGAGTTGGCCGAG (forward, E97K/Q98R/T102R/Q105R/P106R onto the E97K/Q98R/Q105R/P106R mutant),

GAGCCGGTTGAGCCTATGGAGGAG (reverse, E97K/Q98R/T102R/Q105R/P106R onto the E97K/Q98R/Q105R/P106R).

The mutated cDNA with 5 amino acid substitutions (E97K/Q98R/T102R/Q105R/P106R), henceforth refered to as $\alpha1PI_{Pitt\text{-}GAG}$, in the pQE30 vector was transformed into *Escherichia coli* (*E. coli)* XL1-Blue supercompetent cells (Strategene) for cDNA amplification and extracted using MiniPrep Wizard (Qiagen). Sequencing of clones by the UNC-CH Genome Analysis Facility confirmed incorporation of the mutations into the $\alpha1PI_{Pittsburgh}$-pQE30 construct.

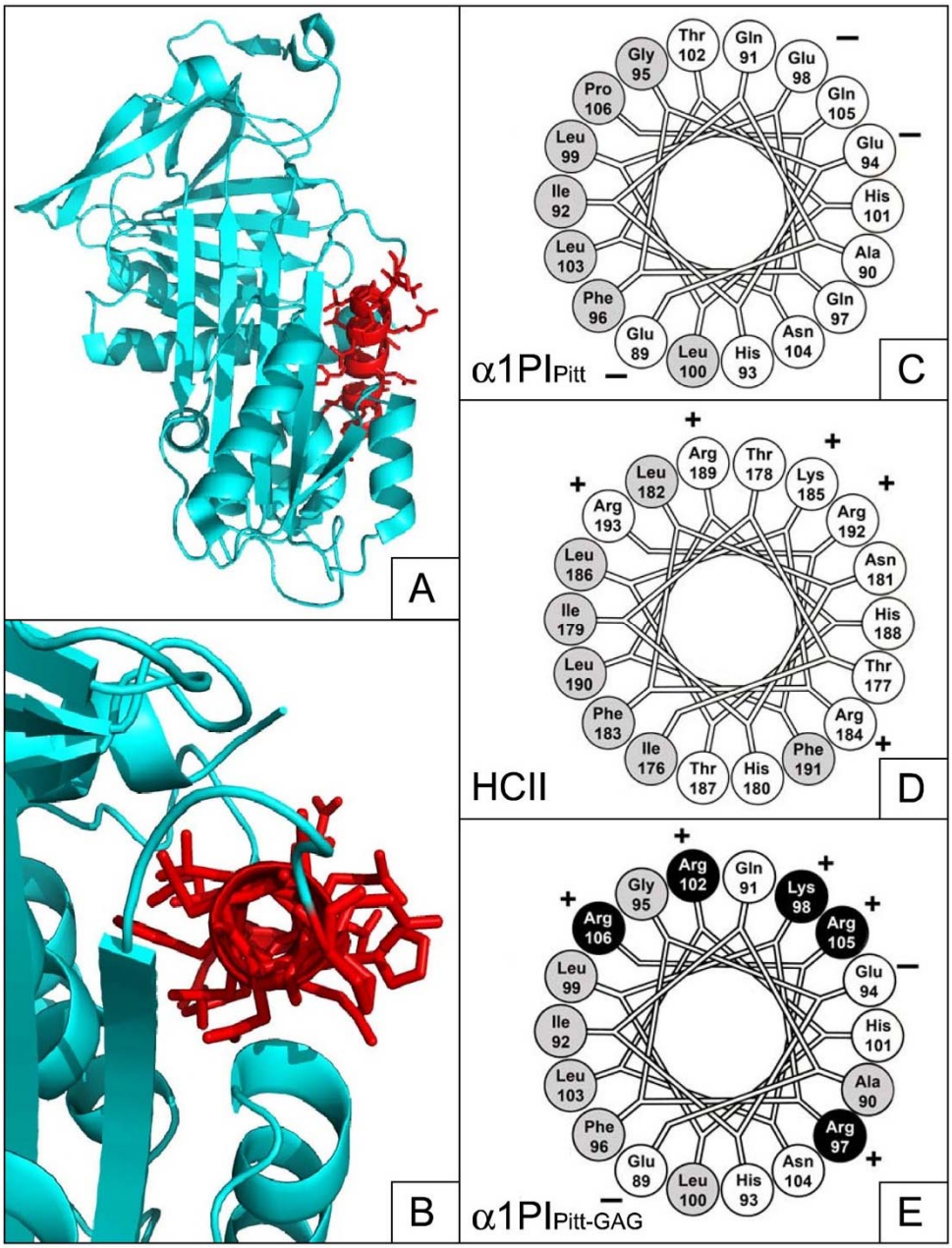


Figure 3.1. *α1PI ribbon structures with highlighted D-helix and helical wheel representations of α1PI, HCII and α1PI$_{Pitt\text{-}GAG}$.* Panel A shows α1PI ribbon structure (PDB #1OPH[286]) in its classical view with D-helix highlighted. Panel B shows α1PI (PDB# 1HP7[285]) rotated 110 degrees around the x axis and zoomed in on the D-helix so that the view is through the bottom of the D-helix displaying the side-chains of the D-helix residues as they are seen in the helical wheel representation in Panel C. Hydrophobic residues are indicated by grey coloring Panels C, D and E. Positively and negatively charged residues are shown with plus and minus signs respectively. Panel D illustrates the helical wheel representation of the HCII D-helix, while Panel E shows the helical wheel representation of α1PI$_{Pitt\text{-}GAG}$ D-helix.

Protein Expression and Purification

$\alpha 1PI_{Pittsburgh}$ and $\alpha 1PI_{Pitt\text{-}GAG}$ proteins were expressed and purified with minor modifications as previously reported[284]. Briefly, cDNA in the pQE30 vector was transformed into competent SG13009 *Escherichia coli* (*E. coli*) (Qiagen) and grown inLuria-Bertani media containing 100 μg/mL ampicillin and 25 μg/mL kanamycin to an absorbance at 600 nm of 0.6. Protein expression was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (Promega) at 27° C for approximately 6 hours. Cell lysate in 20 mM sodium phosphate, 500 mM NaCl pH 7.4 containing 20 mM imidazole was loaded onto the Sepharose immobilized nickel affinity column, HisTrap HP (Amersham Biosciences). The serpin was eluted using the same buffer containing 500 mM imidazole. EDTA was added to the elution to reach a final concentration of 10 mM. The protein was then dialyzed overnight against 20 mM Tris, 50 mM NaCl pH 8.0 for future kinetic studies or 20 mM Hepes, 0.1 % PEG pH 7.4 for affinity chromatography studies. Final concentrations were determined by absorbance at 280 nm (A_{280}) based upon M_r = 45,100 and using an extinction coefficient of 0.43 M^{-1} cm^{-1}[283].

Non-Denaturing Gel Electrophoresis

Seven μg each of $\alpha 1PI_{Pittsburgh}$ and $\alpha 1PI_{Pitt\text{-}GAG}$ were electrophoresed through native gels containing 7.5% polyacrylamide, 375 mM Tris pH 8.0, in 25 mM Tris, 191 mM glycine running buffer. The gel was then stained with Coomassie Brilliant Blue R-250 (GibcoBRL) and destained until sufficient background was removed to visualize bands.

Heparin-Sepharose Affinity Chromatography

The relative affinity of $\alpha 1PI_{Pitt\text{-}GAG}$, $\alpha 1PI_{Pittsburgh}$, and human, plasma-derived HCII for heparin was determined using a FPLC apparatus. Protein was injected onto a 1 mL HiTrap heparin-Sepharose column (Amersham Biosciences) and eluted using NaCl gradient from zero to 500 mM in Hepes buffer pH 7.4. A_{280} was measured for each 1 mL fraction of $\alpha 1PI_{Pitt\text{-}GAG}$ and $\alpha 1PI_{Pittsburgh}$. The peak elution ionic strength was determined by plotting A_{280} versus the fraction number. Alternatively, peak elution ionic strength for HCII as determined by dermatan sulfate activity in each 1 mL fraction (method previously reported[287]).

Inhibition of Proteases in the Presence and Absence of Heparin or Dermatan Sulfate

Second order rates of inhibition were determined by measuring chromogenic development of para-nitroanaline substrate cleavage in a 96-well plate by absorbance at 405nm using a Thermomax microplate reader from Molecular Devices for three hours in the presence of protease, serpin and GAG. Proteases used included α- and γ-thrombin (Haematologic Technologies), R101A-thrombin [(chymotrypsinogen numbering system)[288] also designated R98A (thrombin numbering system)[289] --a gift from the Leung lab at Stanford University School of Medicine], and activated protein C (APC) (Haematologic Technologies) and trypsin (Sigma) in concentrations of 200 pM, 1 nM, 400 pM, 2 nM and 1 nM respectively. $\alpha 1PI_{Pittsburgh}$ or $\alpha 1PI_{Pitt\text{-}GAG}$ was employed in concentrations of 50 nM for thrombins and 100 nM for other proteases. HCII was used at a final concentration of 5 nM. In HCII assays using low concentrations of heparin (67 nM and 667 nM) or dermatan sulfate

(20 nM and 200 nM), only 100 pM thrombin was used. Chromogenic substrates (all purchase from Centerchem) included PefachromeTH (150 μM) for thrombins, PefachromePCA (300 μM) for APC and Tos-Gly-Gly-Pro-Arg-pNA-AcOH substrate (300 mM) for trypsin. Finally, GAG concentrations varied from 66 nM to 133 μM for unfractionated heparin (M_r = 15,000, Diosynth) and from 20 nM to 40 μM for dermatan sulfate (M_r = 50,000, Calbiochem). Solutions were made in buffer containing 20 mM Hepes, 150 mM NaCl, 0.1% PEG 8000, 0.05% NaN_3 and 0.1% ovalbumin. Calcium chloride (2.5 mM) was included in APC assays. (Note, concentrations over 5 μM GAG were not tolerated by the APC assay; the curve of the data was altered and awkward to interpret.)

For each heparin or dermatan sulfate concentration, complete thrombin inhibition was identified by a change of < 0.01 absorbance units over 5 minutes. Each data set was truncated at this point and resultant data was fit to the equation: $abs = ((V_\infty * t)+((V_0 - V_\infty)*(1-\exp(-k_{obs}*t)))/ k_{obs})+A_0$ where *abs* is the absorbance at 405 nm, t is time in seconds, V_0 is the initial velocity of substrate cleavage and V_∞ is the final (infinite) rate of substrate cleavage and k_{obs} is the observed rate of complex formation[110]. Second order rates of inhibition, k_2, were calculated using the equation: $k_2 = k_{obs}/ [\text{serpin}] * (1+ K_m/[\text{substrate}])$[290] where K_m is equal to the half-maximal rate of substrate cleavage by protease. K_m values for all chromogenic substrate-protease pairs were determined in our laboratory using the same buffers used for inhibition assays. Each condition was performed in duplicate in each experiment and each experiment was performed three times. Second order rates of inhibition were plotted versus heparin or dermatan sulfate concentration. The rate of thrombin inhibition by HCII in the absence of GAG is too slow to be determined by this assay. It was determined by a previously described method[291].

Stoichiometry of Inhibition of Protease by Serpin in the Presence and Absence of Heparin or Dermatan Sulfate

The stoichiometry of inhibition (SI) value for each protease-serpin pair in the presence and absence of heparin (6.7 μM), and for each thrombin-derivative-serpin pair in the presence of dermatan sulfate (20 μM), was determined by incubating 1 nM protease (α-thrombin, γ-thrombin, R101A-thrombin, trypsin or APC) with varying concentrations of $\alpha 1PI_{Pittsburgh}$ or $\alpha 1PI_{Pitt\text{-}GAG}$ from 0.25 – 10 nM at room temperature overnight in 96 well plates and utilizing the same buffer as used for kinetic assays. Chromogenic substrate (150 μM PefachromeTH for thrombins or 100 μM PefachromePCA for APC and trypsin) was then added to the solutions. Residual protease activity was measured on the Thermomax by absorbance at 405 nm. Residual protease activity for each concentration of serpin was divided by the activity in a control well containing no serpin and plotted versus serpin concentration (data not shown). A straight line was fit through points at concentrations still possessing protease activity. The value for the stoichiometry of inhibition was considered to be the x value of this line when y = 0.

GAG-Binding Affinity Determined by Extrinsic Fluorescence

Affinity of $\alpha 1PI_{Pittsburgh}$, $\alpha 1PI_{Pitt\text{-}GAG,}$ or HCII for either heparin or dermatan sulfate were investigated as previously reported for HCII and AT[100,292]. Briefly, fluorescence emission spectra (400 – 500 nm) resulting from the excitation of 5 μM toluidino-2-naphthalenesulfonic acid (TNS) at 330 nm in buffer containing 50 mM Tris, 1% PEG 8000, 50 mM NaCl, pH 7.4 with the addition of 250 nM serpin were measured, at each titration of heparin, dermatan sulfate or buffer using a Perkins Elmer Instruments LS55 luminescence

spectrometer. Excitation slit width and emission slit width were set to 10 nm and 15 nm respectively. The fluorescence spectrum of TNS alone was subtracted from all TNS-serpin and TNS-serpin-GAG spectra. TNS-serpin fluorescence for each GAG titration was divided by TNS-serpin fluorescence for each buffer titration to control for dilution effect and machine drift. Dissociations constant (K_d) was determined by plotting the average percentage change in fluorescence at 435 nm versus GAG concentration and curve-fit using SlideWrite Plus version 6.0 software to the equation: F/F_0= *j*-((F_{max}*([P_0]+[GAG]+ K_d -(([P_0]+[GAG]+ K_d)^2-4*[P_0]*X)^0.5))/(2*[P_0])). F/F_0 is the ratio of fluorescence emission upon titration to initial fluorescence emission of TNS-serpin in the absence of GAG. [P_0] is the initial concentration of serpin, set at 250 nM, and [GAG] is the heparin or dermatan sulfate concentration. The variable *j* is included to accommodate minor deviations of F/F_0 in the absence of GAG that were introduced by mathematical controls and instrument variability. The value for *j* never deviated more than 0.04 from 1. F_{max} and K_d (calculated by curve fit) represent maximal fluorescent change at saturation and heparin dissociation constant, respectively. Each assay was performed at least twice in duplicate.

RESULTS

Alignment of α1PI and HCII D-helices

Overlay of the crystal structures of α1PI (PDB#1OPH[286]) (Figure 3.1A) and HCII (PDB# 1JMO[152]) showed structural alignment of the D-helices from α1PI residue Glu 89 to Pro 106 with HCII residues Ile 176 to Arg 193. Rotating α1PI backwards ~110 around the x-axis (Figure 3.1B) showed the approximate alignment of the residues in the D-helix as they are represented in helical wheel projections (Figure 3.1C). Comparison of the HCII and

α1PI D-helices shows a conserved hydrophobicity along the inside of the D-helix and a distinct divergence of charge on the outer portion of the D-helix (Figure 3.1C & ID). Residues on α1PI at the same position as the basic residues on HCII were mutated to become homologous with HCII. The resultant D-helix structural alignment is shown in Figure 3.1E. This molecule, $\alpha1PI_{Pitt\text{-}GAG}$ has a net charge difference of + 6 as compared to $\alpha1PI_{Pittsburgh}$.

Expression and Purity of $\alpha1PI_{Pittsburgh}$ and $\alpha1PI_{Pitt\text{-}GAG}$

Expression levels of $\alpha1PI_{Pittsburgh}$ were not altered by mutations in $\alpha1PI_{Pitt\text{-}GAG}$ and as expected both proteins ran as single bands with molecular weights of ~45kD on Coomassie Brilliant Blue R-250 stained SDS-PAGE (data not shown). In order to illustrate the difference in charge, these proteins were subjected to non-denaturing gel electrophoresis. Figure 3.2 (inset) shows that $\alpha1PI_{Pittsburgh}$ and $\alpha1PI_{Pitt\text{-}GAG}$ each ran as a single band and that $\alpha1PI_{Pitt\text{-}GAG}$ migrated farther toward the cationic pole than $\alpha1PI_{Pitt\text{-}GAG}$ indicating a lesser net positive charge $\alpha1PI_{Pittsburgh}$ and $\alpha1PI_{Pitt\text{-}GAG}$.

Heparin Binding Characterization

In order to evaluate heparin binding capacity, the sodium chloride concentration required to elute $\alpha1PI_{Pittsburgh}$ and $\alpha1PI_{Pitt\text{-}GAG}$ from heparin-Sepharose was determined. Peak $\alpha1PI_{Pittsburgh}$ concentration was eluted ~200 mM NaCl, while peak $\alpha1PI_{Pitt\text{-}GAG}$ was eluted in ~300 mM NaCl (Figure 3.2). Under the same conditions, plasma HCII was eluted in ~450 mM NaCl. These results indicate that $\alpha1PI_{Pitt\text{-}GAG}$ has gained increased heparin binding capacity over $\alpha1PI_{Pittsburgh}$, but has not achieved the heparin binding capacity of HCII.

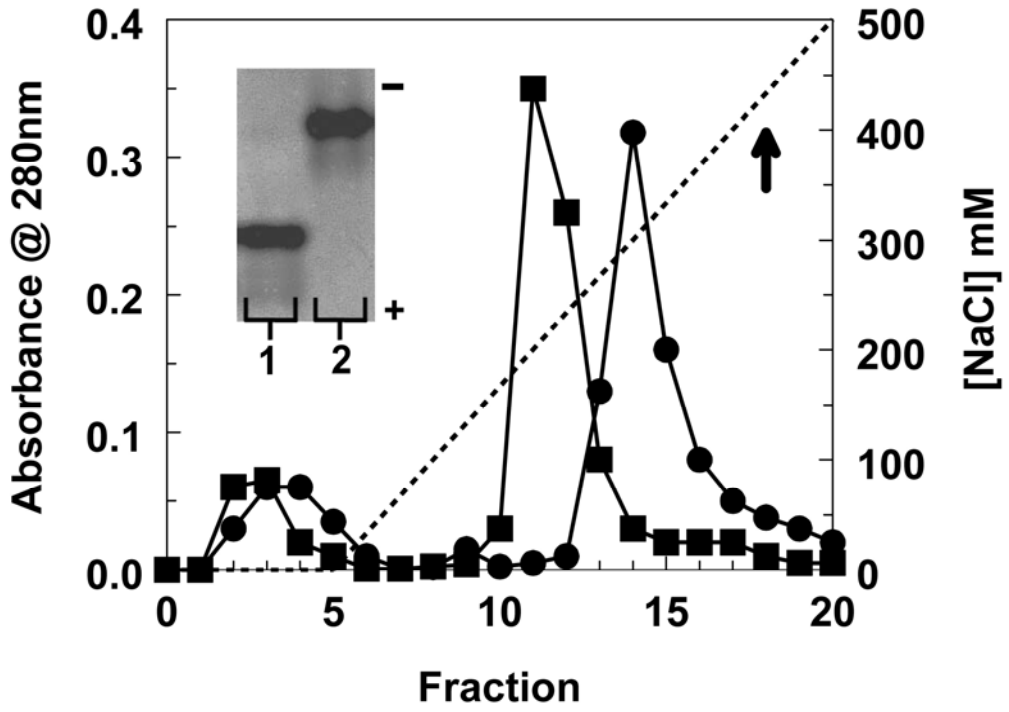


Figure 3.2. *Gel and column chromatography of α1PI$_{Pitt}$ and α1PI$_{Pitt\text{-}GAG}$.* Squares and circles show the elution profiles measured by absorbance at 280 nm of α1PI$_{Pittsburgh}$ and α1PI$_{Pitt\text{-}GAG}$ respectively from a HiTrap heparin-Sepharose column by NaCl gradient (dotted line and right axis). Arrow indicates the peak elution of HCII under similar conditions. Inset depicts the electrophoretic migration of purified α1PI$_{Pittsburgh}$ (Lane 1) and α1PI$_{Pitt\text{-}GAG}$ (Lane 2) through a non-denaturing polyacrylamide gel.

Second Order Rates of Inhibition (k_2) in the Presence and Absence of Heparin and Dermatan Sulfate

Because α1PI$_{Pittsburgh}$ readily inhibits thrombin, trypsin and APC, we calculated the k_2's of these proteases by both α1PI$_{Pittsburgh}$ and α1PI$_{Pitt\text{-}GAG}$ in the absence of GAGs in order to determine if the mutation affected the serpin's activities. No substantial difference in rates was seen between α1PI$_{Pitt\text{-}GAG}$ and α1PI$_{Pittsburgh}$ for any of these protease pairs (Table 3.1). Upon discovering GAG accelerated thrombin inhibition (see below) we also measured the rates of inhibition of two additional thrombin derivatives to examine thrombin-exosite interactions. Certain structural components of thrombin are important for serpin activity and

GAG-binding, including its active site and two electropositive areas, exosite-1 and exosite-2[293]. Exosite-1 binds the N-terminal acidic domain of HCII and exosite-2 binds heparin and other GAGs[294-296]. Derivatives of thrombin deficient in exosite activity such as γ-thrombin, a proteolytically modified thrombin with reduced exosite-1 activity[95,297] and a mutant thrombin (R101A) with reduced exosite-2 activity[289,298,299], have been used to explore both serpin and GAG interactions. Similarily, results showed little change for these thrombins between $\alpha1PI_{Pitt\text{-}GAG}$ and $\alpha1PI_{Pittsburgh}$ in the absence of GAGs (Table 3.1).

Table 3.1. *Second order rates of inhibition (k_2) of protease by $\alpha1PI_{Pittsburgh}$ or $\alpha1PI_{Pitt\text{-}GAG}$ in the absence of GAGs.* Average values are shown ± the standard error based on at least three independent assays performed in duplicate.

$k_2 \times 10^5\ M^{-1}s^{-1}$	**α-thrombin**	**γ-thrombin**	**R101A-thrombin**	**APC**	**trypsin**
$\alpha1PI_{Pittsburgh}$	0.99 ± 0.17	1.18 ± 0.21	1.13 ± 0.03	0.19 ± 0.011	0.25 ± 0.009
$\alpha1PI_{Pitt\text{-}GAG}$	1.25 ± 0.14	1.28 ± 0.13	1.41 ± 0.14	0.20 ± 0.003	0.24 ± 0.008

Next, we measured the k_2's in the presence of varying concentrations of heparin and dermatan sulfate to evaluate the ability of GAGs to alter rates of inhibition for the serpin-protease pairs. As expected, the inhibition of α-thrombin by HCII in the presence of GAGs (Figure 3.3A) was accelerated approximately 6,700 fold by 5 μM heparin and 7,500 fold by 10 μM dermatan sulfate with diminishing rates at higher concentrations. The inhibitory activity of $\alpha1PI_{Pitt\text{-}GAG}$ was accelerated by both heparin (Figure 3.3B) and dermatan sulfate (Figure 3.3C) for all thrombin derivatives. In the presence of heparin, the rate enhancement of α-thrombin was the greatest and also took the least amount of heparin to achieve (Table

3.2). This was followed by γ-thrombin, then R101A-thrombin both of which had increasingly reduced rates and took increasingly more heparin to achieve maximal inhibition. At high levels of heparin, a decrease in the rate of the inhibition of all the thrombin derivatives was observed. Although the fold-stimulation was reduced for dermatan sulfate as compared to heparin (Table 3.2), the data consistently showed a reliable pattern of acceleration (Figure 3.3C). Maximal inhibition of α-thrombin was achieved at 20 μM dermatan sulfate and a subsequent decrease in rate was observed at increasing concentrations. Maximal inhibition of γ-thrombin and R101A-thrombin were observed at the highest concentrations of dermatan sulfate (40 μM) utilized in these experiments.

Table 3.2. *Second order rates of inhibition (k_2) $\alpha 1PI_{Pitt\text{-}GAG}$ inhibition of thrombin derivatives in the prescence or absence of heparin or dermatan sulfate, and the fold acceleration at [peak concentration of GAG].* Average values are shown ± the standard error based on at least three independent assays performed in duplicate.

k_2 x 10^5 $M^{-1}s^{-1}$	**- GAG**	**+ heparin**	**Fold acceleration [heparin]**	**+ dermatan sulfate**	**Fold acceleration [dermatan sulfate]**
α-thrombin	1.25 ± 0.14	19.8 ± 4.15	15.8 [3.33 μM]	2.11 ± 0.23	1.69 [20.0 μM]
γ-thrombin	1.28 ± 0.13	13.8 ± 1.57	10.8 [5.00 μM]	1.83 ± 0.03	1.43 [40.0 μM]
R101A-thrombin	1.41 ± 0.14	4.05 ± 0.31	2.87 [6.7 μM]	1.97 ± 0.01	1.40 [40.0 μM]

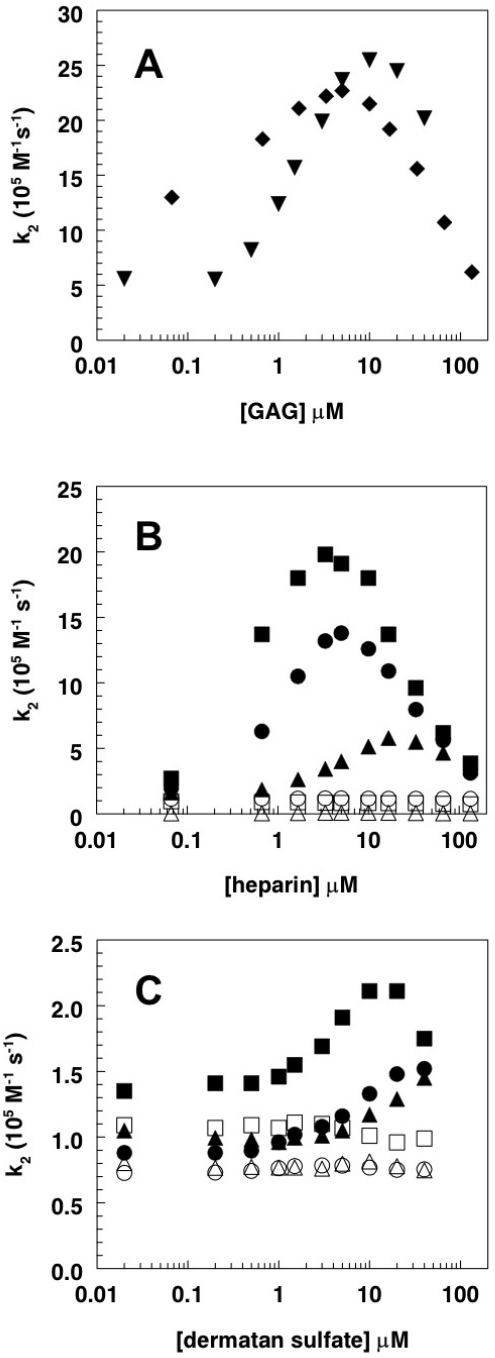


Figure 3.3. *Second order rates of inhibition (k_2) $\alpha 1PI_{Pitt}$, $\alpha 1PI_{Pitt\text{-}GAG}$ or HCII in the presence and absence of heparin or dermatan sulfate.* Panel A depicts the change second order rates of inhibition of α-thrombin by HCII in the presence of heparin (diamonds) or dermatan sulfate (inverted triangles). Panels B and C illustrates the change in the second order rates of inhibition of α-thrombin (squares), γ-thrombin (circles) or R101A-thrombin (triangles) by $\alpha 1PI_{Pitt,}$ (open symbols) and $\alpha 1PI_{Pitt\text{-}GAG}$ (filled symbols) in the presence of increasing concentrations of heparin (Panel A) or dermatan sulfate (Panel B).

No change was observed in the inhibitory activity of thrombins by $\alpha 1PI_{Pittsburgh}$ in the presence of either heparin (Figure 3.3B) or dermatan sulfate (Figure 3.3C). Likewise, neither inhibition of APC nor trypsin by $\alpha 1PI_{Pitt\text{-}GAG}$ showed an enhancement in rate in the presence of heparin. Rather, for all protease-serpin pairs there appeared to be a slight but steady decrease in the rates with increasing amount of heparin (data not shown).

Collectively, these results confirm that not only did the D-helix mutations confer GAG binding to both heparin and dermatan sulfate, they also conferred an HCII-like, GAG-accelerated pattern of thrombin inhibition that approached maximal HCII rates. Furthermore, they indicate that reduction in exosite-2 activity in thrombin (the heparin binding region) has a profound effect on heparin-accelerated activity indicating the need for an intact exosite-2, or bridging of heparin to thrombin, for the majority of the acceleration seen here.

Stoichiometry of Inhibition (SI) in the Presence and Absence of Heparin and Dermatan Sulfate

Stoichiometries of inhibition of proteases by $\alpha 1PI_{Pittsburgh}$ or $\alpha 1PI_{Pitt\text{-}GAG}$ in the presence and absence of GAGs were measured to ensure that the D-helix mutations did not alter the substrate characteristics of $\alpha 1PI_{Pittsburgh}$ for the proteases nor did GAGs binding to $\alpha 1PI_{Pitt\text{-}GAG}$ effect the substrate capacity of the serpin. Results shown in Table 3 indicate that there no substantial difference between the stoichiometry of inhibition for most of the protein pairs, nor do GAGs affect this relationship. The one exception is that the SI of R101A-thrombin is somewhat lower for $\alpha 1PI_{Pitt\text{-}GAG}$ than for $\alpha 1PI_{Pittsburgh}$ both with and without heparin. However, since the drop in SI is very similar both in the absence and presence of heparin (1.53 and 1.78, respectively), it cannot account for the acceleration of R101A-inhibition seen at peak heparin concentrations. Overall, these results indicate that mutating

the D-helix of $\alpha 1PI_{Pittsburgh}$ does not increase or decrease its propensity to act as a substrate for these specific proteases, and that minor variations in SI values are not responsible for the increased rates of thrombin inhibition observed in the presence of GAGs.

Table 3.3. *Stoichiometry of inhibition values for $\alpha 1PI_{Pittsburgh}$ or $\alpha 1PI_{Pit\text{-}GAG}$ and protease in the presence or absence of heparin or dermatan sulfate.* Average values are shown ± the standard error based on at least three independent assays performed in duplicate. *(N.D. = not determined).*

	NO GAG		**heparin**		**dermatan sulfate**	
	$\alpha 1PI_{Pittsburgh}$	**$\alpha 1PI_{Pitt\text{-}GAG}$**	**$\alpha 1PI_{Pittsburgh}$**	**$\alpha 1PI_{Pitt\text{-}GAG}$**	**$\alpha 1PI_{Pittsburgh}$**	**$\alpha 1PI_{Pitt\text{-}GAG}$**
α-thrombin	1.53 ± 0.07	1.74 ± 0.11	1.79 ± 0.06	2.11 ± 0.08	2.72 ± 0.11	2.61 ± 0.17
γ-thrombin	2.58 ± 0.35	2.03 ± 0.21	2.48 ± 0.24	1.86 ± 0.06	0.88 ± 0.02	0.84 ± 0.04
R101A-thrombin	2.03 ± 0.08	1.33 ± 0.19	2.13 ± 0.07	1.20 ± 0.16	1.71 ± 0.13	1.73 ± 0.07
APC	1.61 ± 0.16	1.55 ± 0.25	1.70 ± 0.19	1.63 ± 0.29	N. D.	N. D.
trypsin	1.41± 0.14	1.46± 0.06	1.32± 0.19	1.50± 0.14	N. D.	N. D.

GAG-Binding Affinity Determined by Extrinsic Fluorescence

Finally, we used TNS extrinsic fluorescence to examine the binding affinity of the $\alpha 1PI_{Pitt\text{-}GAG}$ mutant for heparin and dermatan sulfate and to investigate their potential allosteric effects on the serpins. TNS has been used to measure the GAG-binding affinity of serpin-GAG in instances where the introduction of GAG causes a conformational change in the serpin, consequently altering the TNS environment and thus its fluorescence spectrum[100,292]. HCII-heparin is one such serpin-GAG pair[100]. In the current study, the addition of HCII to TNS alone caused an increase in peak fluorescence emission from ~200 to ~600 units and induced a blue shift from a peak of ~450 nm to ~440 nm. The addition of

either $\alpha 1PI_{Pittsburgh}$ or $\alpha 1PI_{Pitt\text{-}GAG}$ to TNS produced very similar emission spectra with an increase to ~300 fluorescence units and a mild blue shift to ~450 nm. The titration of heparin to HCII-TNS caused a 48% reduction in fluorescence signal and red shift of ~5 nM, while the titration of dermatan sulfate to HCII-TNS resulted in a 13% drop in fluorescence signal and with no shift in the peak emission wavelength. The titration of heparin to $\alpha 1PI_{Pitt\text{-}GAG}$ also caused a 13% quench in fluorescence signal without a shift in peak emission wavelength. Titration of dermatan sulfate into $\alpha 1PI_{Pitt\text{-}GAG}$ and titrations of either heparin or dermatan sulfate into $\alpha 1PI_{Pittsburgh}$ failed to produce a systematic change in TNS-serpin spectra (data not shown).

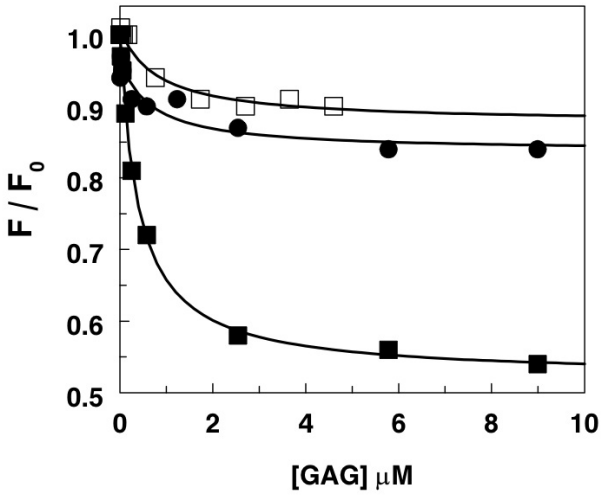


Figure 3.4. *Change in TNS fluorescence emission at 435 nm.* Data points depict HCII (squares) or $\alpha 1PI_{Pitt\text{-}GAG}$ (circles) with titration of heparin (filled symbols) or dermatan sulfate (open symbols). Lines are fit to the equation $F / F_0 = (j - (F_{max} * ([P_0] + [GAG] + K_d - (([P_0] + [GAG] + K_d)^2 - 4 * [P_0] * [GAG])^{0.5})) / (2 * [P_0])$, where $[P_0] = 250$ nM and *j* is a variable for the accommodation of $F/F_0 \neq 1$ due to instrument and human variability.

The half-maximal binding equilibrium constant or dissociation constant, K_d, for HCII and heparin was measured to be 397 nM ($r^2 = 0.99$, $F_{max} = 0.48$) (Figure 3.4). This is consistent with previously reported K_d measured by other methods[300], and while they cannot

be directly compared with data from O'Keefe et al.[100] due to different ionicity reported for HCII and unfractionated heparin in that study, they are consistent with their data ranges and patterns. Similarily, titration of dermatan sulfate into TNS-HCII resulted in a K_d, 814 nM (r^2 = 0.97, F_{max} = 0.13) (Figure 3.4), consistent with that determined by other methods[300], but which has not been previously reported using this method. Under these same conditions the K_d for $\alpha 1PI_{Pitt\text{-}GAG}$ with heparin was calculated to be 645 nM (r^2 = 0.87, F_{max} = 0.13) (Figure 3.4). These results might be interpreted to conclude that heparin is able to induce a conformational change in $\alpha 1PI_{Pitt\text{-}GAG}$ and that $\alpha 1PI_{Pitt\text{-}GAG}$ binds heparin with slightly less affinity than HCII binds heparin but with better affinity than HCII binds dermatan sulfate. However, we should note that the increase in fluorescence emission of TNS-$\alpha 1PI_{Pitt\text{-}GAG}$ over TNS alone is small. Therefore, once background TNS is subtracted from the $\alpha 1PI_{Pitt\text{-}GAG}$ fluorescence spectra, the signal-to-noise ratio is increased. So while a consistent systematic quenching of fluorescence signal was observed, the noise limited the accuracy of the observed curve-fit.

DISCUSSION

This study illustrates three purposes for using gain-of-function manipulations in serpin study: 1) to determine whether specific functions can be transferred with their purported associated structures; 2) to isolate and examine the relationship between a structure and its activity; 3) to develop promising new proteins for therapeutic purposes. We endeavored to engineer this novel serpin primarily for the first two reasons: to determine whether HCII-like GAG-binding can be conferred to a non-GAG-binding serpin and explore

what this tells us about GAG-binding in HCII and other serpins, with the understanding that the manipulation of GAG-binding capacity could have therapeutic applications.

The engineering of $\alpha 1PI_{Pitt\text{-}GAG}$ achieved successful transfer of heparin and dermatan sulfate binding and associated functional characteristics, specifically, the acceleration of thrombin inhibition and allows for the examination of HCII GAG-binding outside the context of the unique HCII structure. This gain-of-function approach is rarely employed in the study of serpins outside the mutation of RCL residues to alter protease specificity. Exceptions in the literature include several studies with the three primary goals described above. Grasberger et al.[281] were the first to pioneer the concept of transferring foreign functionality to one serpin by replacing entire structural domains with those from a different serpin. These investigators substituted the thyroxine binding domain of the serpin, thyroxine binding globulin onto $\alpha 1PI$ to produce a chimeric serpin that gained thryoxine binding capacity, but did not lose its primary neutrophil elastase inhibitory activity. The primary purpose of this experiment was to examine the modularity of serpins and determine if bifunctional serpins could be created from structurally homologous but functionally diverse family members. McCarthy et al.[301] created chimeric proteins in order to investigate the functionality of a specific structural region in serpins. These investigators transferred increasing percentages of the C-terminus of the inhibitory serpin, plasminogen activator inhibitior-2 (PAI-2) to the non-inhibitory serpin, ovalbumin. Inhibitory activity observed in a chimera with 64% PAI-2, but was not seen in the chimera with only 35%. They were thus able to pinpoint the structural differences between these chimeras to identify the critical region for inhibition. Finally, Sutherland et al.[302] added the N-terminal extension including the acidic domain of HCII onto $\alpha 1PI_{Pittsburgh}$. They were able to investigate the role of the N-terminus outside the

context of the HCII molecule. Additionally, the appendage bestowed $\alpha 1PI_{Pittsburgh}$ the ability to interact with thrombin exosite-1, thus increasing its inhibition of thrombin, but having little effect on its inhibition of APC. The overall result was the creation of a chimeric serpin with an increased ratio of thrombin to APC inhibition, which is beneficial in the development of potential therapeutic anticoagulants.

The results from our study further confirm the notion that the homology of serpins affords not only the exchange of structural components between serpins, but also their associated activity. Specifically, it demonstrates that with the substitution of only 5 amino acids, GAG-binding can be conferred to a non-GAG binding serpin without diminishing the inhibitory activity of the parent protein. $\alpha 1PI_{Pitt\text{-}GAG}$ bound heparin-Sepharose with greater affinity than $\alpha 1PI_{Pittsburgh}$ and eluted in a salt concentration greater than physiologic strength. Additionally, heparin and dermatan sulfate were both able to accelerate $\alpha 1PI_{Pitt\text{-}GAG}$ inhibition of thrombin indicating that activity was conferred along with the addition of the basic charges in the D-helix. The increase in thrombin inhibition by $\alpha 1PI_{Pitt\text{-}GAG}$ in the presence of dermatan sulfate is also evidence of binding and hence, indicates HCII-specific qualities in $\alpha 1PI_{Pitt\text{-}GAG.}$

HCII shows up to 20,000 fold stimulation of thrombin inhibition in the presence of heparin and dermatan sulfate, while $\alpha 1PI_{Pitt\text{-}GAG}$ demonstrated only a 15 fold increase with heparin and approximately two fold increase in the presence of dermatan sulfate. The unique N-terminal extension of HCII is responsible for the majority of its GAG-accelerated activity. GAG-binding works through an allosteric mechanism whereby a global conformational change is induced, extending the RCL while releasing the N-terminal acidic domain to interact with thrombin exosite-1. Mutants of HCII with the N-terminal extension

truncated[287,297,303] are more akin to the $\alpha 1PI_{Pitt\text{-}GAG}$ molecule created here. These mutants show 5-40 fold stimulation of thrombin inhibition in the presence of heparin and 0-3 fold acceleration in the presence of dermatan sulfate, ranges very like to those found in this study. Similarly, when exosite-1 is eliminated in thrombin (e.g., γ-thrombin), so that HCII cannot utilize exosite-1 binding, the remaining GAG-accelerated inhibition of the exosite-1-deficient thrombin is along the first order of magnitude; 3-10 fold acceleration is seen with heparin[95,101,303,304] and ~10 fold acceleration is seen with dermatan sulfate[101].

Although $\alpha 1PI_{Pitt\text{-}GAG}$ has no HCII- like N-terminal extension to interact with thrombin exosite-1, we still see a mild drop in acceleration in the presence of heparin (15 fold to 10 fold) and dermatan sulfate (1.7 fold to 1.4 fold) from α-thrombin to γ-thrombin inhibition, but with no difference between the inhibition of the two thrombins in the absence of GAGs. While this might be interpreted as exosite-1 involvement in GAG-binding, it is much more likely that there may be a small amount of non-specific GAG-binding to thrombin in effect for the GAG-acceleration seen in these assays or that the ablation of exosite-1 has an indirect effect on exosite-2[305,306] binding and reflects thrombin-GAG binding effects rather than serpin-thrombin interactions. This is supported by the fact that a drop in acceleration with γ-thrombin as compared to α-thrombin inhibition in the presence of heparin is also seen with antithrombin[304] even though the crystal structure of the ternary complex (AT-heparin-thrombin) shows no exosite-1 involvement[118,134].

The GAG-accelerated HCII activity reported both with N-terminal truncated HCII and γ-thrombin is generally attributed to the bridging mechanism, from the D-helix region to the protease. Mutations in thrombin exosite-2, thrombin's heparin and dermatan sulfate binding region[298], shows a small reduction in heparin accelerated activity by HCII (<10

fold)[298,299,303]. Results with dermatan sulfate varied, showing no change between HCII peak rates of inhibition of exosite-2-diminished thrombin and α-thrombin[298] using a quadruple mutation (R89E, R245E, K248E, K252E – thrombin numbering system) in exosite-2, or a ~2 fold reduction using a single point mutation (R101A)[299]. In this study, we observe a 7 fold reduction in heparin accelerated inhibition of R101A-thrombin and α-thrombin by $\alpha 1PI_{Pitt-GAG}$, but no change in the acceleration in the presence of dermatan sulfate. Together, the results indicate that the majority (~7 fold) of heparin acceleration of thrombin inhibition by $\alpha 1PI_{Pitt-GAG}$ is due to bridging between the positively charged D-helix and thrombin's exosite-2. The remaining 2 fold stimulation seen in $\alpha 1PI_{Pitt-GAG}$ in the presence of heparin and dermatan sulfate, cannot be accounted for by bridging or, not surprisingly, by exosite-1 interactions. These results mimic the GAG-acceleration of HCII's thrombin inhibition when exosite-1 interactions have been eliminated.

The logical explanation for the above results is that both heparin and dermatan sulfate induce a conformational change in $\alpha 1PI_{Pitt-GAG}$ that increases its thrombin inhibition approximately two-fold. This is supported by the evidence that heparin titration results in a reduction of TNS-$\alpha 1PI_{Pitt-GAG}$ fluorescence. This cannot be accounted for by TNS competing with heparin for the positively charged D-helix as both $\alpha 1PI_{Pitt-GAG}$ and $\alpha 1PI_{Pittsburgh}$ showed the same increase and shift in peak fluorescence emission when added to TNS. However, only a quenching was observed with TNS-$\alpha 1PI_{Pitt-GAG}$. Titration with dermatan sulfate did not exhibit this same quenching. However, while HCII-TNS showed a dramatic reduction in fluorescence emission with heparin titration, the same degree quenching was not observed with dermatan sulfate titration. This is not to say that HCII does not undergo conformational change in the presence of dermatan sulfate, indeed it does. It does demonstrate that the

changes in the TNS-HCII environment brought about by dermatan sulfate are different than those engendered by heparin. If dermatan sulfate has a similarly reduced effect on $\alpha 1PI_{Pitt-GAG}$-TNS, given the maximal fluorescence change was already small, it may not be detected in this assay.

The GAG-induced conformational change that we have reasoned to account for a portion of the acceleration of thrombin inhibition by $\alpha 1PI_{Pitt-GAG}$ is perplexing. Both HCII and AT exhibit structural modifications upon GAG-binding; their RCLs are expelled from insertion into β-sheet A which exposes the previously protected P1 arginine in AT[267,307] and simultaneously liberates the N-terminal acidic domain in HCII[152,303]. Because the RCL of α1PI is not inserted into the β-sheet A, nor does it have an N-terminal extension[285], neither can describe the conformational change presumably occurring when GAGs bind the D-helix of $\alpha 1PI_{Pitt-GAG.}$ GAG-binding also generates new exosites on AT for factors IXa and Xa interaction[96]. The formation of novel exosites on $\alpha 1PI_{Pitt-GAG}$ for thrombin interface is one explanation which could account for both the TNS-fluorescence quenching and accelerated thrombin inhibition upon GAG-binding. However, the complex and exquisite nature of serpin-protease interactions intimates a protracted evolutional development of their complementary structures. It is quite improbable that generation of an exosite for thrombin would be a consequence of GAG binding to $\alpha 1PI_{Pitt-GAG}$. Thrombin is not a physiological target-protease for α1PI, α1PI does not bind GAGs and $\alpha 1PI_{Pittsburgh}$ is a rare variant with severe health consequences, thus, there is no evolutionary pressure for the development of GAG-induced thrombin exosites.

One plausible GAG-induced conformational change which could be occurring in $\alpha 1PI_{Pitt-GAG}$ is the elongation of the D-helix. Antithrombin exhibits elongation of its D-helix

upon GAG binding that contributes to the expulsion of the RCL and translates into increased inhibitory activity[307-309]. It is undetermined whether this occurs in HCII or other GAG-binding serpins. Thus we can only speculate that D-helix elongation could be the structural change that results in quenching of the TNS-α1PI$_{Pitt\text{-}GAG}$ fluorescent signal. Even so, it is unclear how this might result in increased thrombin inhibition by α1PI$_{Pitt\text{-}GAG}$.

Although we are unable to provide a cogent description of the conformational change that may take place in α1PI$_{Pitt\text{-}GAG}$ when it binds heparin or dermatan sulfate, we are able to assert that any transformation that occurs can be attributed to binding at the D-helix. What is particularly interesting about this is that α1PI$_{Pitt\text{-}GAG}$, whose parent protein α1PI does not bind GAGs, harbors the ability to change its conformation when GAGs interact with its D-helix. This speaks to the conservation of structure in serpins and the centrality of the D-helix region to GAG-acceleration of thrombin inhibitory serpins. Virtually all of the naturally occurring human serpins that inhibit thrombin utilize the D-helix for GAG-binding. We hypothesize that the conserved structural environment of serpins' D-helices fetters their conformational rearrangement and that engagement of the D-helix region with GAGs eases the constraint. If you will, the D-helix region acts as a lock on the serpin, GAGs—the key(s). Binding of a GAG unlocks the serpin, permitting or promoting the other structural changes to take place, such as release of the N-terminus, expulsion of the RCL, generation of new exosites, some undetermined structural rearrangement or perhaps just facilitated insertion of the RCL into β-sheet A. Using further manipulations and this same gain-of-function strategy could prove useful in investigating the D-helix region as potential serpin lock.

While developing a therapeutic anticoagulant was not the goal of this study, the addition of GAG-binding to α1PI$_{Pittsburgh}$ has potential usefulness for the treatment of

thrombosis. The chimeric serpin created by Sutherland et al.[302], which appended the N-terminus including the acidic domain of HCII onto $\alpha 1PI_{Pittsburgh}$ showed 21 fold augmentation of thrombin inhibition with little effect on APC inhibition or stoichiometries of inhibition. The authors point out the benefit that such an increase in thrombin: APC inhibitory ratio could have if pursued as an anticoagulant. In the presence of GAGs, $\alpha 1PI_{Pitt\text{-}GAG}$ achieved similar enhancement in thrombin inhibition (up to 15 fold) while no changes were observed in APC inhibition or stoichiometries of inhibition. As $\alpha 1PI_{Pitt\text{-}GAG}$ is able to bind both heparin and dermatan sulfate, manipulation of such a GAG-binding mutant has the added potential to be targeted to specific locations in the intra- and extra- vasculature where heparan sulfate and dermatan sulfate are found. Finally, GAG binding is also an important element in the function of an array of proteins in addition to serpins including many growth factors (e.g., acidic and basic fibroblast growth factors, insulin-like growths factor and insulin-like growth factor binding proteins, EGF-like growth factors) and the heparin binding proteins that affect DNA synthesis. Therefore, the ability to engineer GAG-binding and confer a functional benefit may be a novel and useful tool for the development of chimeric proteins to combat not just thrombosis, but numerous other pathologies.

ACKNOWLEDGEMENTS

We are grateful to the following individuals for their contributions to this manuscript. Steve Olson, Ph.D. (University of Illinois at Chicago) provided indispensable guidance for the optimization and analysis of the extrinsic-fluorescence assay. James Huntington, Ph.D. and Denis O'Keeffe, M.D. (Cambridge Institute for Medical Research) provided individual training in the protocol of the extrinsic-fluorescence assay. John Sondek, Ph.D. (University

of North Carolina at Chapel Hill) kindly granted the use of the Perkins Elmer Instruments LS55 luminescence spectrometer. Lawrence Leung, M.D. (Stanford University) graciously provided R101A thrombin. Peter Gettins, Ph.D. (University of Illinois at Chicago) generously donated $\alpha 1PI_{Pittsburgh}$ cDNA along with expression and purification protocols.

CHAPTER 4

Engineering of the thrombin-inhibiting chimeric serpin, HATpin

SUMMARY

In addition to its central role in thrombosis, thrombin also has pro-inflammatory functions that contribute to cardiovascular disease (CVD). Interestingly, thrombin can also act in anti-thrombotic and anti-inflammatory capacities by activating protein C. Serine protease inhibitors (serpins) are a family of proteins with high structural homology, such that it is possible to transfer individual domains between serpins and confer related activity. Heparin cofactor II (HCII) and antithrombin (AT) are serpins with unique structures regulating thrombin inhibition. The goal of this project was to engineer a chimeric serpin, HATpin, by transferring HCII and AT structural elements to α1-protease inhibitor-M358R ($\alpha 1PI_{Pittsburgh}$) that specifically inhibits the coagulant and atherogenic functions of thrombin without altering its Protein C activating function with purpose of creating a novel preventative therapy for CVD.

The N-terminal region of HCII (comprising its unique acidic domain through its glycosaminoglycan-binding domain) and the reactive center loop (RCL) of AT were substituted onto $\alpha 1PI_{Pittsburgh}$. The presence of specific structural components was confirmed by immunoblot. RCL integrity was determined by visualization of HATpin-thrombin complex. HATpin was observed to have heparin binding capacity greater than $\alpha 1PI_{Pittsburgh}$

but less than HCII as measured by elution from a heparin column. Inhibition of thrombin and activated protein C (APC) and factor Xa and their stoichiometries of inhibition by HATpin were measured using chromogenic assays in the presence and absence of glycosaminoglycans. Results indicate that despite having all of the elements that would predict the creation of a superior thrombin inhibitor for anticoagulation, HATpin fell short of this goal. Potential explanations for these unexpected results are provided.

INTRODUCTION

Cardiovascular Disease and Its Pharmaceutical Treatment

Cardiovascular disease (CVD) is responsible for the death and debilitation of millions of people each year and billions of dollars in healthcare costs[1]. Therefore it is imperative that we continue to develop new and better preventative pharmaceuticals to diminish its devastating effects.

Strategies for treatment and prevention of CVD are targeted at its underlying pathophysiologic mechanisms. Two of the primary pathological processes causing CVD are atherosclerosis and thrombosis[310]. Atherosclerosis is a chronic inflammatory process marked by a focal thickening of the arterial intima[14]. Stenosis of coronary and carotid or cerebral blood vessels from this vascular pathology can result in acute coronary syndrome or ischemic stroke. However, more often atherosclerosis leads to these conditions from rupture of the lesion which triggers the coagulation cascade leading to occlusive thrombi or emboli[310]. Additionally, imbalance of the elements of coagulation and fibrinolysis can lead to venous thrombosis and subsequent emboli with severe and sometimes fatal consequences. Thus, targeting atherosclerosis and thrombosis are amongst the strategies for prevention of CVD.

Preventative treatment guidelines for CVD are derived from the evidence of reduced risk in controlled trials. Recommendations are based on individual patient's risk factors which include both non-modifiable risk factors such as age, race, gender, and previous cardiovascular events and modifiable risk factors including atherosclerotic disease, atrial fibrillation, hypertension, etc. Antithrombotic therapies comprise anti-platelet and anti-coagulant treatments with different risk factors dictating the recommended use of either or both. Anti-coagulant treatment is recommended in patients with atrial fibrillation, acute myocardial ischemia, left ventricular thrombus, extracranial arterial dissection, cerebral venous sinus thrombosis, deep vein thrombosis, pulmonary embolism and certain hypercoagulable states[4,311-314]. Anticoagulants specifically target elements of the coagulation cascade with the ultimate goal of attenuating the action of thrombin.

Thrombin

Thrombin is a multifunctional serine protease. It acts as a pro-coagulant by cleaving fibrinogen to fibrin and activating factor XIII, which crosslinks fibrin to form a clot. Thrombin also activates factors V and VIII upstream in the coagulation cascade, thus perpetuating its own activation. (See Chapter 1, Figure 1.1.) Thrombin is a potent platelet agonist through its cleavage and activation of protease-activated receptors (PARs)[44,57]. Conversely, thrombin possesses some anti-coagulant functionality since it feeds into an auto-regulatory loop, activating zymogen protein C to activated protein C (APC) (see below). Finally, thrombin has many non-hemostatic pro-inflammatory functions, which include cytokine-like activities that contribute to the pathological processes of atherosclerosis[42].

Therapeutically, thrombin attenuation can be accomplished in three ways: by reducing the amount of active thrombin generated in the system, by directly inhibiting thrombin's proteolytic activity, or by enhancing the activity of inhibitors in any part of the cascade. Accordingly, there are currently three types of anticoagulation medications available: vitamin K antagonist (warfarin); glycosaminoglycans (unfractionated heparin (UFH), low molecular weight heparin (LMWH) and the heparin pentasaccharide, Fondaparinux); and direct thrombin inhibitors (recombinant hirudins, bivalirudin, argatroban, ximelagatran and dabigatran). Vitamin K antagonists inhibit coagulation by preventing the synthesis of the vitamin K-dependent coagulation factors VII, IX, X, prothrombin, protein S, and protein C. The glycosaminoglycans act as cofactors to accelerate antithrombin's (AT) inhibition of coagulation. UFH and LMWH accelerate the inhibition of both thrombin and factor Xa (an upstream coagulation factor) by AT. LMWH has considerably more anti-factor-Xa than anti-thrombin activity, and Fondaparinux catlayzes only AT- factor Xa. Finally, direct thrombin inhibitors inactivate thrombin by binding tightly to the protein structures in thrombin responsible for cleaving fibrinogen to fibrin[310].

Activated Protein C

While thrombin is crucial in coagulation, activated protein C (APC) another serine protease, is also imperative for proper hemostasis[315-317]. In the presence of thrombomodulin (TM), a receptor found on endothelial cells, thrombin activates protein C[59]. APC in turn, when bound to its cofactor protein S, inactivates factors Va and VIIIa upstream from thrombin in the coagulation cascade, thus regulating the activation of thrombin in an auto-regulatory feedback loop. APC also acts to reduce endothelial inflammation[70]. It has been

proposed that through such mechanisms APC protects against ischemic stroke[182]. Recombinant APC is used to treat severe sepsis and has been shown to be effective in reducing mortalities[318]. Currently, the use of recombinant APC as an adjunct to thrombolytic therapies is being investigated[319,320]. Because of the protective effects of APC, an ideal anticoagulant would inhibit thrombin from cleaving fibrinogen and activating platelets, but would not inhibit it from activating protein C. Nor would it inhibit APC itself. Of the current anti-coagulant therapies, only LMWHs avoid inhibiting the generation of APC.

Thrombin Structures

Therapeutic strategies for inhibition of thrombin, either directly or through enhancement of endogenous inhibitors requires an understanding of the structure and kinetic mechanisms of all of the molecules involved. The ability of thrombin to specifically recognize substrates is dependent not only its active site, but also two other regions on the molecule. Thrombin utilizes two electropositive areas located at opposite poles of the molecule. These anion-binding regions, known as exosite 1 and exosite 2, mediate thrombin's interactions with many cofactors and substrates[52]. Exosite 1 is the region responsible for the binding of fibrinogen, fibrin[321], thrombomodulin, hirudin's acidic C-terminal tail, and the acidic domain of HCII[293,299,322] Exosite 2 binds heparin and other glycosaminoglycans (GAGs) to mediate reactions with GAG-binding inhibitors[52,296,299]. Many ligands bind thrombin in these regions and their spatiotemporal availability and relative affinities define the local thrombin function and its inhibitors.

Serine Protease Inhibitors

As serine proteases, thrombin and APC are inhibited by serine protease inhibitors (serpins). Serpins are a superfamily of proteins with high structural homology. They contain 9 α-helices, 3 β-sheets and a reactive center loop (RCL)[51]. Unique sequences in the RCL define target protease activity while variations in the other structural domains confer cofactor activity. Amongst others, three serpins, antithrombin (AT), heparin cofactor II (HCII) and α1-protease inhibtor (α1PI) inhibit proteases in the coagulation cascade[304,323]. Understanding how the unique structures of these serpins determine their specific function can help to create a novel anti-coagulant therapy.

Antithrombin

Antithrombin is the serpin most responsible for regulation of the coagulation cascade. The GAGs heparin or heparan sulfate are necessary to accelerate AT activity to physiologically relevant rates. GAG binding results in accelerated thrombin inhibition by a bridging mechanism from AT to thrombin exosite 2 and by causing an allosteric conformation change in AT that exposes its reactive center loop (RCL) for enhanced inhibition of factor Xa[133,324,325]. The primary structure of the RCL of antithrombin is such that it is simultaneously a potent thrombin and factor Xa inhibitor and poor APC inhibitor[109,326]. However, because AT-heparin utilizes exosite 2 on thrombin, which is available when thrombin is bound to TM, AT (in the presence of heparin) inhibits thrombin bound to TM (thrombin-TM). This subsequently reduces the activation of APC. Furthermore, when thrombin is released rapidly from a clot through lytic therapy, it is bound

to fibrin at exosite, but still enzymatically active. Because AT requires heparin binding through exosite 2 on thrombin for inhibition, AT cannot inhibit clot-bound thrombin[127].

Heparin Cofactor II

While HCII is known to inhibit thrombin *in vitro* and has been found in complex with thrombin *in vivo*[237], it does not appear to play a significant role in normophysiologic coagulation[153]. However, HCII is capable of inhibiting clot-bound thrombin[327-329]. HCII has a unique N-terminus which is able to bind thrombin's exosite 1 when HCII is bound to GAGs; the GAG-bridging mechanism is not necessary for thrombin inhibition[101]. Therefore, this region of HCII may be important for limiting reocclusion after lytic therapy.

Elevated HCII levels have been shown to be associated with decreased atherosclerosis and restenosis, while reduced levels have been associated with increased cardiac events[50,154,156,247]. Although the inhibition of thrombin by HCII can be accelerated by many GAGs, dermatan sulfate exclusively accelerates the inhibition of thrombin by HCII. Dermatan sulfate, is abundant in vessel walls[330], is increased in atherosclerotic tissue and has been shown to regulate the antithrombotic activity of HCII in mice after vascular injury[46]. Thus, it is the current scientific premise that HCII's action against thrombin in atherogenesis is due to its unique ability to bind dermatan sulfate which targets it to the vascular intima.

The GAG-associated acceleration of thrombin inhibition by HCII has been attributed to the binding of positively charged residues on and around the D-helix of HCII and to subsequent transposition of its N-terminal acidic domain[275-279]. GAG binding accelerates thrombin inhibition primarily by an allosteric/conformation change mechanism whereby the acid region of HCII binds thrombin exosite 1. There is also a small additional acceleration

that can be accounted for by GAGs bridging. Because HCII and TM both utilize exosite 1 for binding to thrombin, and TM has a much higher binding affinity, HCII cannot inhibit thrombin-TM and thus has no effect on the APC system.

α1-Protease Inhibitor

α1PI is considered the protoypical serpin[107,331]. It is able to inhibit APC but is an extremely poor thrombin inhibitor. A naturally occurring mutation in the RCL of α1PI from a methionine to an arginine (M358R) ($\alpha1PI_{Pittsburgh}$) increases its affinity for both thrombin and APC[184]. The inhibitory effects of $\alpha1PI_{Pittsburgh}$ were so much enhanced that the variant resulted in the hemorrhagic death of the person in whom it was discovered[183]. Neither α1PI nor $\alpha1PI_{Pittsburgh}$ show any cofactor associated acceleration of thrombin inhibition.

Serpin Structural Homology for Protein Engineering

The significant homology of serpin structure makes it possible to substitute structural elements of one serpin for another[332] and transfer the specific activity associated with the structural domain[302,326]. In this study, we designed a chimeric serpin with the goal of harnessing the beneficial properties of $\alpha1PI_{Pittsburgh}$, AT and HCII. The chimera was designed to have the following biochemical properties: 1) to possess the potent thrombin inhibition of $\alpha1PI_{Pittsburgh}$; 2) to have the specificity of AT for thrombin and factor Xa over APC; 3) to have the N-terminal region of HCII which prevents it from inhibiting APC generation (thrombin-TM) and permits it to inhibit clot-bound thrombin; and 4) to posses the dermatan sulfate binding capability of HCII.

HATpin Design

Specifically, we used $\alpha 1PI_{Pittsburgh}$ as the scaffolding on which to build our novel thrombin-inhibitory serpin. α1PI is often used as the structural framework for serpin engineering in order to learn about structure-function relationships[302,326,333]. The acidic domain (without the D-helix, GAG binding region) of HCII has been added to $\alpha 1PI_{Pittsburgh}$[302] and increased thrombin inhibition. Additionally, as seen in Chapter 3, addition of several basic residues to the D-helix resulted in HCII-like GAG binding of $\alpha 1PI_{Pittsburgh.}$ Upon the $\alpha 1PI_{Pittsburgh}$ backbone, we chose to replace the N-terminus of $\alpha 1PI_{Pittsburgh}$ for the N-terminus of HCII, from the acidic domain through the D-helix, in order to incorporate the GAG-binding properties of HCII. A component of the design of this chimeric serpin is to utilize endogenous GAGs to target the chimera's activity to sites of atherosclerosis and diseased vascular endothelium predisposed to atherosclerosis and venous thrombosis. This region of HCII incorporates all of the residues that have been shown to be crucial for dermatan sulfate and heparin binding as well as the N-terminal extension which binds exosite 1 of thrombin. This N-terminus of HCII was included to increase the thrombin specificity, facilitate the inhibition of clot-bound thrombin and decrease the inhibition of thrombin-TM. Next, the RCL, from P7-P3′, was substituted with that of AT. These AT residues were included because they have been shown to be those responsible for AT's specificity of thrombin over APC and promote the inhibition of factor Xa[109,326,334]. Through this understanding of the specific activities of these serpin's structures, we purposefully designed the chimeric serpin, HATpin (Heparin cofactor II, AntiThrombin, α1-Protease INhibitor), to possess the anti-atherogenic and anti-reocclusion properties of HCII and the anti-coagulant properties of

antithrombin without the drawback of inhibiting the antithrombotic, and anti-inflammatory and neuroprotective effects of APC.

MATERIALS AND METHODS

Design of HATpin

Figure 4.1 depicts a schematic of the critical features of the HATpin's parental molecules that were incorporated into its structure.

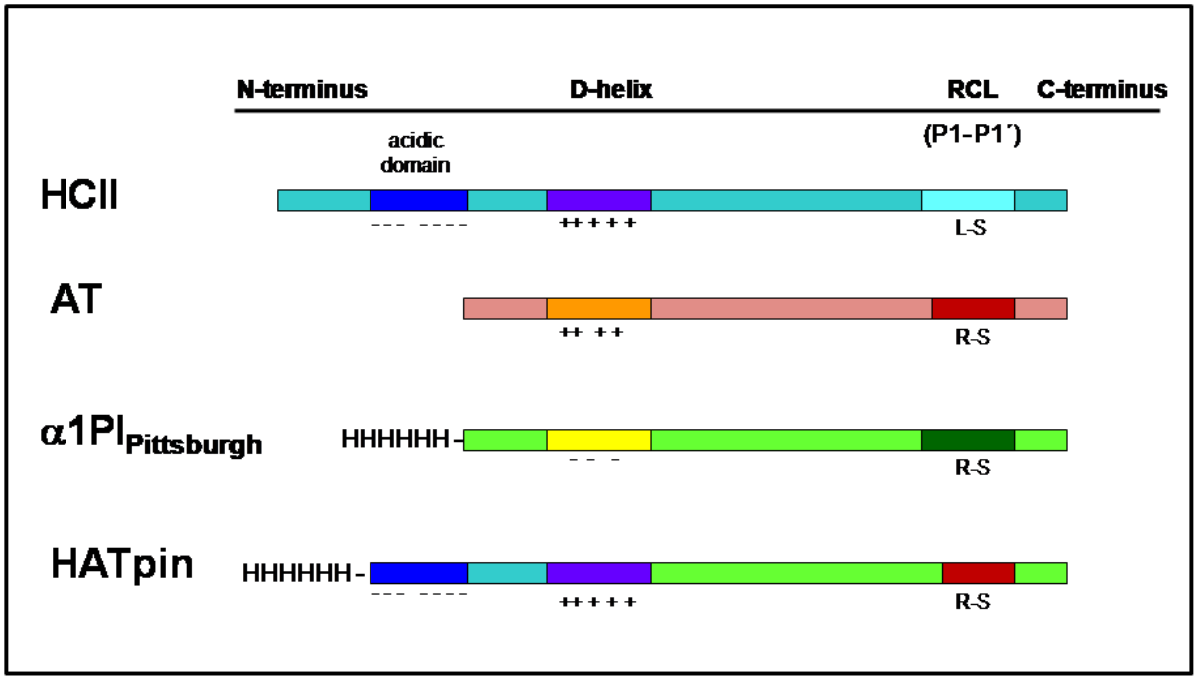


Figure 4.1 *Linear Schematic indicating critical features in wildtype HCII, AT and $\alpha 1PI_{Pittsburgh}$ for incorporation into the chimeric serpin, HATpin.*

Substitution of HCII cDNA Coding Sequence from its N-Terminal Acidic Domain Region through its D-Helix for the Coding Sequence Of $\alpha 1PI_{Pittsburgh}$

Residues 193 and 106 represent the 3´ end of the D-helix of HCII and $\alpha 1PI_{Pittsburgh}$, respectively. This was confirmed by structural alignment using Pymol Molecular

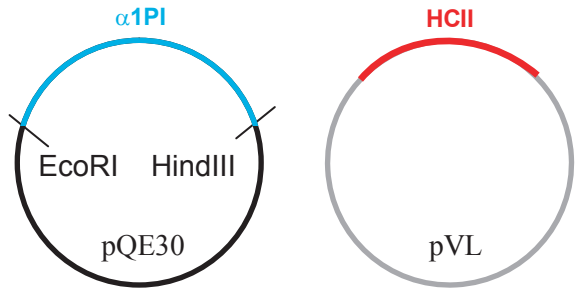
Visualization System and published crystal structures of HCII[152] and α1PI[335]. HCII residues 1-51 have been shown to be inconsequential to its ability to inhibit thrombin in the presence or absence of GAGs[101,287]. Furthermore, expression and purification of HCII from *Escherichia coli* (*E. coli*) is notoriously finicky. Therefore, to reduce extraneous HCII sequence, we substituted only from the beginning of the acidic domain of HCII (residue 52) through the end of its D-helix (residue 193) for the N-terminus of $\alpha1PI_{Pittsburgh}$ (residue 4) through the end of its D-helix (residue 106). $\alpha1PI_{Pittsburgh}$ cDNA containing an N-terminal hexahistidine tag for purification, 7 stabilizing mutations (F51L, T59A, T68A, A70G, M374I, S381A, K387R)[283] and a cysteine to serine (C232S) mutation to prevent dimerization[284] in the pQE30 vector (Strategene) was generously donated by the Gettins laboratory (Department of Biochemistry and Molecular Genetics, University of Chicago, IL). These mutations affect the heat stability of the $\alpha1PI_{Pittsburgh}$ protein without altering its inhibitory properties[283]. HCII cDNA in the pVL1392 vector (BD Biosciences Pharmingen) was a gift from the Tollefsen Laboratory (Division of Hematology, Department of Medicine, Washington University School of Medicine, St. Louis, MO).

Chimeric cDNA was created using the oligonucliotide primers purchased from Integrated DNA Technologies, denoted in Table 4.1, and a series of polymerase chain reactions (PCR) outlined in Figures 4.2a, 4.2b and 4.2c and described below. PCR comprised thirty cycles each of 30 seconds at 95° C, followed by 30 seconds at 55° C and 2 minutes at 72° C. All reactions utilized Pfu Turbo DNA polymerase (Stratagene) in 1 x Cloned Pfu DNA polymerase reaction buffer (Stratagene), 200μM dNTP mix (Invitrogen), 50nM each of forward and reverse primer, and 1 μL of a 1 to 10 dilution of purified plasmid cDNA or purified DNA product.

Table 4.1. *Oligonucleotide primers used in PCR reactions for engineering the chimeric serpin, HATpin.*

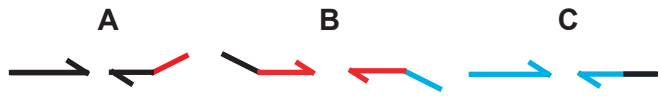
Primer Name	Primer Sequence	Direction	Coverage
Forward A	GTG AGC GGA TAA CAA TTA TAA TAG ATT C	5' - 3'	**pQE30 vector**
Reverse A	CCT CTG GAA TGG ATC CGT GAT GGT GAT GGT GAT GCG	3' - 5'	**HCII / pQE30 vector**
Forward B	CAC GGA TCC ATT CCA GAG GGC GAG GAG	5' - 3'	**pQE30 vector / HCII**
Reverse B	CAG CTG GAG CTG GCT GTC CCT CCT GAA GAG GCG ATG	3' - 5'	**α1PI / HCII**
Forward C	GAC AGC CAG CTC CAG CTG ACC ACC GGC	5' - 3'	**α1PI**
Reverse C	CCA AGC TCA GCT AAT TAA GC	3' - 5'	**pQE30 vector**
AT-RCL-Fwd	GAA GCT GCT GGG GCC ATG GCT GTA GTG ATC GCA GGC AGA TCT CTC AAC CCC GAG GTC AAG TTC	5' - 3'	**α1PI + AT RCL mutations**
AT-RCL-Rvs	GAA CTT GAC CTC GGG GTT GAG AGA TCT GCC TGC GAT CAC TAC AGC CAT GGC CCC AGC AGC TTC	3' - 5'	**α1PI + AT RCL mutations**

Step 1. Obtain plasmids with HCII and α1PI cDNA

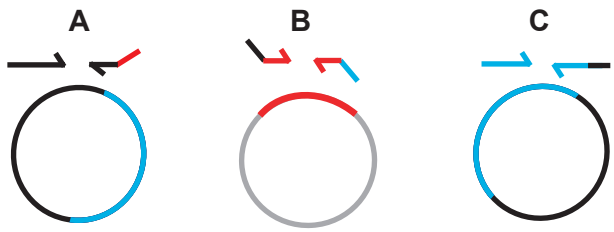


Step 2. Design three sets of primers.

Forward A: pQE30 vector
Reverse A: HCII / pQE30 vector
Forward B: pQE30 vector / HCII
Reverse B: α1PI / HCII
Forward C: α1PI
Reverse C: vector / α1PI

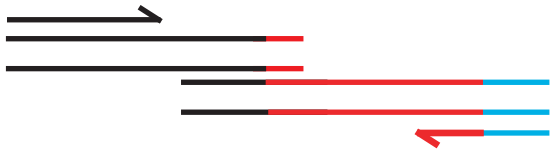


Step 3. PCR primers with α1PI or HCII cDNA.



Step 4. Purify PCR products: A, B and C.

Step 5: Join products A and B using forward primer A and reverse primer B.



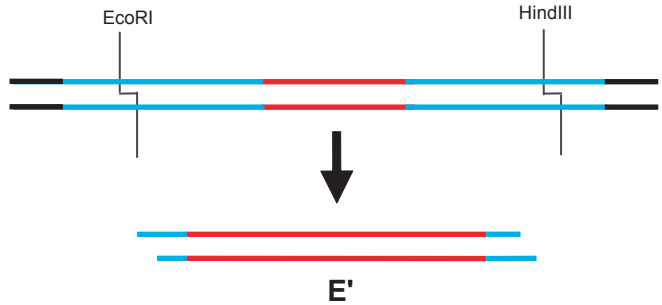
Step 6. Purify PCR product, D.

Step 7: Join products C and D using forward primer A and reverse primer C.

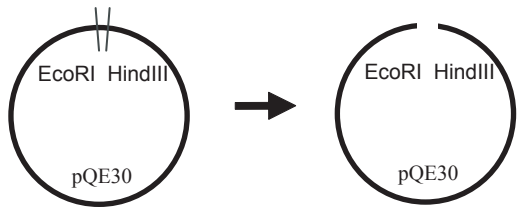
Figure 4.2a. *Protocol for Domain Engineering Using Polymerase Chain Reaction. Steps 1 - 7.*

Step 8. Purify PCR product, E.

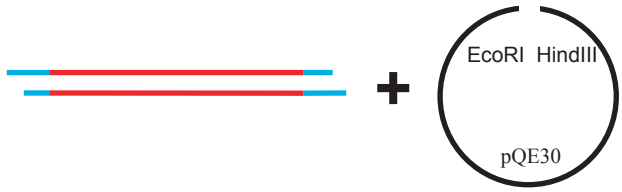
Step 9. Digest PCR product E and pQE30 vector with EcoRI and HindIII restriction enzymes.



Step 10. Purify digestions.



Step 11. Ligate PCR product and pQE30 vector.



Steps 12 +: Transform plasmid into supercompetent *E. coli*. Amplify plasmid and purify. Confirm coding sequence integrity using UNC Genome Analysis Facility.

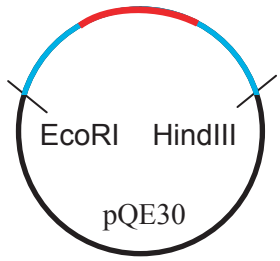
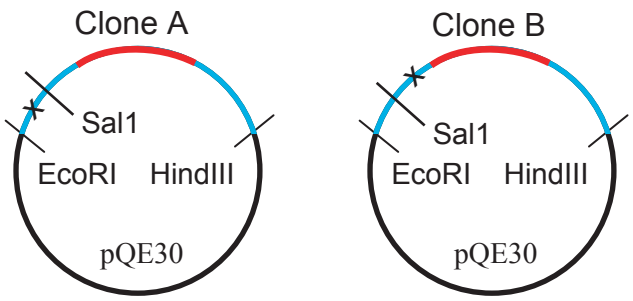


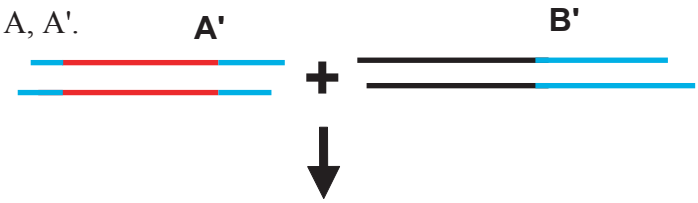
Figure 4.2b. *Protocol for Domain Engineering Using Polymerase Chain Reaction. Steps 8 - 12.*

Step 13. Find two clones with mutations on opposite sides of Sal1 restriction site.

Step 14. Digest Clone A and B with Sal1 and HindIII.

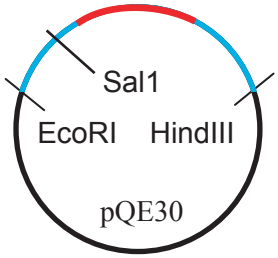


Step 15. Purify smaller band of Clone A, A'. Purify larger band of Clone B, B'.



Step 16. Ligate A' and B'.

Step 17. Transform plasmid into supercompetent *E. coli*. Amplify plasmid and purify. Confirm coding sequence integrity using UNC Genome Facility.



Step 18. Mutate reactive site loop residues of α1PIPittsburgh to **AT** homologous residues using complimentary forward and reverse primers with modifications in the α1PIPittsburgh sequence to result in coding sequence for AT RCL. The reactive center is boxed in red.

```
344  T  E  A  A  G  A  M  F  L  E  A  I  P  R  S  I  P  P  E  V  K  F  N   367
     ACTGAAGCTGCTGGGGCCATGTTTTTAGAGGCCATACCCAGATCTATCCCCCCCGAGGTCAAGTTCAAC

344  T  E  A  A  G  A  M  A  V  V  I  A  G  R  S  L  N  P  E  V  K  F  N   367
     ACTGAAGCTGCTGGGGCCATGGCTGTAGTGATCGCAGGCAGATCTCTCAACCCCGAGGTCAAGTTCAAC
```

Steps 19 + . Digest parental DNA. Transform plasmid into supercompetent *E. coli*. Amplify plasmid and purify. Confirm coding sequence

Figure 4.2c. *Protocol for Domain Engineering Using Polymerase Chain Reaction. Steps 13 - 19.*

Initially, three separate double stranded DNA products (A, B and C) were created using PCR. The resultant PCR products (A, B and C) were subjected to electrophoresis through a 1.5% agarose gel. Desired bands were evaluated by molecular weight and were purified using QIAquick PCR Purification Kit (Qiagen). Product A contained vector cDNA which included an ECORI restriction site, the signal peptide sequence, the polyhistidine tag and part of the coding region for the desired HCII amino acid sequence. Product B contained the entire HCII coding sequence for HCII residues 52 - 193, flanked 5' by a portion of pQE30 vector sequence that overlapped with Product A, and 3' by coding sequence for $\alpha 1PI_{Pittsburgh}$ that overlapped with that of Product C (below). Product C comprised cDNA from $\alpha 1PI_{Pittsburgh}$ coding for sequence just 3' of its D-helix through its C-terminus and some of the pQE30 vector that followed (including the HINDIII restriction enzyme cleavage site). Purified Products A and B were joined by PCR using primers Forward A, Reverse B and purified Products A and B. Product AB resulted. This was then purified and joined with Product C using primers Forward A and Reverse C. After purification, this product (D) was digested with restriction endonucleases EcoRI (New England BioLabs) and HINDIII (New England BioLabs). An empty pQE30 vector was separately digested with the same enzymes simultaneously. The digested product and vector were each purified and then ligated together using T4 DNA ligase (New England BioLabs) at 37° C for 1 hour. Calf intestinal alkaline phosphatase (CIAP) (Invitrogen) was included in both the digestions and the ligation to prevent circularization of the cDNA. The ligation mixture was transformed into XL1-Blue supercompetent *E.coli* (Strategene) for plasmid amplification. The cDNA from thirteen individual *E. coli* colonies was extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega), and cDNA sequences were determined by the UNC Genome

Analysis Facility. Each of these colonies exhibited at least one point-mutation in its cDNA sequence. Therefore, we devised the following strategy to circumvent the errors arising from the PCR.

The cDNA of the desired chimeric serpin, HATpin, was determined to have a SAL1 restriction endonuclease cleavage site located within the $\alpha 1PI_{Pittsburgh}$ coding region. We selected the cDNA of two sequenced clones: one contained a point mutation 5' to the SAL1 restriction site and the other contained a point mutation 3' to the SAL1 restriction site. We digested each clone with the restriction endonucleases SAL1 (New England BioLabs) and HINDIII (including CIAP in the mixture to prevent circularization). The digestions were electrophoresed through 0.8% agarose gel and the desired fragment from each clone (without the point mutation) was determined based on its molecular weight. The cDNA from these bands was purified from the agarose and the fragments were ligated using T4 DNA ligase plus CIAP. The ligation mixture was transformed into XL1-Blue supercompetent *E.coli*. The plasmid cDNA was amplified, purified and the sequence was resolved by the UNC Genome Facility. This resulted in a pQE30 plasmid containing the desired cDNA sequence for an intermediate chimeric protein containing a polyhistadine tag, the HCII acidic domain through the HCII D-helix and $\alpha 1PI_{Pittsburgh}$ from 3' of its D-helix through its N-terminus.

Substitution of the RCL of AT onto the Intermediate Chimeric cDNA

Multiple basepair mutagenesis using PCR was directed to the coding sequence for P7 – P3' (residues 352 - 361) of the intermediate chimeric sequence. The primers for this reaction were designed to incorporate as much complementary sequence as possible, with mutations only to the basepairs needed to code for the AT RCL between P7-P3' (residues 387

– 396). The primers used, AT-RCL-Fwd and AT-RCL-Rvs, are reported in Table 4.1. The PCR mix contained the same proportions of ingredients as listed above. The thermocycling conditions for this mutagenesis comprised 18 cycles, each consisting of 30 seconds at 95° C, followed by 1 minute at 55° C and 10 minutes at 68° C. Thereafter, parental cDNA was digested with Dpn1 restriction enzyme (Strategene). The resultant product was transformed into XL1-Blue supercompetent *E. coli*. Amplified plasmid was purified and its cDNA sequence was the confirmed (through the UNC Genome Analysis Facility) to be the complete HATpin coding sequence, without mutation, in the pQE30 vector.

Protein Expression and Purification

The HATpin protein was expressed and purified with modifications as using methods previously reported[284]. Briefly, cDNA in the pQE30 vector was transformed into competent SG13009 *E. coli* (Qiagen) and grown in Luria-Bertani media containing 100 μg/mL ampicillin and 25 μg/mL kanamycin to an absorbance at 600 nm of 0.6. Protein expression was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (Promega) at 22° C for approximately 8 hours. Cells were pelleted by centrifugation ~ 10,000 x g at 4° C for 20 minutes. Pellets were resuspended in 20 mM MES (2-(*N*-morpholino)ethanesulfonic acid), 500 mM NaCl. 0.1% PEG-8000 and 0.05% NaN_3, pH 6.0 and frozen at -80° C. Thawed cells were lysed using sonication (Sonicator Dismembrator, Fisher Scientific) in 30 second bursts with 15 seconds rest for a total of 3 minutes of sonication at an output of ~10-15 watts. Cell debris and insoluble contaminants were eliminated by centrifugation at 10,000 x g for 20 minutes at 4° C. Imidazole was added to a final concentration of 20 mM and the cell lysate solution was filtered through a 0.45 μm nylon filter disc (Whatman). This was loaded onto

the Sepharose immobilized nickel affinity column, HisTrap HP (Amersham Biosciences). HATpin was eluted using the same buffer as above, containing 500 mM imidazole. EDTA was added to the elution to reach a final concentration of 10 mM. The protein was then dialyzed overnight against 20 mM Hepes, 50 mM NaCl, 0.1% PEG-8000, 0.05% NaN_3, pH 7.6 for future kinetic studies or 20 mM Hepes, 0.1 % PEG pH, 7.4 for affinity chromatography studies. HATpin concentration was initially estimated by absorbance at 280 nm (A_{280}) based upon M_r = 50,000 and using an extinction coefficient of 0.46 M^{-1} cm^{-1}. These values were determined by entering the amino acid sequence of HATpin into the Peptide Property Calculator on the Northwestern University Medical School Basic Sciences website: http://www.basic.northwestern.edu/biotools/proteincalc.html. The principles underlying the calculations made have been published in the peer–reviewed journal, *Analytical Biochemistry*[336].

Verification of Structural Components of HATpin

In order to confirm the presence of each of the structural components of HATpin's parent serpins in the expressed and purified HATpin protein, it was probed for antigenic activity of polyhistidine, HCII, α1PI and AT. HATpin, human plasma HCII and AT (purified as previously described[254]) and recombinant human $\alpha1PI_{Pittsburgh}$ (purified as described in Chapter 3) were electrophoresed through a 4-15% gradient SDS-polyacrylamide PhastGel (General Electric Healthcare) and transferred to Immobilon-B transfer membrane, 45 μm pore-size (Millipore) on the PhastGel System. Membranes were blocked for one hour at room temperature with 5% milk powder (Carnation) in Dulbecco's phosphate buffered saline (PBS) (Gibco) containing 0.1% Tween 20 (Sigma) ($PBStw_{0.1\%}$) and then incubated in

the same solution for one hour at 37° C with the primary antibodies, either affinity purified goat anti-human HCII IgG polyclonal antibody (1:1000, Affinity Biologicals), goat anti-human α1PI IgG polyclonal antibody (1:1000, Fitzgerald Industries), or goat anti-human AT IgG polyclonal antibody (1:100, Diasorin). The membranes were then washed three times for 10 minutes each with $PBStw_{0.1\%}$ and incubated for one hour with the secondary antibody, HRP conjugated donkey anti-goat IgG (1:5000, Santa Cruz Biotechnology). Antigen was detected by incubating the membranes in Pierce ECL Western Blotting Substrate (Pierce Biotechnology), exposing them to Kodak Scientific Processing film and developing using a SRX-101A tabletop film processor (Konica Minolta). The membranes were then stripped of antibodies by soaking them in 7 M guanidine HCl solution with 10 mM dithiothreitol (Sigma) for one hour at room temperature. The membrane was rinsed in tap water until opaque and the process described above was repeated with the exception that the primary antibody used for all membranes was monoclonal mouse anti-polyhistidine IgG (1:1000, Novagen) and the secondary antibody used was HRP conjugated rabbit anti-mouse IgG (1:5000, Sigma).

Verification of HATpin-Thrombin Complex Formation

To confirm the integrity of the RCL and the ability of HATpin to complex with thrombin (Haematologic Technologies), HATpin was incubated with thrombin in the presence of unfractionated heparin (UFH) (Diosynth) and visualized by Coomassie Blue R-250 (Bio-Rad Laboratories) stained 10% SDS-polyacrylamide gel along with other serpin-thrombin complexes. Complexes for HATpin, HCII and $\alpha 1PI_{Pittsburgh}$ with thrombin were created using the same protocol used in Chapter 2, although heparin was not included in the

incubation with $\alpha 1PI_{Pittsburgh}$. Gel electrophoresis and Coomassie staining also followed the technique described in Chapter 3.

Heparin-Sepharose Affinity Chromatography of HATpin

The relative affinity of HATpin compared to plasma-derived HCII and $\alpha 1PI_{Pittsburgh}$ for heparin was determined using a FPLC apparatus. HATpin was loaded onto a 1 mL HiTrap heparin-Sepharose column (Amersham Biosciences) and eluted using NaCl gradient from zero to 500 mM in Hepes buffer pH 7.4. Peak elution ionic strength was determined by the inhibition of thrombin (in the presence of 100 μg/mL UFH) by each 1 mL fraction. This was compared to the elution profile of HCII and $\alpha 1PI_{Pittsburgh}$ as determined in Chapter 3.

Determination of HATpin Protein Concentration

For the above studies, HATpin concentration was estimated by absorbance at 280 nm, and the quantity of protein loaded onto each gel was based accordingly. In examining the gels and blots generated by these studies, we noted that our HATpin stock appeared of a considerably smaller concentration than originally estimated. Thus, we re-evaluated the concentration of HATpin by another method. Using the Odyssey Infrared Imaging System (LI-COR) the band intensity of HATpin was compared to serial dilutions of a known concentration of $\alpha 1PI_{Pittsburgh}$ on an immunoblot probed with polyclonal anti-human α1PI IgG (Fitzgerald Industries) and detected with donkey anti-goat Alexa Fluor 680 secondary antibody (Invitrogen). Immunoblots were generated using the PhastGel system and the method described in Chapter 2. A standard curve of $\alpha 1PI_{Pittsburgh}$ was generated and HATpin concentration was calculated based on the relative intensity of its band on the immunoblot.

Inhibition of Serine Proteases in the Presence and Absence of GAGs

To determine if HATpin has the postulated inhibitory profile, we measured its second order rates of inhibition against α-thrombin, γ-thrombin (Haematologic Technologies), factor Xa and APC (Haematologic Technologies) in the presence and absence of UFH, low molecular weight heparin (LMWH) (Calbiochem), and / or dermatan sulfate (Calbiochem). In a 96-well plate 20 nM HATpin along with increasing concentrations of GAG was incubated with 1nM protease for varying lengths of time. The reaction was quenched by the addition of para-nitroaniline chromogenic substrate. Remaining protease activity was measured by the development of substrate cleavage product assessed by the change in absorbance at 405 nm. The rate of substrate cleavage was plotted against the incubation time in seconds and fit to the equation: $A = A_0 * e^{(-kt)}$, where A is activity as determined by substrate cleavage, A_0 is the activity at time zero, *t* is time in seconds and *k* is the observed rate of protease inhibition. Second order rates of inhibition (k_2) were determined by dividing *k* by HATpin concentration. GAG concentrations used ranged from 0 to 1000 μg/mL for UFH and dermatan sulfate and from 0 to 2000 μg/mL for LMWH. The estimated molecular weight for UFH, dermatan sulfate and LMWH used to determine their molarity was Mr = 15,000, 50,000 and 3,700 respectively. The following chromogenic substrates were used in a final concentration of 150 μM each: Pefachrome TH (Pentapharm) for α-thrombin and γ-thrombin, Pefachrome PCa (Pentapharm) and Pefachrome FXa (Pentapharm).

Stoichiometries of Inhibition of Serine Proteases in the Presence and Absence of GAGs

To evaluate if the second order rates of inhibition were being affected by alteration in the protein structure making it a substrate rather than inhibitor of the serine proteases, the stoichiometries of inhibition for each of the serine proteases with HATpin was determined in the presence and absence of UFH, dermatan sulfate, and LMWH. Increasing concentrations of HATpin were incubated for 12 hours at room temperature with 1 nM α-thrombin, γ-thrombin, factor Xa or 2 nM APC in the presence of either no GAG, UFH (25 μg/mL for α-thrombin and γ-thrombin or 500 μg/mL for factor Xa and APC), dermatan sulfate (1000 μg/mL for α-thrombin and γ-thrombin) or LMWH (125 μg/mL for factor Xa). Remaining protease activity was evaluated by chromogenic substrate cleavage as described above. The ratio of substrate cleavage in the presence of each HATpin concentration to substrate cleavage in the absence of HATpin was plotted against HATpin concentration. The intersection with the x-axis of a line fit to data points with remaining protease activity indicated the value of the stoichiometry of inhibition.

RESULTS

Immunoblot Analysis of the Structural Components of HATpin

Figure 4.3 depicts immunoblot analysis of the antigenic activity of HATpin. Panel A depicts Fermentas PageRuler Plus Prestain Protein Ladder. In each of the Panels B, C, and D, HATpin is depicted in the left lane, HCII in the center lane and $\alpha 1PI_{Pittsburgh}$ in the right lane. Panel B clearly shows a single band of HATpin protein migrating between 35 and 55 kDa with polyhistidien antigenic activity. HCII and $\alpha 1PI_{Pittsburgh}$ serve as negative and positive controls respectively; HCII was purified from human plasma and therefore has no

polyhistidine tag while the recombinant $\alpha 1PI_{Pittsburgh}$ does contain a polyhistidine tag. Panel C shows multiple bands with HCII antigenic activity in the HATpin lane. The top band indicates a molecular weight of just over 55 kDa. The second band which has the most antigenic activity, appears in the same location as the single HATpin band in Panel B.

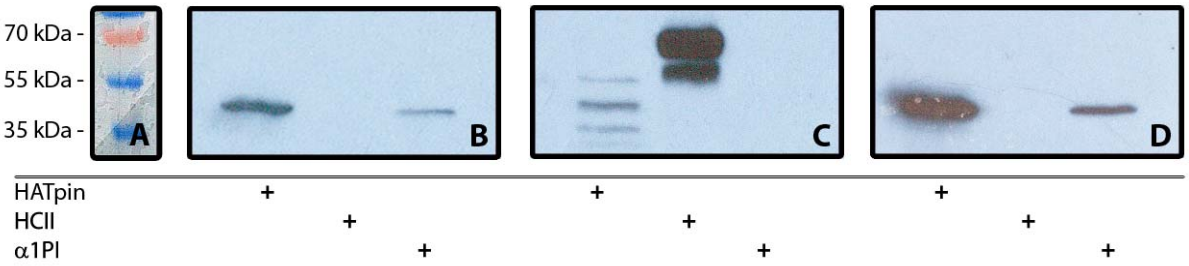


Figure 4.3 *Immunoblots of HATpin compared with HCII and $\alpha 1PI_{Pittsburgh}$.* Panel A shows the molecular weight ladder from the original transfer membrane. Panel B shows protein bands with poly-histidine antigenic activity. Panel C shows protein bands with HCII antigenic activity. Panel D shows protein bands with α1PI antigenic activity. Note that Panel D shows the same transfer membrane as Panel C, but stripped and reprobed. Furthermore, the transfer membrane depicted in Panel B was also stripped and reprobed for α1PI antigenic activity and the resultant blot was indistinguishable to that shown in Panel D.

Because Panel B depicts the exact same transfer membrane stripped and reprobed, it is certain that this second HATpin band in the left lane of Panel C, corresponds with the single band of HATpin in Panel B. Two additional bands with molecular weight below 35 kDa exhibit HCII antigenic activity in the HATpin preparation. HCII is depicted in the center lane of Panel C. The lower molecular weight band is cleaved HCII. The right lane of Panel C contains $\alpha 1PI_{Pittsburgh}$ and therefore no band is evident when probing for HCII antigen. Panel D depicts the serpins probed for α1PI antigenic activity. HATpin exhibits α1PI antigenic activity and again runs as a single band with a molecular weight of between 35 and

55 kDa in the same location as the prominent bands in Panels B and C. As expected, HCII does not show α1PI antigenic activity, while $\alpha 1PI_{Pittsburgh}$ does. The immunoblot probed for AT antigenic activity is not shown. HATpin was not visualized on this blot. This is not unexpected as HATpin only possesses a ten residue sequence unique to AT and it is probable that this region does not make up an epitope for the AT antibody.

Formation of HATpin-Thrombin Complex

In order to confirm the integrity of the RCL of HATpin and and visualize its potential interaction with thrombin, HATpin-thrombin complex was formed and compared with HCII, $\alpha 1PI_{Pittsburgh}$ and their thrombin complexes using Coomasie Blue stained SDS-PAGE (Figure 4.4). Lane 1 contains Fermentas PageRuler Plus Prestain Protein Ladder. Lane 2 depicts HATpin as a single band migrating between 35 kDa and 55 kDa and at the same location as $\alpha 1PI_{Pittsburgh}$ (MW = 45.1 kDa) shown in Lane 3. Lane 4 shows the formation of HATpin-thrombin complex migrating just below the 70 kDa marker. In this lane the band corresponding to HATpin is not evident, indicating the consumption of HATpin in complex formation. Thrombin (MW = 36.6 kDa) that is not complexed to HATpin can be visualized as the lowest band in Lane 3 and compared to thrombin alone in Lane 6. Lanes 5 and 7 show HCII and HCII-thrombin complex respectively. Lane 8 depicts $\alpha 1PI_{Pittsburgh}$ complex with thrombin. These results indicate that like HCII and $\alpha 1PI_{Pittsburgh,}$ HATpin is able to form stable complex with thrombin. This also indicates that the RCL HATpin has maintained its integrity through the protein expression and purification procedures.

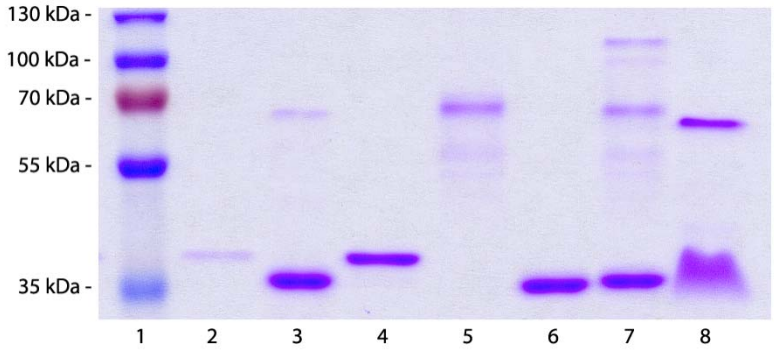


Figure 4.4 *Coomassie Blue Stained SDS-Polyacrylamide Gel.* Depicts molecular weight marker in column 1, HATpin in column 2, HATpin-thrombin complex in column 3, $\alpha 1PI_{Pittsburgh}$ 4, HCII in column 5, thrombin in column 6, HCII-thrombin complex in column 7 and $\alpha 1PI_{Pittsburgh}$-thrombin complex column 8.

Heparin-Sepharose Binding Affinity

HATpin eluted from heparin-Sepharose with ~ 250 mM NaCl (Figure 4.5). This is slightly higher than $\alpha 1PI_{Pittsburgh}$ eluted under the same conditions (~200 nM NaCl), but less than the NaCl concentration at which HCII eluted from the column (~ 450 nM). While this data suggests that HATpin had not gained the full heparin binding capacity of HCII, it has improved heparin binding capacity over $\alpha 1PI_{Pittsburgh}$.

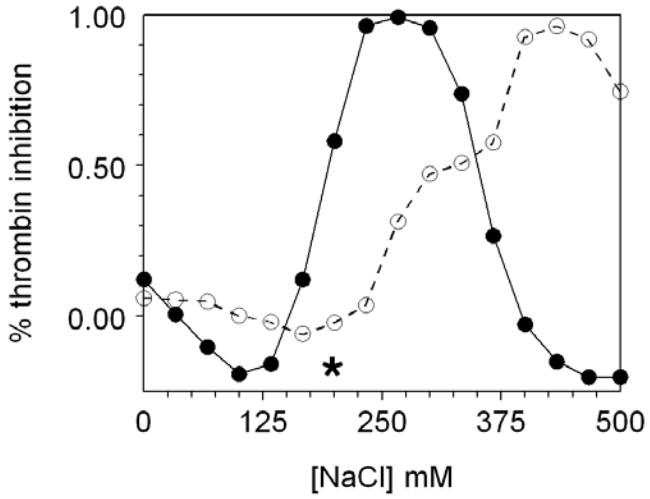


Figure 4.5 *Elution of HATpin and HCII from HiTrap heparin-Sepharose column.* Closed circles (●) represent HATpin. Open circles (○) represent HCII. The star (*) indicates the NaCl elution of $\alpha 1PI_{Pittsburgh}$ determined in Chapter 3 by absorbance at 280 nm.

Determination of HATpin Concentration by Immunoblot Band Comparison to $\alpha 1PI_{Pittsburgh}$

Based on concentration estimations determined by A_{280} , the same quantity of total protein was loaded into Lanes 2 and 4 of the Coomassie Blue stained gel depicted by Figure 4.4. The band intensity suggests that there is far less HATpin present (Lane 2) than there is $\alpha 1PI_{Pittsburgh}$ (Lane 4). An explanation for this that is supported by Figure 4.3, is that there are multiple protein bands (Panel C) in the HATpin preparation that are not visualized by Coomassie Blue staining. These proteins and proteins parts will have contributed to the total protein estimation by A_{280}. Therefore, to more accurately determine the concentration of HATpin, we compared the intensity of the antigenic activity of HATpin to that of $\alpha 1PI_{Pittsburgh}$ on immunoblot. Figure 4.6, Lanes 1 – 5, depict serial dilutions of 750 nM, 500nM, 200 nM, 100 nM and 50 nM of $\alpha 1PI_{Pittsburgh}$, respectively. Lane 6 contains HATpin. The intensity of the major bands in Lanes 1 - 6 was evaluated by the Odyssey Infrared Imaging System to emit 317.8, 266.0, 168.9, 127.5, 84.8 and 136.1 infrared units.

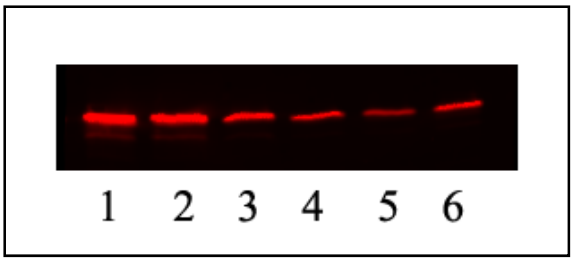


Figure 4.6 *Determination of HATpin concentration by immunoblot and standard curve with $\alpha 1PI_{Pittsburgh}$.* Columns 1-5 contain serial dilutions $\alpha 1PI_{Pittsburgh}$ (750 nM, 500 nM, 200 nM, 100 nM and 50 nM, respectively). Column 6 contains HATpin, determined by fluorescence intensity to be 116 nM.

A plot of infrared intensity of a1PI versus concentration fit the equation I = 406.8 * *conc*/ (242 + *conc*), where I is infrared intensity and *conc* is nM of serpin. Substituting the value of 136.1 infrared units for I and solving for *conc* resulted in the concentration of 115.8 nM for the HATpin, over four times less than estimated by A_{280}. This measurement of HATpin concentration permits the more accurate evaluation of its kinetic inhibitory properties.

Inhibition of Serine Proteases in the Presence and Absence of GAGs

The goal of this project was to engineer a serpin with the GAG-binding characteristics and thrombin specificity of both HCII and AT, that was able to inhibit factor Xa, but was a poor APC inhibitor. To evaluate these properties, we measured the inhibition of α-thrombin, γ-thrombin, factor Xa and APC in the presence and absence of UFH, dermatan sulfate and LMWH. HATpin inhibits α-thrombin and γ-thrombin in the absence of heparin at the respectable second order rates (k_2) of 1.89×10^5 M^{-1} s^{-1} and 1.19×10^5 M^{-1} s^{-1}, respectively. Similar to AT and HCII, the rates of α-thrombin and γ-thrombin inhibition by HATpin exhibit a heparin template curve with increasing concentrations of UFH. The second order rate constants of inhibition increased at moderate concentrations and decreasing with high concentrations (Figure 4.7). However, the peak acceleration is less than two-fold (Table 4.2), so the heparin acceleration is considerably diminished compared to either HCII or AT with UFH. Furthermore, if the acidic domain of HATpin was contributing to thrombin inhibition in the same way that it does with HCII, we would expect to see a reduction in the fold-acceleration of heparin with γ-thrombin as compared to α-thrombin which we do not observe. HATpin did not show accelerated thrombin inhibition in the presence of dermatan

sulfate. Increasing concentrations systematically *decreased* HATpin's inhibition of α-thrombin at maximum dermatan sulfate tested (~ 40 μM) to approximately 75% of the rate of thrombin inhibition seen in the absence of GAGs (data not shown). Dermatan sulfate had no effect on the rate of γ-thrombin inhibition by HATpin. Factor Xa was inhibited by HATpin. In the absence of GAGs, factor Xa was inhibited with a k_2 of ~ 3.5 x 10^5 M^{-1} s^{-1}. This rate was enhanced ~ 2-fold by the presence of either UFH or LWMH (Table 4.2), but required more heparin than the peak acceleration of α- or γ-thrombin by HATpin (Figure 4.7). HATpin inhibited APC with a second order rate of inhibition of 4.6 x 10^4 M^{-1} s^{-1}. UFH did not accelerate this inhibition. Rather, the rate of APC inhibition decreased with increasing UFH concentrations to 41% of it inhibition of APC in the absence of GAGs.

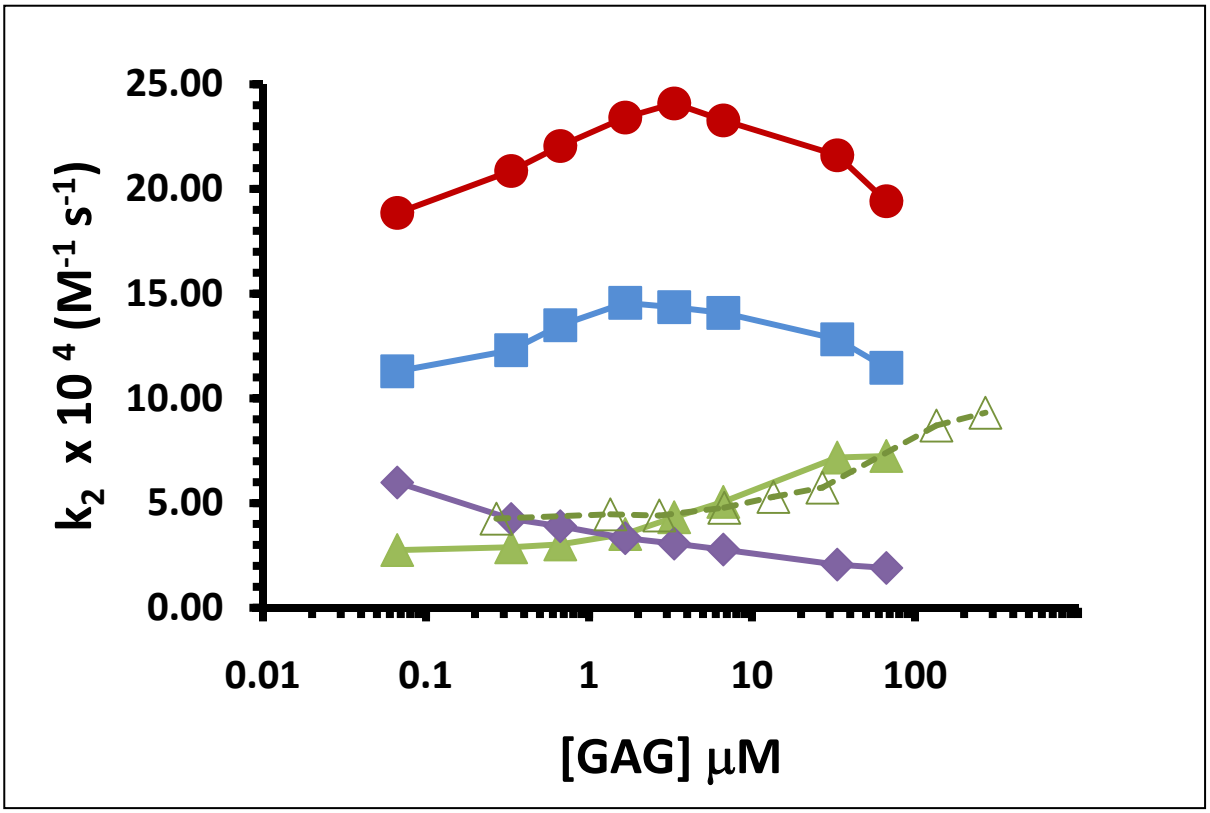


Figure 4.7 *Inhibition of serine proteases by HATpin in the presence of GAGs.* Closed symbols represent data with UFH and open symbols represent data with LMWH. Red circles (●) show inhibition of α-thrombin, blue squares (■) show inhibition of γ-thrombin, green triangles (▲,△) show inhibition of factor Xa, and purple diamonds (♦) show inhibition of APC.

Table 4.2 *Acceleration of HATpin Inhibition of Serine Proteases by Unfractionated and Low Molecular Weight Heparin.*

	k_2 (M^{-1} s^{-1}) no GAG	k_2 (M^{-1} s^{-1}) at peak	fold acceleration	[GAG] (μM)
α-thrombin (UFH)	1.89E+05	2.41E+05	1.3	3.33
γ-thrombin (UFH)	1.11E+05	1.46E+05	1.3	6.67
factor Xa (UFH)	2.96E+04	7.25E+04	2.4	33.3
factor Xa (LMWH)	4.11E+04	9.32E+04	2.3	66.7

Stoichiometries of Inhibition of Serine Proteases in the Presence and Absence of GAGs

In order to determine whether the structural combinations we engineered on HATpin had caused it to become a substrate for any of the serine proteases of interest, either in the presence or absence of GAGs, we determined the stoichiometries of inhibition. Results are shown in Table 4.3. The stoichiometries of inhibition for HATpin with all of the serine proteases measured is either with or without GAG is close to 4 except for γ-thrombin which is nearer 3. GAGs did not affect the stoichiometries. This data suggests that HATpin is acting to some degree as a substrate for these serine proteases.

Table 4.3 *Stoichiometries of inhibition for HATpin with serine proteases in the presence and absence of GAGs.*

GAG	α-thrombin	γ-thrombin	factor Xa	APC
None	4.0	3.3	4.3	3.7
heparin	4.2	3.0	3.8	4.1
dermatan sulfate	4.0	3.1		
low molecular weight heparin			3.8	

DISCUSSION

The overall goal of this project was to create a novel serpin, a chimera with structural elements from HCII, AT, and α1PI, with specifically enhanced protease inhibitory activities in the presence of GAGs. After much effort, the recombinant protein, HATpin, was successfully created, expressed, purified, and characterized. The expected unique inhibitory properties of HATpin were never realized, and while the results showed it to be a formidable protease inhibitor in its own right, the unique selectivity for thrombin and factor Xa, and not for APC, in the presence of either heparin or dermatan sulfate were not apparent. We discuss the evidence for having successfully engineered HATpin and we present a logical supposition for the deviation of its inhibitory profile from that which was anticipated.

The results indicate that while HATpin has heparin binding capacity and is able to inhibit thrombin and factor Xa, it does not fit the inhibitory profile for which it was designed. HATpin possesses a D-helix homologous to HCII. This was included so that the inhibition of thrombin would be accelerated by both heparin and dermatan sulfate. Based on the results presented in Chapter 3, we expected that the incorporation of the D-helix of HCII would allow for heparin acceleration of α-thrombin inhibition of no less than 15-fold. Additionally, it was presumed that including the entire D-helix sequence and other critical heparin binding residues such as $Lys173^{279}$, Arg106 and $Lys101^{337}$ that heparin binding and therefore HATpin activation, would increase compared to the mutant made in Chapter 3. This was not the case. Furthermore, while the ability of HATpin to bind Heparin-Sepharose as a function of ionic strength was increased over α1PI, it was still less able to bind heparin compared to both $\alpha 1PI_{Pitt\text{-}GAG}$ (See Chapter 3.) and HCII. Moreover, HATpin shows no dermatan sulfate

accelerated activity at all; dermatan sulfate causes the rate of HATpin inhibition of thrombin to decrease.

The N-terminal acidic domain of HCII was included to increase HATpin's specificity for thrombin and specifically, to utilize thrombin's exosite 1. Results from the inhibition of HATpin with α– and γ-thrombins suggest little difference in the inhibition of the proteases in the absence of GAGs and no difference in the acceleration by UFH. This would suggest that the N-terminal acidic domain is not free, even in the presence of UFH to interact with exosite 1 of thrombin. The reduced interaction of HATpin with Heparin-Sepharose compared to HCII might also imply that the acidic domain might be more closely associated with its D-helix region than in HCII itself .

The RCL sequence of HATpin was designed based on a study[109] which showed that mutating the P7 - P3' residues of $\alpha 1PI_{Pittsburgh}$ to those of AT reduced the inhibition of APC over 12,000-fold, while having a miniscule effect on thrombin inhibition. On the contrary, the rate of APC inhibition by HATpin is comparable to that reported for $\alpha 1PI_{Pittsburgh}$ (Table 4.4). One possible explanation is the use of wild-type α1PI in the former study and the use of a stabilized a1-protease inhibitor cDNA coupled with the acidic and D-helix domains of HCII in our study. It is possible that these different scaffolding somehow altered the equilibrium between the chimera and APC so as to maintain its ability to inhibit APC.

Such a discrepancy in the expected inhibitory profile versus actual inhibitory profile of HATpin, along with the protein migrating at a slightly lower molecular weight (~45 kDa) than predicted (~50 kDa), did prompt the question, "Did we really express and purify HATpin or is the protein that we purified, some cleaved or incomplete version of HATpin?" The evidence strongly supports that our protein is indeed HATpin.

First, sequence analysis confirmed that the cDNA we synthesized coded for HATpin. The cDNA had been ligated into an empty vector, and therefore, any protein expressed by the *E. coli* must be some form of HATpin or an *E. coli* protein. Second, our purified protein shows anti-polyhistidine antigenic activity. This indicates that the N-terminus is intact. Third, the HATpin protein reacted with antibodies directed at human proteins, specifically, HCII and α1PI. This confirms that the protein we have purified is indeed some version of HATpin, including structural elements of both HCII and α1PI. Fourth, our protein complexes with, and is able to inhibit thrombin, demonstrating the structural integrity of the RCL. Finally, the rates of protease inhibition measured indicate that the C-terminus is also intact. The RCL sequence is near (35 residues from) the C-terminus of HATpin. Although the apparent molecular weight of HATpin as estimated by gel mobility was ~ 45 kDa, which is remarkable similar to the theoretical weight of HATpin deleted after the RCL (~46 kDa), the residues in the C-terminus contribute to major serpin structures (the C-Sheet, the B-sheet and the I-helix)[264]. Deletion of these residues has been shown to drastically reduce serpin activity[338,339]. Based on our kinetic data, HATpin is a very active serpin which substantiates our conviction that HATpin is full-length. Additionally, it is well documented that protein characteristics other than molecular weight influence migration on SDS-PAGE[340,341]. Collectively, these results establish that our protein has a full-length N-terminus, components of HCII and α1PI and an intact reactive site loop that is capable of inhibiting thrombin, factor Xa and APC.

Confident that we expressed and purified an intact HATpin, it is interesting to consider its protease inhibitory profile. HATpin is a better thrombin inhibitor in the absence of GAGs than both HCII and AT, and only slightly less active than $\alpha 1PI_{Pittsburgh}$ (Table 4.4).

Moreover, it is a better inhibitor of factor Xa than HCII, $\alpha 1PI_{Pittsburgh}$ and AT in the absence of GAGs. Nonetheless, HATpin falls short of its desired profile primarily in two realms, GAG-accelerated activity and APC inhibition. Despite the presence of the entire region of HCII that has been identified as critical for both heparin and dermatan sulfate binding, HATpin is miserly in its response to GAGs.

Table 4. 4. *Comparison of second order rates of inhibition of serine proteases by HATpin and other serpins in the presence and absence of GAGs.* Rates for HATpin are gathered from this study. Other rates, unless specified have been extracted Chapter 1, Table 1.1[51].

Protease	GAG	Serpin			
		HATpin	$\alpha 1PI_{Pittsburgh}$	HCII	AT
α – thrombin	-	1.89 E+05	4.8E+05	6 E+02	1 E+04
	UFH	2.41 E+05		5 E+06	2 E+07
	dermatan sulfate			1 E+07	5.3 E+06
factor Xa	-	3.54 E+04	1.07 E+04‡	0[229]*	6 E+03
	UFH	7.25 E+04		1.7 E+02[229]**	6.6 E+06
	LMWH	9.32 E+04			1.3 E+06
APC	-	4.69 E+04	3.54 E+04	4.4 E+01[229]*	0.014 E+01[342]

* at 37° C. ‡ J. Rau data not published.

The D-helix-based $\alpha 1PI_{Pittsburgh}$ mutant, $\alpha 1PI_{Pitt\text{-}GAG}$ (See Chapter 3.) showed 15-fold acceleration of thrombin inhibition with heparin with some evidence of conformational change. In a different study, Sutherland et al.[302] fused the N-terminal extension of HCII onto $\alpha 1PI_{Pittsburgh}$ and saw a 21-fold increase in thrombin inhibition. We postulated that the addition of the entire N-terminal acidic domain along with the HCII heparin binding region would result in at least the additive if not multiplicative acceleration of thrombin inhibition in

the presence of heparin. It seems that the interaction of these elements (and perhaps the RCL sequence) has eliminated the benefits of both structural elements when placed together on a serpin without their native additional components.

The spatial relationship of the acidic domain of HCII has not been confirmed by crystallography[152]. However, based on molecular biological and biochemical experiments with HCII, it has been proposed that negatively charged residues of the acidic domain interact with the positively charged residues in and around the D-helix. In authentic HCII this is a powerful combination that triggers an allosteric mechanism when GAGs bind to the D-helix, releasing the N-terminal acidic domain for the binding of thrombin exosite 1. We speculated that a similar relationship would exist in HATpin. However, HATpin may not contain necessary structural elements of HCII that facilitate this mechanism, perhaps maintaining a stronger ionic interaction between acidic domain and its D-helix region than in HCII. We conjecture that the acidic domain of HATpin may have tighter or more substantial interactions with the D-helix which limit its heparin binding and also restrict its full molecular flexibility. This would explain its reduced protease inhibitory profile and also its GAG-binding properties.

The APC inhibition by HATpin in the face of the P7-P3' RCL substitutions is puzzling. Hopkins et al.[109] originally substituted the RCL of AT from P7-P3' into $\alpha 1PI_{Pittsburgh}$ and showed over 12,000-fold reduction in APC inhibition. However, Sutherland et al.[343] substituted the P7-P2' onto a $\alpha 1PI_{Pittsburgh}$ mutant containing the N-terminus of HCII through the acidic domain, and observed only a 140-fold decrease in APC activity. While still a large reduction in APC inhibition, it is closer to our results than those of Hopkins et al.[109] We speculate that that the acidic region itself has an influence on the

difference in altered rates for APC inhibition compared to the substitution of RCL onto $\alpha 1PI_{Pittsburgh}$ with no acidic domain. We therefore reason that not only the HCII acidic domain, but also the additional HCII structure in HATpin, acts to reduce the effect of the mutated RCL resulting in the negligible reduction in APC activity that we observed.

Overall, the inhibitory profile of HATpin indicates that there is still much to be learned about the structure-activity relationships in serpins. While substitution of one domain or small stretches of amino acids for another between molecules is sometimes successful, replacement of large portions may distort either or both the donor and recipient proteins to the end that the domains do not function as expected. It would be interesting to continue to pursue the reasons why HATpin failed to meet its inhibitory potential, and continue to assess both the strategy and the process used to engineer this chimera. It would likely provide us more knowledge about HCII, AT, α1PI and serpin engineering in general. In closing, one must consider that it may be best to embrace the adage reaffirmed by HATpin and the strategy to improve on serpin activity, "Mother," Mother Nature that is, "knows best."

CHAPTER 4 SUPPLEMENTAL MATERIAL

```
   1 atgagaggatcgcatcaccatcaccatcacggatccattccagagggcgaggaggacgac    60
   1  M  R  G  S  H  H  H  H  H  H  G  S  I  P  E  G  E  E  D  D    20
  61 gactatctggacctggagaagatattcagtgaagacgacgactacatcgacatcgtcgac    120
  21  D  Y  L  D  L  E  K  I  F  S  E  D  D  D  Y  I  D  I  V  D    40
 121 agtctgtcagtttccccgacagactctgatgtgagtgctgggaacatcctccagcttttt    180
  41  S  L  S  V  S  P  T  D  S  D  V  S  A  G  N  I  L  Q  L  F    60
 181 catggcaagagccggatccagcgtcttaacatcctcaacgccaagttcgctttcaacctc    240
  61  H  G  K  S  R  I  Q  R  L  N  I  L  N  A  K  F  A  F  N  L    80
 241 taccgagtgctgaaagaccaggtcaacactttcgataacatcttcatagcacccgttggc    300
  81  Y  R  V  L  K  D  Q  V  N  T  F  D  N  I  F  I  A  P  V  G    100
 301 atttctactgcgatgggtatgatttccttaggcctgaagggagagacccatgaacaagtg    360
 101  I  S  T  A  M  G  M  I  S  L  G  L  K  G  E  T  H  E  Q  V    120
 361  cactcgattttgcattttaaagactttgttaatgctagcagcaagtatgaaatcacgacc   420
 121  H  S  I  L  H  F  K  D  F  V  N  A  S  S  K  Y  E  I  T  T    140
 421  attcataatctcttccgtaagctgactcatcgcctcttcaggagggacagccagctccag   480
 141  I  H  N  L  F  R  K  L  T  H  R  L  F  R  R  D  S  Q  L  Q    160
 481  ctgaccaccggcaatggcctgttcctcagcgagggcctgaagctagtggataagtttttg   540
 161  L  T  T  G  N  G  L  F  L  S  E  G  L  K  L  V  D  K  F  L    180
 541  gaggatgttaaaaagttgtaccactcagaagccttcactgtcaacttcggggacaccgaa   600
 181  E  D  V  K  K  L  Y  H  S  E  A  F  T  V  N  F  G  D  T  E    200
 601  gaggccaagaaacagatcaacgattacgtggagaagggtactcaagggaaaattgtggat   660
 201  E  A  K  K  Q  I  N  D  Y  V  E  K  G  T  Q  G  K  I  V  D    220
 661  ttggtcaaggagcttgacagagacacagtttttgctctggtgaattacatcttctttaaa   720
 221  L  V  K  E  L  D  R  D  T  V  F  A  L  V  N  Y  I  F  F  K    200
 721  ggcaaatgggagagacccttttgaagtcaaggacaccgaggaagaggacttccacgtggac   780
 241  G  K  W  E  R  P  F  E  V  K  D  T  E  E  E  D  F  H  V  D    260
 781  caggtgaccaccgtgaaggtgcctatgatgaagcgtttaggcatgtttaacatccagcac   840
 261  Q  V  T  T  V  K  V  P  M  M  K  R  L  G  M  F  N  I  Q  H    280
 841 agcaagaagctgtccagctgggtgctgctgatgaaatacctgggcaatgccaccgccatc   900
 281  S  K  K  L  S  S  W  V  L  L  M  K  Y  L  G  N  A  T  A  I    300
 901  ttcttcctgcctgatgaggggaaactacagcacctggaaaatgaactcacccacgatatc   960
 301  F  F  L  P  D  E  G  K  L  Q  H  L  E  N  E  L  T  H  D  I    320
 961  atcaccaagttcctggaaaatgaagacagaaggtctgccagcttacatttacccaaactg   1020
 321  I  T  K  F  L  E  N  E  D  R  R  S  A  S  L  H  L  P  K  L    340
1021  tccattactggaacctatgatctgaagagcgtcctgggtcaactgggcatcactaaggtc   1080
 341  S  I  T  G  T  Y  D  L  K  S  V  L  G  Q  L  G  I  T  K  V    360
1081  ttcagcaatggggctgacctctccggggtcacagaggaggcacccctgaagctctccaag   1140
 361  F  S  N  G  A  D  L  S  G  V  T  E  E  A  P  L  K  L  S  K    380
1141  gccgtgcataaggctgtgctgaccatcgacgagaaagggactgaagctgctggggccatg   1200
 381  A  V  H  K  A  V  L  T  I  D  E  K  G  T  E  A  A  G  A  M    400
1201  gctgtagtgatcgcaggcagatctctcaaccccgaggtcaagttcaacaaacccttttgtc   1260
 401  A  V  V  I  A  G  R  S  L  N  P  E  V  K  F  N  K  P  F  V    420
1261  ttcttaattattgaccaaaataccaaggctcccctcttcatgggaagagtggtgaatccc   1320
 421  F  L  I  I  D  Q  N  T  K  A  P  L  F  M  G  R  V  V  N  P    440
1321  acccaaaaataa
 441  T  Q  K  -
```

Figure 4. S1. *cDNA for coding region of HATpin with corresponding amino acid sequence.* pQE30 vector sequence is in black. Homologous HCII sequence (residues 13 - 155) is in red. Homologous $\alpha 1PI_{Pittsburgh}$ sequence (residues 156 - 400 and 411 - 442) is in blue. Homologous AT sequence (residues 401 - 410) is in green.

CHAPTER 5

Future Directions

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them." Sir William Bragg

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" (I found it!) but "That's funny..." Isaac Asimov

Science has the habit of creating as many questions as it answers and the science performed for this dissertation is no exception. This Chapter recapitulates the overall findings of this dissertation and presents future directions that would be useful to perpetuate the science described in the preceding Chapters.

Immunologic Detection of Plasma Proteins in Normal and Atherosclerotic Vessels

Chapter 2 asked the questions, "Is heparin cofactor II (HCII) present in atherosclerotic lesions, does it co-localize with thrombin and is there a relationship between these proteins and the severity of the atherosclerosis?" The results of immunohistochemical and immunoblot analyses indicated that HCII is present in atherosclerotic lesions, and it co-localizes with (pro)thrombin in lipid-rich regions and necrotic cores of the atheromas. A positive correlation between plaque severity and protein staining for both HCII and

(pro)thrombin was observed. Interestingly, it was discovered that antithrombin (AT) is also present in the same location and its staining intensity was also positively correlated with plaque severity. Because prior scientific evidence suggests that AT does not act in atherosclerosis, this result was surprising. Therefore, to further investigate the specificity of the presence of HCII in the atheroma, we probed for another serpin α1-protease inhibitor (α1PI) and another liver-derived protein of similar molecular weight, albumin; both of these proteins were present in the same location as HCII, (pro)thrombin and AT.

A search of the literature revealed that many additional plasma proteins are reportedly found in the same region of the atheroma. This lead us to the hypothesis that due to increased permeability of the endothelium in atherosclerosis, the lipid-rich and necrotic core regions of atheromas act as a watershed, trapping circulating plasma proteins in the degraded matrix and extracellular debris. Additionally, fibrin has been detected in atheromas. Because fibrin is synthesized as a zymogen in the liver and must be generated through activation of the coagulation pathway, it follows that one possibility for the fibrin deposition in the atherosclerotic plaques is activation of the coagulation cascade. If our watershed hypothesis is true, this follows as coagulation factors would permeate the atheroma coming into contact with tissue factor that is present in the intima, and coagulation would be initiated. This does suggest however, that fibrinogen, having a molecular weight of over 300 kDa can enter the atheroma. Confirmation of this hypothesis would have implications for the thrombogenicity of non-ruptured atherosclerotic plaques and for their treatment and prevention.

The results of Chapter 2 beget the questions "Are the clotting factors, either in their active or zymogen form, present in atherosclerotic lesions?" "Are additional serpins present in atheromas?" To address these questions, one could probe additional sections for

coagulation factors, in particular factors VII(a), X(a), and V(a) as these are directly involved in tissue factor binding and thrombin activation. Regarding serpins, probing for α1-antichymotrypsin, angiotensinogen, plasminogen activator inhibitor-type 1 and type 2, and protein C inhibitor might offer additional clues supporting the watershed versus specificity issues for serpins in atheromas. Additionally, for the curious, one could probe for fibrinogen to address the question, "Does fibrinogen enter the plaque through the endothelium, or is it deposited as fibrin through another mechanism such as by scavenger macrophages?" Alternatively, fibrinogen synthesis by the inflammatory cells of the atheroma could be investigated by in situ hybridization and the polymerase chain reaction (PCR) applied to mRNA in frozen sections.

The utility of fixed histological slides is somewhat limited. Because of this, we secured fresh aortic samples containing either normal or atherosclerotic lesions for examination. Protein extracted from these vessels was subjected to SDS-PAGE and analyzed by immunoblot, providing the opportunity to distinguish between thrombin, prothrombin and complexed thrombin. Results from this study definitively showed prothrombin in all the samples, and thrombin in one sample. HCII was also detected, but no HCII complex could be visualized.

The lack of serpin-protease complex detection does not indicate that HCII does not inhibit thrombin in atherosclerosis. Rather, it is simply an indication that HCII-thrombin complex is undetectable using the method employed. However, having the vascular tissue homogenized and the proteins solubilized provides additional opportunities to approach this problem. With the solubilized normal and atherosclerotic vessel one could perform Enzyme Linked ImmunoSorbent Assays (ELISA) probing specifically for HCII,

thrombin and also HCII-thrombin combinations. This method would not be subject to total-protein concentration that can be loaded into a gel and therefore should be more sensitive to antigenic detection. Finally, the preparation of an antibody specific for the neo-antigenic epitope expressed by the HCII-thrombin complex would be an extraordinarily helpful reagent to further probe these tissues.

Transfer of GAG-binding Properties to a Non-GAG -Binding Serpin

Chapter 3 asked the questions, "Can the glycosaminoglycan (GAG)-binding properties of HCII be transferred to the non-GAG binding serpin, α1PI, through mutation residues on its D-helix to the homologous basic residues in the D-helix of HCII?" and "Can such gain-of-function approach to the study of serpin structure provide useful information that can be generalized to serpins?" The results of this chapter indicate that HCII-like GAG-binding properties can indeed be conferred upon the non-GAG binding serpin α1PI. Additionally, these results provide useful information regarding the GAG-accelerated activity of thrombin-inhibiting serpins. In this study, we observed a ~15-fold acceleration of thrombin inhibition in the presence of unfractionated heparin (UFH) and ~ 2-fold acceleration in the presence of dermatan sulfate. Based on results using exosite variants of thrombin and extrinsic fluorescence changes, we concluded that 2-fold acceleration could be contributed to conformational changes in the serpin rather than bridging. To further substantiate these conclusions, it would be beneficial to examine the GAG-accelerated inhibition of thrombin by fractionated heparins that are unable to bridge the serpin and thrombin to form a ternary complex.

Only five residues on the D-helix of α1PI were mutated to become positively charged and homologous with those of HCII. Several other residues in HCII have been identified as being important in GAG binding, generating the question, "Can GAG-binding and its acceleration of thrombin-inhibition be enhanced by the mutation of additional residues?" Also, the mutated D-helix of our GAG-binding mutant, $\alpha1PI_{Pitt\text{-}GAG}$, contained one negatively charged residue that HCII does not possess. Thus we ask, "Does the remaining negatively charged residue in $\alpha1PI_{Pitt\text{-}GAG}$ effect the GAG-binding? Finally, we employed $\alpha1PI_{Pittsburgh}$ as the primary scaffolding for our mutations due to its rapid thrombin inhibition and thus ease of inhibitory measurements. However, the rates of thrombin inhibition without GAG-accelerated activity are extremely fast. One must wonder whether an absolute maximum rate of thrombin inhibition has been reached with a 15-fold increase of this already rapid rate of inhibition. "Could the rate of thrombin inhibition be accelerated greater than ~15-fold if the baseline rates of thrombin inhibition were not so fast?" All of these questions could be addressed with molecular studies much like those already performed in Chapter 3. Serial mutations of residues homologous to Lys173, Arg106 and Lys101 would provide additional information regarding the role of these residues in GAG-binding. Mutation of the negatively charged Glu89 in α1PI to its homologous residue in HCII (Ile) would inform us of the role of opposing electric charges in this region. Would mutating the reactive center residue from Arg358 to Leu to make it more "HCII-like" provide another novel manner to study the GAG-activation process in this recombinant serpin? Finally, mutating the D-helix of α1PI in the same way as we did $\alpha1PI_{Pittsburgh}$, would increase our understanding of maximal rates of protease inhibition.

Creation of the Novel Chimeric Serpin, HATpin

Chapter 4 asked the question, "Can we engineer a chimeric serpin with the combined beneficial properties of HCII, AT and $\alpha 1PI_{Pittsburgh}$ through the assembly of specific structural regions from each protein?" While we were able to synthesize the cDNA, and express and purify the HATpin protein, its inhibitory profile disappointed. Three questions roared to mind when examining the data generated in this study, "Why doesn't HATpin exhibit the expected inhibitory profile?", "Could a slightly modified version of HATpin have the desired inhibitory activity?" and "Is the natural scaffolding of HCII with its acidic domain and glycosamoglycan-binding region uniquely dependent on its overall structure?"

We hypothesized that the acidic domain of HATpin interacts too strongly with its D-helix and that without some unknown critical structural component of the HCII native structure, the acidic domain remained bound to the D-helix and impaired GAG-binding. Additionally, we theorized that the large amount of HCII structure somehow decreased the effect of the reactive center loop (RCL) mutations so that activated protein C (APC) inhibition was not decreased. To test both of these hypotheses, one could engineer a serpin based on a combination of $\alpha 1PI_{Pitt\text{-}GAG}$ and the acidic domain fusion protein, HAPI, created by Sutherland et al.[302]. This protein would not contain the large amount of HCII sequence between the D-helix and the acidic domain that is present in HATpin. It would only contain the two separate structural regions that have already been proven to separately accelerate thrombin inhibition. Any deviations from anticipated inhibitory profiles could be more easily attributed to interaction between the two regions rather than some other unidentifiable structural component. The RCL of AT could subsequently be transposed onto this protein

for further analysis and alternative approach to creating an ideal HATpin-like protease inhibitor.

Another consideration would be to recreate HATpin on a wild-type scaffold rather than the stabilized α1PI backbone. In the creation of HATpin we generated the cDNA for an intermediate chimeric protein, it might be informative to examine the inhibitory profile of this protein compared to HATpin to see what the effect the RCL sequence mutation had on the protein as a unit. Finally, we have begun dialog with the UNC-CH Proteomics Core Facility to investigate the peptide components of HATpin and other minor products in the HATpin preparation in an attempt to understand the composition of HATpin, its expression chaperones and its degradation products.

In conclusion, despite the many questions generated by the research carried out for this dissertation, the scientific investigation has proved useful not only in educating and training me for my future career, but also to the advancement of the understanding of serpins in vascular pathophysiology and their manipulation for its treatment.

REEFERENCES CITED

1. Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S.M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M. & Hong, Y. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 117, e25-146 (2008).

2. CDC. Health, United States, 2007, With Chartbook on Trends in the Health of Americans. (National Center for Disease Statistics, Hyattsville, Maryland, 2007).

3. World Health Statistics 2007. (World Health Organization, Geneva, Switzerland, 2007).

4. Goldstein, L.B., Adams, R., Alberts, M.J., Appel, L.J., Brass, L.M., Bushnell, C.D., Culebras, A., Degraba, T.J., Gorelick, P.B., Guyton, J.R., Hart, R.G., Howard, G., Kelly-Hayes, M., Nixon, J.V. & Sacco, R.L. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group: the American Academy of Neurology affirms the value of this guideline. *Stroke* 37, 1583-633 (2006).

5. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama* 285, 2486-97 (2001).

6. Davidson, M.H. Overview of prevention and treatment of atherosclerosis with lipid-altering therapy for pharmacy directors. *Am J Manag Care* 13 Suppl 10, S260-9 (2007).

7. Steinberg, D. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 272, 20963-6 (1997).

8. Traub, O. & Berk, B.C. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol* 18, 677-85 (1998).

9. Davies, P.F. Flow-mediated endothelial mechanotransduction. *Physiol Rev* 75, 519-60 (1995).

10. Ando, J. & Kamiya, A. Blood flow and vascular endothelial cell function. *Front Med Biol Eng* 5, 245-64 (1993).

11. Libby, P. Inflammation in atherosclerosis. *Nature* 420, 868-74 (2002).

12. Gleissner, C.A., Leitinger, N. & Ley, K. Effects of native and modified low-density lipoproteins on monocyte recruitment in atherosclerosis. *Hypertension* 50, 276-83 (2007).

13. Leitinger, N. Oxidized phospholipids as modulators of inflammation in atherosclerosis. *Curr Opin Lipidol* 14, 421-30 (2003).

14. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 352, 1685-95 (2005).

15. Stary, H.C., Chandler, A.B., Glagov, S., Guyton, J.R., Insull, W., Jr., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D. & Wissler, R.W. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 14, 840-56 (1994).

16. Stary, H.C., Chandler, A.B., Dinsmore, R.E., Fuster, V., Glagov, S., Insull, W., Rosenfeld, M.E., Schwartz, C.J., Wagner, W.D. & Wissler, R.W. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler., Thromb., and Vascul. Biol.* 15, 1512-1531 (1995).

17. Farb, A., Burke, A.P., Tang, A.L., Liang, T.Y., Mannan, P., Smialek, J. & Virmani, R. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation* 93, 1354-63 (1996).

18. Rauch, U., Osende, J.I., Fuster, V., Badimon, J.J., Fayad, Z. & Chesebro, J.H. Thrombus formation on atherosclerotic plaques: pathogenesis and clinical consequences. *Ann Intern Med* 134, 224-38 (2001).

19. Simionescu, M. Implications of early structural-functional changes in the endothelium for vascular disease. *Arterioscler Thromb Vasc Biol* 27, 266-74 (2007).

20. Weissberg, P.L. Atherogenesis: current understanding of the causes of atheroma. *Heart* 83, 247-52 (2000).

21. Fernandez-Ortiz, A., Badimon, J.J., Falk, E., Fuster, V., Meyer, B., Mailhac, A., Weng, D., Shah, P.K. & Badimon, L. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. *J Am Coll Cardiol* 23, 1562-9 (1994).

22. Davies, M.J. & Thomas, A.C. Plaque fissuring--the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. *Br Heart J* 53, 363-73 (1985).

23. Falk, E. Coronary thrombosis: pathogenesis and clinical manifestations. *Am J Cardiol* 68, 28B-35B (1991).

24. Horie, T., Sekiguchi, M. & Hirosawa, K. Coronary thrombosis in pathogenesis of acute myocardial infarction. Histopathological study of coronary arteries in 108 necropsied cases using serial section. *Br Heart J* 40, 153-61 (1978).

25. Wakefield, T.W., Myers, D.D. & Henke, P.K. Mechanisms of venous thrombosis and resolution. *Arterioscler Thromb Vasc Biol* 28, 387-91 (2008).

26. Heit, J.A. Venous thromboembolism: disease burden, outcomes and risk factors. *J Thromb Haemost* 3, 1611-7 (2005).

27. Benjamin, E.J., Wolf, P.A., D'Agostino, R.B., Silbershatz, H., Kannel, W.B. & Levy, D. Impact of atrial fibrillation on the risk of death: the Framingham Heart Study. *Circulation* 98, 946-52 (1998).

28. Malone, P.C. & Agutter, P.S. The aetiology of deep venous thrombosis. *Qjm* 99, 581-93 (2006).

29. Mammen, E.F. Pathogenesis of venous thrombosis. *Chest* 102, 640S-644S (1992).

30. Lip, G.Y. Hypercoagulability and haemodynamic abnormalities in atrial fibrillation. *Heart* 77, 395-6 (1997).

31. Goldman, M.E., Pearce, L.A., Hart, R.G., Zabalgoitia, M., Asinger, R.W., Safford, R. & Halperin, J.L. Pathophysiologic correlates of thromboembolism in nonvalvular atrial fibrillation: I. Reduced flow velocity in the left atrial appendage (The Stroke Prevention in Atrial Fibrillation [SPAF-III] study). *J Am Soc Echocardiogr* 12, 1080-7 (1999).

32. Esmon, C.T. Does inflammation contribute to thrombotic events? *Haemostasis* 30 Suppl 2, 34-40 (2000).

33. Esmon, C.T. The impact of the inflammatory response on coagulation. *Thromb Res* 114, 321-7 (2004).

34. van Hinsbergh, V.W. The endothelium: vascular control of haemostasis. *Eur J Obstet Gynecol Reprod Biol* 95, 198-201 (2001).

35. Esmon, C.T. Inflammation and thrombosis. *J Thromb Haemost* 1, 1343-8 (2003).

36. Petralia, G.A. & Kakkar, A.K. Treatment of venous thromboembolism in cancer patients. *Semin Thromb Hemost* 33, 707-11 (2007).

37. Lee, A.Y. Cancer and venous thromboembolism: prevention, treatment and survival. *J Thromb Thrombolysis* 25, 33-6 (2008).

38. Machado, F.R. & Silva, E. Coagulation and sepsis. *Endocr Metab Immune Disord Drug Targets* 6, 175-82 (2006).

39. Caplan, L.R. Thrombolysis 2004: the good, the bad, and the ugly. *Rev Neurol Dis* 1, 16-26 (2004).

40. Gross, P.L. & Weitz, J.I. New anticoagulants for treatment of venous thromboembolism. *Arterioscler Thromb Vasc Biol* 28, 380-6 (2008).

41. Waldo, A.L. Anticoagulation: stroke prevention in patients with atrial fibrillation. *Med Clin North Am* 92, 143-59, xi (2008).

42. Harker, L.A., Hanson, S.R. & Runge, M.S. Thrombin hypothesis of thrombus generation and vascular lesion formation. *Am J Cardiol* 75, 12B-17B (1995).

43. Baykal, D., Schmedtje, J.F. & Runge, M.S. Role of thrombin receptor in restenosis and atherosclerosis. *Am. J. Cardiol.* 75, 82B-87-B (1995).

44. Hirano, K. The roles of proteinase-activated receptors in the vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 27, 27-36 (2007).

45. Pike, R.N., Buckle, A.M., le Bonniec, B.F. & Church, F.C. Control of the coagulation system by serpins. Getting by with a little help from glycosaminoglycans. *Febs J* 272, 4842-51 (2005).

46. He, L., Giri, T.K., Vicente, C.P. & Tollefsen, D.M. Vascular dermatan sulfate regulates the antithrombotic activity of heparin cofactor II. *Blood* (2008).

47. Vicente, C.P., He, L. & Tollefsen, D.M. Accelerated atherogenesis and neointima formation in heparin cofactor II deficient mice. *Blood* 110, 4261-7 (2007).

48. Tollefsen, D.M. Heparin Cofactor II Modulates the Response to Vascular Injury. *Arterioscler Thromb Vasc Biol* 27, 454-60 (2007).

49. Aihara, K., Azuma, H., Akaike, M., Ikeda, Y., Sata, M., Takamori, N., Yagi, S., Iwase, T., Sumitomo, Y., Kawano, H., Yamada, T., Fukuda, T., Matsumoto, T., Sekine, K., Sato, T., Nakamichi, Y., Yamamoto, Y., Yoshimura, K., Watanabe, T., Nakamura, T., Oomizu, A., Tsukada, M., Hayashi, H., Sudo, T., Kato, S. & Matsumoto, T. Strain-dependent embryonic lethality and exaggerated vascular remodeling in heparin cofactor II-deficient mice. *J Clin Invest* 117, 1514-26 (2007).

50. Aihara, K., Azuma, H., Takamori, N., Kanagawa, Y., Akaike, M., Fujimura, M., Yoshida, T., Hashizume, S., Kato, M., Yamaguchi, H., Kato, S., Ikeda, Y., Arase, T., Kondo, A. & Matsumoto, T. Heparin cofactor II is a novel protective factor against carotid atherosclerosis in elderly individuals. *Circulation* 109, 2761-5 (2004).

51. Rau, J.C., Beaulieu, L.M., Huntington, J.A. & Church, F.C. Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 5 Suppl 1, 102-15 (2007).

52. Bode, W. The structure of thrombin: a janus-headed proteinase. *Semin Thromb Hemost* 32 Suppl 1, 16-31 (2006).

53. Hoffman, M. & Monroe, D.M. Coagulation 2006: a modern view of hemostasis. *Hematol Oncol Clin North Am* 21, 1-11 (2007).

54. von Hundelshausen, P. & Weber, C. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circ Res* 100, 27-40 (2007).

55. Leger, A.J., Covic, L. & Kuliopulos, A. Protease-activated receptors in cardiovascular diseases. *Circulation* 114, 1070-7 (2006).

56. Zheng, B., Clarke, J.B., Busby, W.H., Duan, C. & Clemmons, D.R. Insulin-like growth factor-binding protein-5 is cleaved by physiological concentrations of thrombin. *Endocrinology* 139, 1708-14 (1998).

57. Macfarlane, S.R., Seatter, M.J., Kanke, T., Hunter, G.D. & Plevin, R. Proteinase-activated receptors. *Pharmacol Rev* 53, 245-82 (2001).

58. Sandset, P.M. Tissue factor pathway inhibitor (TFPI)--an update. *Haemostasis* 26 Suppl 4, 154-65 (1996).

59. Dahlback, B. & Villoutreix, B.O. The anticoagulant protein C pathway. *FEBS Lett* 579, 3310-6 (2005).

60. Broze, G.J., Jr. Protein Z-dependent regulation of coagulation. *Thromb Haemost* 86, 8-13 (2001).

61. Cooper, S.T., Whinna, H.C., Jackson, T.P., Boyd, J.M. & Church, F.C. Intermolecular interactions between protein C inhibitor and coagulation proteases. *Biochemistry* 34, 12991-12997 (1995).

62. van Meijer, M., Smilde, A., Tans, G., Nesheim, M.E., Pannekoek, H. & Horrevoets, A.J. The suicide substrate reaction between plasminogen activator inhibitor 1 and thrombin is regulated by the cofactors vitronectin and heparin. *Blood* 90, 1874-82 (1997).

63. Esmon, C.T. The protein C pathway. *Chest* 124(3 Suppl), 26S-32S (2003).

64. Fukudome, K., Kurosawa, S., Stearns-Kurosawa, D.J., He, X., Rezaie, A.R. & Esmon, C.T. The endothelial cell protein C receptor. Cell surface expression and direct ligand binding by the soluble receptor. *J Biol Chem* 271, 17491-8 (1996).

65. Fuentes-Prior, P., Iwanaga, Y., Huber, R., Pagila, R., Rumennik, G., Seto, M., Morser, J., Light, D.R. & Bode, W. Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex. *Nature* 404, 518-25 (2000).

66. Stearns-Kurosawa, D.J., Kurosawa, S., Mollica, J.S., Ferrell, G.L. & Esmon, C.T. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc Natl Acad Sci U S A* 93, 10212-6 (1996).

67. Lu, D., Kalafatis, M., Mann, K.G. & Long, G.L. Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. *Blood* 87, 4708-17 (1996).

68. Feistritzer, C., Mosheimer, B.A., Sturn, D.H., Riewald, M., Patsch, J.R. & Wiedermann, C.J. Endothelial protein C receptor-dependent inhibition of migration of human lymphocytes by protein C involves epidermal growth factor receptor. *J Immunol* 176, 1019-25 (2006).

69. Yuda, H., Adachi, Y., Taguchi, O., Gabazza, E.C., Hataji, O., Fujimoto, H., Tamaki, S., Nishikubo, K., Fukudome, K., D'Alessandro-Gabazza, C.N., Maruyama, J., Izumizaki, M., Iwase, M., Homma, I., Inoue, R., Kamada, H., Hayashi, T., Kasper, M., Lambrecht, B.N., Barnes, P.J. & Suzuki, K. Activated protein C inhibits

bronchial hyperresponsiveness and Th2 cytokine expression in mice. *Blood* 103, 2196-204 (2004).

70. Joyce, D.E., Gelbert, L., Ciaccia, A., DeHoff, B. & Grinnell, B.W. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *J Biol Chem* 276, 11199-203 (2001).

71. Feistritzer, C. & Riewald, M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation

10.1182/blood-2004-10-3985. *Blood* 105, 3178-3184 (2005).

72. Mosnier, L.O., Zlokovic, B.V. & Griffin, J.H. The cytoprotective protein C pathway. *Blood* (2006).

73. Cheng, T., Liu, D., Griffin, J.H., Fernandez, J.A., Castellino, F., Rosen, E.D., Fukudome, K. & Zlokovic, B.V. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. *Nat Med* 9, 338-42 (2003).

74. Guo, H., Liu, D., Gelbard, H., Cheng, T., Insalaco, R., Fernandez, J.A., Griffin, J.H. & Zlokovic, B.V. Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. *Neuron* 41, 563-72 (2004).

75. Bernard, G.R., Vincent, J.L., Laterre, P.F., LaRosa, S.P., Dhainaut, J.F., Lopez-Rodriguez, A., Steingrub, J.S., Garber, G.E., Helterbrand, J.D., Ely, E.W. & Fisher, C.J., Jr. Efficacy and Safety of Recombinant Human Activated Protein C for Severe Sepsis. *N. Engl. J. Med.* 344, 699-709 (2001).

76. Rezaie, A.R. Vitronectin functions as a cofactor for rapid inhibition of activated protein C by plasminogen activator inhibitor-1. Implications for the mechanism of profibrinolytic action of activated protein C. *J Biol Chem* 276, 15567-70 (2001).

77. Heeb, M.J. & Griffin, J.H. Physiologic inhibition of human activated protein C by alpha 1-antitrypsin. *J Biol Chem* 263, 11613-6 (1988).

78. Cesarman-Maus, G. & Hajjar, K.A. Molecular mechanisms of fibrinolysis. *Br J Haematol* 129, 307-21 (2005).

79. Wiman, B. & Collen, D. Molecular mechanism of physiological fibrinolysis. *Nature* 272, 549-550 (1978).

80. Loskutoff, D.J. & Quigley, J.P. PAI-1, fibrosis, and the elusive provisional fibrin matrix. *J Clin Invest* 106, 1441-3 (2000).

81. Levi, M., van der Poll, T. & Buller, H.R. Bidirectional relation between inflammation and coagulation. *Circulation* 109, 2698-704 (2004).

82. Miles, L.A., Hawley, S.B., Baik, N., Andronicos, N.M., Castellino, F.J. & Parmer, R.J. Plasminogen receptors: the sine qua non of cell surface plasminogen activation. *Front Biosci* 10, 1754-62 (2005).

83. Dano, K., Behrendt, N., Hoyer-Hansen, G., Johnsen, M., Lund, L.R., Ploug, M. & Romer, J. Plasminogen activation and cancer. *Thromb Haemost* 93, 676-81 (2005).

84. Galis, Z.S. & Khatri, J.J. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 90, 251-62 (2002).

85. Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A. & Dolo, V. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol* 160, 673-80 (2002).

86. Andreasen, P.A., Egelund, R. & Peterson, H.H. The plasminogen activation system in tumor growth, invasion, and metastasis. *Cellular and Molecular Life Sciences* 57, 25-40 (2000).

87. Manders, P., Tjan-Heijnen, V.C., Span, P.N., Grebenchtchikov, N., Foekens, J.A., Beex, L.V. & Sweep, C.G. Predictive impact of urokinase-type plasminogen activator: plasminogen activator inhibitor type-1 complex on the efficacy of adjuvant systemic therapy in primary breast cancer. *Cancer Res* 64, 659-64 (2004).

88. Coughlin, P.B. Antiplasmin: the forgotten serpin? *Febs J* 272, 4852-7 (2005).

89. Vaughan, D.E. Angiotensin, fibrinolysis, and vascular homeostasis. *Am J Cardiol* 87, 18C-24C (2001).

90. Mosnier, L.O. & Bouma, B.N. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. *Arterioscler Thromb Vasc Biol* 26, 2445-53 (2006).

91. Espana, F., Berrettini, M. & Griffin, J.H. Purification and characterization of plasma protein C inhibitor. *Thromb. Res.* 55, 369-384 (1989).

92. Heeb, M.J., Espana, F., Geiger, M., Collen, D., Stump, D.C. & Griffin, J.H. Immunological identity of heparin-dependent plasma and urinary protein C inhibitor and plasminogen activator inhibitor-3. *J. Biol. Chem.* 262, 15813-15816 (1987).

93. Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G.A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I. & Whisstock, J.C. An overview of the serpin superfamily. *Genome Biol* 7, 216 (2006).

94. Silverman, G.A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., Gettins, P.G.W., Irving, J.A., Lomas, D.A., Luke, C.J., Moyer, R.W., Pemberton, P.A., Remold-O'Donnell, E., Salvesen, G.S., Travis, J. & Whisstock, J.C. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins: Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J. Biol. Chem.* 276, 33293-33296 (2001).

95. Rogers, S.J., Pratt, C.W., Whinna, H.C. & Church, F.C. Role of thrombin exosites in inhibition by heparin cofactor II. *J. Biol. Chem.* 267, 3613-3617 (1992).

96. Izaguirre, G., Zhang, W., Swanson, R., Bedsted, T. & Olson, S.T. Localization of an antithrombin exosite that promotes rapid inhibition of factors Xa and IXa dependent on heparin activation of the serpin. *J Biol Chem* 278, 51433-40 (2003).

97. Olson, S.T., Swanson, R., Raub-Segall, E., Bedsted, T., Sadri, M., Petitou, M., Herault, J.P., Herbert, J.M. & Bjork, I. Accelerating ability of synthetic oligosaccharides on antithrombin inhibition of proteinases of the clotting and fibrinolytic systems. Comparison with heparin and low-molecular-weight heparin. *Thromb Haemost* 92, 929-39 (2004).

98. Björk, I. & Olson, S.T. Antithrombin: A bloody important serpin. *Adv. Exp. Med. Biol.* 425, 17-33 (1997).

99. Mauray, S., de Raucourt, E., Talbot, J.C., Dachary-Prigent, J., Jozefowicz, M. & Fischer, A.M. Mechanism of factor IXa inhibition by antithrombin in the presence of unfractionated and low molecular weight heparins and fucoidan. *Biochim Biophys Acta* 1387, 184-94 (1998).

100. O'Keeffe, D., Olson, S.T., Gasiunas, N., Gallagher, J., Baglin, T.P. & Huntington, J.A. The heparin binding properties of heparin cofactor II suggest an antithrombin-like activation mechanism. *J Biol Chem* 279, 50267-73 (2004).

101. VanDeerlin, V.M.D. & Tollefsen, D.M. The N-terminal acidic domain of heparin cofactor II mediates the inhibition of a-thrombin in the presence of glycosaminoglycans. *J. Biol. Chem.* 266, 20223-20231 (1991).

102. Rezaie, A.R., Manithody, C. & Yang, L. Identification of factor Xa residues critical for interaction with protein Z-dependent protease inhibitor: both active site and exosite interactions are required for inhibition. *J Biol Chem* 280, 32722-8 (2005).

103. Han, X., Fiehler, R. & Broze, G.J., Jr. Characterization of the protein Z-dependent protease inhibitor. *Blood* 96, 3049-55 (2000).

104. Rezaie, A.R., Cooper, S.T., Church, F.C. & Esmon, C.T. Protein C inhibitor is a potent inhibitor of the thrombin-thrombomodulin complex. *J. Biol. Chem.* 270, 25336-25339 (1995).

105. Pratt, C.W., Macik, B.G. & Church, F.C. Protein C inhibitor: purification and proteinase reactivity. *Thromb. Res.* 53, 595-602 (1989).

106. Shen, L., Villoutreix, B.O. & Dahlback, B. Involvement of Lys 62(217) and Lys 63(218) of human anticoagulant protein C in heparin stimulation of inhibition by the protein C inhibitor. *Thromb. Haemost.* 82, 72-79 (1999).

107. Travis, J. & Johnson, D. Human a1-proteinase inhibitor. *Meth. Enzymol.* 80, 754-764 (1981).

108. Glasscock, L.N., Gerlitz, B., Cooper, S.T., Grinnell, B.W. & Church, F.C. Basic residues in the 37-loop of activated protein C modulate inhibition by protein C inhibitor but not by alpha(1)-antitrypsin. *Biochim Biophys Acta* 1649, 106-17 (2003).

109. Hopkins, P.C.R., Crowther, D.C., Carrell, R.W. & Stone, S.R. Development of a novel recombinant serpin with potential antithrombotic properties. *J. Biol. Chem.* 270, 11866-11871 (1995).

110. Longstaff, C. & Gaffney, P.J. Serpin-serine protease binding kinetics: alpha 2-antiplasmin as a model inhibitor. *Biochemistry* 30, 979-86 (1991).

111. Rezaie, A.R. Role of exosites 1 and 2 in thrombin reaction with plasminogen activator inhibitor-1 in the absence and presence of cofactors. *Biochemistry* 38, 14592-9 (1999).

112. Hekman, C.M. & Loskutoff, D.J. Kinetic analysis of the interactions between plasminogen activator inhibitor 1 and both urokinase and tissue plasminogen activator. *Arch Biochem Biophys* 262, 199-210 (1988).

113. Im, H., Ahn, H.Y. & Yu, M.H. Bypassing the kinetic trap of serpin protein folding by loop extension. *Protein Sci* 9, 1497-502 (2000).

114. Carrell, R.W., Evans, D.L. & Stein, P. Mobile reactive centre of serpins and the control of thrombosis. *Nature* 353, 576-578 (1991).

115. Gettins, P.G. Serpin structure, mechanism, and function. *Chem Rev* 102, 4751-804 (2002).

116. Huntington, J.A. Shape-shifting serpins--advantages of a mobile mechanism. *Trends Biochem Sci* 31, 427-35 (2006).

117. Olson, S.T., Swanson, R., Day, D., Verhamme, I., Kvassman, J. & Shore, J.D. Resolution of Michaelis complex, acylation, and conformational change steps in the reactions of the serpin, plasminogen activator inhibitor-1, with tissue plasminogen activator and trypsin. *Biochemistry* 40, 11742-56 (2001).

118. Dementiev, A., Petitou, M., Herbert, J.M. & Gettins, P.G. The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. *Nat Struct Mol Biol* 11, 863-7 (2004).

119. Huntington, J.A., Read, R.J. & Carrell, R.W. Structure of a serpin-protease complex shows inhibition by deformation. *Nature* 407(2000).

120. Bock, P.E., Olsen, S.T. & Bjork, I. Inactivation of thrombin by antithrombin is accompanied by inactivation of regulatory exosite I. *J. Biol. Chem.* 272, 19837-19845 (1997).

121. Huntington, J.A. Mechanisms of glycosaminoglycan activation of the serpins in hemostasis. *J Thromb Haemost* 1, 1535-49 (2003).

122. Johnson, D.J., Langdown, J., Li, W., Luis, S.A., Baglin, T.P. & Huntington, J.A. Crystal structure of monomeric native antithrombin reveals a novel reactive center loop conformation. *J Biol Chem* (2006).

123. Quinsey, N.S., Greedy, A.L., Bottomley, S.P., Whisstock, J.C. & Pike, R.N. Antithrombin: in control of coagulation. *Int J Biochem Cell Biol* 36, 386-9 (2004).

124. Egeberg, O. Inherited antithrombin III deficiency and thromboembolism. *Thromb. Diath. Haemorrh.* 13, 516-530 (1965).

125. Haverkate, F. Levels of haemostatic factors, arteriosclerosis and cardiovascular disease. *Vascul Pharmacol* 39, 109-12 (2002).

126. Hogg, P.J. & Jackson, C.M. Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: implications for heparin efficacy. *Proc Natl Acad Sci U S A* 86, 3619-23 (1989).

127. Weitz, J.I. Activation of blood coagulation by plaque rupture: mechanisms and prevention. *Am J Cardiol* 75, 18B-22B (1995).

128. Weitz, J.I. Low-molecular-weight heparins. *N. Engl. J. Med.* 337, 688-698 (1997).

129. Lindahl, U. & Kjellen, L. Heparin or heparan sulfate: What is the difference? *Thromb. Haemost.* 66, 44-48 (1991).

130. Rosenberg, R.D., Armand, G. & Lam, L. Structure-function relationships of heparin species. *Proc. Natl. Acad. Sci. USA* 75, 3065-3069 (1978).

131. Belzar, K.J., Dafforn, T.R., Petitou, M., Carrell, R.W. & Huntington, J.A. The effect of reducing-end extension on pentasaccharide binding by antithrombin. *JBC* 275, 8733-8741 (2000).

132. McCoy, A.J., Pei, X.Y., Skinner, R., Abrahams, J.P. & Carrell, R.W. Structure of beta-antithrombin and the effect of glycosylation on antithrombin's heparin affinity and activity. *J Mol Biol* 326, 823-33 (2003).

133. Johnson, D.J., Li, W., Adams, T.E. & Huntington, J.A. Antithrombin-S195A factor Xa-heparin structure reveals the allosteric mechanism of antithrombin activation. *Embo J* 25, 2029-37 (2006).

134. Li, W., Johnson, D.J., Esmon, C.T. & Huntington, J.A. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat Struct Mol Biol* 11, 857-62 (2004).

135. Wiedermann Ch, J. & Romisch, J. The anti-inflammatory actions of antithrombin--a review. *Acta Med Austriaca* 29, 89-92 (2002).

136. O'Reilly, M.S., Pirie-Shepherd, S., Lane, W.S. & Folkman, J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. *Science* 285, 1926-8 (1999).

137. Zhang, W., Swanson, R., Xiong, Y., Richard, B. & Olson, S.T. Antiangiogenic antithrombin blocks the heparan sulfate-dependent binding of proangiogenic growth factors to their endothelial cell receptors: evidence for differential binding of antiangiogenic and anticoagulant forms of antithrombin to proangiogenic heparan sulfate domains. *J Biol Chem* 281, 37302-10 (2006).

138. van Boven, H.H. & Lane, D.A. Antithrombin and its inherited deficiency states. *Semin Hematol* 34, 188-204 (1997).

139. Lane, D.A., Bayston, T., Olds, R.J., Fitches, A.C., Cooper, D.N., Millar, D.S., Jochmans, K., Perry, D.J., Okajima, K., Thein, S.L. & Emmerich, J. Antithrombin mutation database: 2nd (1997) update. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost* 77, 197-211 (1997).

140. Corral, J., Huntington, J.A., Gonzalez-Conejero, R., Mushunje, A., Navarro, M., Marco, P., Vicente, V. & Carrell, R.W. Mutations in the shutter region of antithrombin result in formation of disulfide-linked dimers and severe venous thrombosis. *J Thromb Haemost* 2, 931-9 (2004).

141. Corral, J., Rivera, J., Guerrero, J.A., Minano, A., Alberca, I., Hernandez-Espinosa, D., Ordonez, A., Martinez, C., Navarro-Nunez, L., Gonzalez-Conejero, R., Lozano, M.L. & Vicente, V. Latent and polymeric antithrombin: clearance and potential thrombotic risk. *Exp Biol Med (Maywood)* 232, 219-26 (2007).

142. Corral, J., Hernandez-Espinosa, D., Soria, J.M., Gonzalez-Conejero, R., Ordonez, A., Gonzalez-Porras, J.R., Perez-Ceballos, E., Lecumberri, R., Sanchez, I., Roldan, V., Mateo, J., Minano, A., Gonzalez, M., Alberca, I., Fontcuberta, J. & Vicente, V. Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis. *Blood* (2007).

143. Kristensen, S.R., Rasmussen, B., Pedersen, S. & Bathum, L. Detecting antithrombin deficiency may be a difficult task - more than one test is necessary. *J Thromb Haemost* (2007).

144. Bates, S.M. & Weitz, J.I. The status of new anticoagulants. *Br J Haematol* 134, 3-19 (2006).

145. Arbit, E., Goldberg, M., Gomez-Orellana, I. & Majuru, S. Oral heparin: status review. *Thromb J* 4, 6 (2006).

146. Daneschvar, H.L. & Daw, H. Heparin-induced thrombocytopenia (an overview). *Int J Clin Pract* 61, 130-7 (2007).

147. Petitou, M., Herault, J.P., Bernat, A., Driguez, P.A., Duchaussoy, P., Lormeau, J.C. & Herbert, J.M. Synthesis of thrombin-inhibiting heparin mimetics without side effects. *Nature* 398, 417-22 (1999).

148. Weitz, J.I. Emerging anticoagulants for the treatment of venous thromboembolism. *Thromb Haemost* 96, 274-84 (2006).

149. Hampton, T. Agents to control bleeding show promise. *Jama* 297, 349-50 (2007).

150. Patel, S., Berry, L.R. & Chan, A.K. Covalent antithrombin-heparin complexes. *Thromb Res* (2006).

151. Maimone, M.M. & Tollefsen, D.M. Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *J Biol Chem* 265, 18263-71 (1990).

152. Baglin, T.P., Carrell, R.W., Church, F.C., Esmon, C.T. & Huntington, J.A. Crystal structures of native and thrombin-complexed heparin cofactor II reveal a multistep allosteric mechanism. *Proc Natl Acad Sci U S A* 99, 11079-84 (2002).

153. Tollefsen, D.M. Heparin cofactor II deficiency. *Arch Pathol Lab Med* 126, 1394-400 (2002).

154. He, L., Vicente, C.P., Westrick, R.J., Eitzman, D.T. & Tollefsen, D.M. Heparin cofactor II inhibits arterial thrombosis after endothelial injury. *J Clin Invest* 109, 213-9 (2002).

155. Schillinger, M., Exner, M., Sabeti, S., Mlekusch, W., Amighi, J., Handler, S., Quehenberger, P., Kalifeh, N., Wagner, O. & Minar, E. High plasma heparin cofactor II activity protects from restenosis after femoropopliteal stenting. *Thromb Haemost* 92, 1108-13 (2004).

156. Takamori, N., Azuma, H., Kato, M., Hashizume, S., Aihara, K., Akaike, M., Tamura, K. & Matsumoto, T. High plasma heparin cofactor II activity is associated with reduced incidence of in-stent restenosis after percutaneous coronary intervention. *Circulation* 109, 481-6 (2004).

157. Tollefsen, D.M. Insight into the mechanism of action of heparin cofactor II. *Thromb. Haemost.* 74, 1209-1214 (1995).

158. Buchanan, M.R. & Brister, S.J. Anticoagulant and antithrombin effects of intimatan, a heparin cofactor II agonist. *Thromb Res* 99, 603-12 (2000).

159. Mungall, D. Desmin 370 (Opocrin SpA/Alfa Wassermann). *IDrugs* 2, 579-83 (1999).

160. Pavao, M.S., Aiello, K.R., Werneck, C.C., Silva, L.C., Valente, A.P., Mulloy, B., Colwell, N.S., Tollefsen, D.M. & Mourao, P.A. Highly sulfated dermatan sulfates

from Ascidians. Structure versus anticoagulant activity of these glycosaminoglycans. *J Biol Chem* 273, 27848-57 (1998).

161. Fonseca, R.J. & Mourao, P.A. Fucosylated chondroitin sulfate as a new oral antithrombotic agent. *Thromb Haemost* 96, 822-9 (2006).

162. Church, F.C., Meade, J.B., Treanor, R.E. & Whinna, H.C. Antithrombin activity of fucoidan: The interaction of fucoidan with heparin cofactor II, antithrombin III, and thrombin. *J. Biol. Chem.* 264, 3618-3623 (1989).

163. Water, N., Tan, T., Ashton, F., O'Grady, A., Day, T., Browett, P., Ockelford, P. & Harper, P. Mutations within the protein Z-dependent protease inhibitor gene are associated with venous thromboembolic disease: a new form of thrombophilia. *Br J Haematol* 127, 190-4 (2004).

164. Al-Shanqeeti, A., van Hylckama Vlieg, A., Berntorp, E., Rosendaal, F.R. & Broze, G.J., Jr. Protein Z and protein Z-dependent protease inhibitor. Determinants of levels and risk of venous thrombosis. *Thromb Haemost* 93, 411-3 (2005).

165. Corral, J., Gonzalez-Conejero, R., Soria, J.M., Gonzalez-Porras, J.R., Perez-Ceballos, E., Lecumberri, R., Roldan, V., Souto, J.C., Minano, A., Hernandez-Espinosa, D., Alberca, I., Fontcuberta, J. & Vicente, V. A nonsense polymorphism in the protein Z-dependent protease inhibitor increases the risk for venous thrombosis. *Blood* 108, 177-83 (2006).

166. Kemkes-Matthes, B., Nees, M., Kuhnel, G., Matzdorff, A. & Matthes, K.J. Protein Z influences the prothrombotic phenotype in Factor V Leiden patients. *Thromb Res* 106, 183-5 (2002).

167. Huntington, J.A., Kjellberg, M. & Stenflo, J. Crystal structure of protein C inhibitor provides insights into hormone binding and heparin activation. *Structure (Camb)* 11, 205-15 (2003).

168. Laurell, M., Christensson, A., Abrahamsson, P.A., Stenflo, J. & Lilja, H. Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system. *J Clin Invest* 89, 1094-101 (1992).

169. Aznar, J., Espana, F., Estelles, A. & Royo, M. Heparin stimulation of the inhibition of activated protein C and other enzymes by human protein C inhibitor--influence of the molecular weightof heparin and ionic strength. *Thromb Haemost* 76, 983-8 (1996).

170. Espana, F., Estelles, A., Fernandez, P., Gilabert, J., Sanchez-Cuenca, J. & Griffin, J. Evidence for the regulation of urokinase and tissue type plasminogen activators by the serpin, protein C inhibitor, in semen and blood plasma. *Thrombosis and Hemostasis* 70, 989 (1993).

171. Pratt, C.W. & Church, F.C. Heparin binding to protein C inhibitor. *J. Biol. Chem.* 267, 8789-8794 (1992).

172. Ecke, S., Geiger, M., Resch, I., Jerabek, I., Sting, L., Maier, M. & Binder, B.R. Inhibition of tissue kallikrein by protein C inhibitor. Evidence for identity of protein C inhibitor with the kallikrein binding protein. *J Biol Chem* 267, 7048-52 (1992).

173. Zechmeister-Machhart, M., Hufnagl, P., Uhrin, P., Xu, J., Geiger, M. & Binder, B.R. Molecular cloning and tissue distribution of mouse protein C inhibitor (PCI). *Immunopharmacology* 32, 96-8 (1996).

174. Nishii, Y., Gabazza, E.C., Fujimoto, H., Nakahara, H., Takagi, T., Bruno, N., D'Alessandro-Gabazza, C.N., Maruyama, J., Maruyama, K., Hayashi, T., Adachi, T., Suzuki, K. & Taguchi, O. Protective role of protein C inhibitor in monocrotaline-induced pulmonary hypertension. *J. Thromb. Haemost.*, in press (2006).

175. Uhrin, P., Dewerchin, M., Hilpert, M., Chrenek, P., Schofer, C., Zechmeister-Machhart, M., Kronke, G., Vales, A., Carmeliet, P., Binder, B.R. & Geiger, M. Disruption of the protein C inhibitor gene results in impaired spermatogensis and male infertility. *J Clin Invest* 106, 1531-1539 (2000).

176. Kolbel, T., Strandberg, K., Mattiasson, I., Stenflo, J. & Lindblad, B. Activated protein C-protein C inhibitor complex: a new biological marker for aortic aneurysms. *J Vasc Surg* 43, 935-9 (2006).

177. Bhiladvala, P., Strandberg, K., Stenflo, J. & Holm, J. Early identification of acute myocardial infarction by activated protein C--protein C inhibitor complex. *Thromb Res* 118, 213-9 (2006).

178. Nilsson, G., Strandberg, K., Astermark, J., Vernersson, E., Stenflo, J. & Berntorp, E. The APC-PCI complex concentration predicts outcome of aortic surgery. *Thromb Res* (2006).

179. Strandberg, K., Stenflo, J., Nilsson, C. & Svensson, P.J. APC-PCI complex concentration is higher in patients with previous venous thromboembolism with Factor V Leiden. *J Thromb Haemost* 3, 2578-80 (2005).

180. Carroll, V.A., Griffiths, M.R., Geiger, M., Merlo, C., Furlan, M., Lammle, B. & Binder, B.R. Plasma protein C inhibitor is elevated in survivors of myocardial infarction. *Arterioscler Thromb Vasc Biol* 17, 114-8 (1997).

181. Beatty, K., Bieth, J. & Travis, J. Kinetics of association of serine proteinases with native and oxidized alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin. *J Biol Chem* 255, 3931-4 (1980).

182. Burghaus, B., Langer, C., Thedieck, S. & Nowak-Gottl, U. Elevated alpha1-antitrypsin is a risk factor for arterial ischemic stroke in childhood. *Acta Haematol* 115, 186-91 (2006).

183. Owen, M.C., Brennan, S.O., Lewis, J.H. & Carrell, R.W. Mutation of antitrypsin to antithrombin. Alpha1-antitrypsin Pittsburgh (358 Met to Arg), a fatal bleeding disorder. *N Engl J Med* 309, 694-698 (1983).

184. Travis, J., Matheson, N.R., George, P.M. & Carrell, R.W. Kinetic studies on the interaction of alpha1-proteinase inhibitor (Pittsburgh) with trypsin-like serine proteinases. *Biol Chem Hoppe Seyler* 367, 853-859 (1986).

185. Vidaud, D., Emmerich, J., Alhenc-Gelas, M., Yvart, J., Fiessinger, J.N. & Aiach, M. Met 358 to Arg mutation of alpha 1-antitrypsin associated with protein C deficiency in a patient with mild bleeding tendency. *J Clin Invest* 89, 1537-43 (1992).

186. Heeb, M.J., Bischoff, R., Courtney, M. & Griffin, J.H. Inhibition of activated protein C by recombinant alpha-1-antitrypsin variants with substitution of arginine or leucine for methionine(356). *J. Biol. Chem.* 265, 2365-2369 (1990).

187. Aoki, N. Genetic abnormalities of the fibrinolytic system. *Semin Thromb Hemost* 10, 42-50 (1984).

188. Collen, D. & Wiman, B. Turnover of antiplasmin, the fast-acting plasmin inhibitor of plasma. *Blood* 53, 313-24 (1979).

189. Miles, L.A., Plow, E.F., Donnelly, K.J., Hougie, C. & Griffin, J.H. A bleeding disorder due to deficiency of alpha 2-antiplasmin. *Blood* 59, 1246-51 (1982).

190. Holmes, W.E., Nelles, L., Lijnen, H.R. & Collen, D. Primary structure of human alpha 2-antiplasmin, a serine protease inhibitor (serpin). *J Biol Chem* 262, 1659-64 (1987).

191. van Mourik, J.A., Lawrence, D.A. & Loskutoff, D.J. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J Biol Chem* 259, 14914-21 (1984).

192. Sprengers, E.D. & Kluft, C. Plasminogen activator inhibitors. *Blood* 69, 381-387 (1987).

193. Fay, W.P. Plasminogen activator inhibitor 1, fibrin, and the vascular response to injury. *Trends Cardiovasc Med* 14, 196-202 (2004).

194. Lawrence, D.A., Palaniappan, S., Stefansson, S., Olson, S.T., Francis-Chmura, A.M., Shore, J.D. & Ginsburg, D. Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J Biol Chem* 272, 7676-80 (1997).

195. Keijer, J., Ehrlich, H.J., Linders, M., Preissner, K.T. & Pannekoek, H. Vitronectin Governs the Interaction between Plasminogen Activator Inhibitor 1 and Tissue-type Plasminogen Activator. *JBC* 266, 10700-10707 (1991).

196. Zhou, A., Huntington, J.A., Pannu, N.S., Carrell, R.W. & Read, R.J. How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat Struct Biol* 10, 541-4 (2003).

197. Heimark, R.L., Kurachi, K., Fujikawa, K. & Davie, E.W. Surface activation of blood coagulation, fibrinolysis and kinin formation. *Nature* 286, 456-460 (1980).

198. Sharp, A.M., Stein, P.E., Pannu, N.S., Carrell, R.W., Berkenpas, M.B., Ginsburg, D., Lawrence, D.A. & Read, R.J. The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. *Structure* 7, 111-8 (1999).

199. Nar, H., Bauer, M., Stassen, J.M., Lang, D., Gils, A. & Declerck, P.J. Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and implications for serpin inactivation. *J Mol Biol* 297, 683-95 (2000).

200. Stout, T.J., Graham, H., Buckley, D.I. & Matthews, D.J. Structures of active and latent PAI-1: a possible stabilizing role for chloride ions. *Biochemistry* 39, 8460-9 (2000).

201. Hagglof, P., Bergstrom, F., Wilczynska, M., Johansson, L.B. & Ny, T. The reactive-center loop of active PAI-1 is folded close to the protein core and can be partially inserted. *J Mol Biol* 335, 823-32 (2004).

202. de Fouw, N.J., van Hinsbergh, V.W.M., de Jong, Y.F., Haverkate, F. & Bertina, R.M. The interaction of activated protein C and thrombin with the plasminogen activator inhibitor released from human endothelial cells. *Thromb. Haemost.* 57, 176-182 (1987).

203. Sakata, Y., Loskutoff, D.J., Gladson, C.L., Hekman, C.M. & Griffin, J.H. Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. *Blood* 68, 1218-23 (1986).

204. Mertens, I., Verrijken, A., Michiels, J.J., Van der Planken, M., Ruige, J.B. & Van Gaal, L.F. Among inflammation and coagulation markers, PAI-1 is a true component of the metabolic syndrome. *Int J Obes (Lond)* (2006).

205. Bourcier, T. & Libby, P. HMG CoA reductase inhibitors reduce plasminogen activator inhibitor-1 expression by human vascular smooth muscle and endothelial cells. *Arterioscler Thromb Vasc Biol* 20, 556-62 (2000).

206. Wolfrum, S., Jensen, K.S. & Liao, J.K. Endothelium-dependent effects of statins. *Arterioscler Thromb Vasc Biol* 23, 729-36 (2003).

207. Oishi, K., Shirai, H. & Ishida, N. Identification of the circadian clock-regulated E-box element in the mouse plasminogen activator inhibitor-1 gene. *J Thromb Haemost* 5, 428-31 (2007).

208. Kaikita, K., Fogo, A.B., Ma, L., Schoenhard, J.A., Brown, N.J. & Vaughan, D.E. Plasminogen activator inhibitor-1 deficiency prevents hypertension and vascular fibrosis in response to long-term nitric oxide synthase inhibition. *Circulation* 104, 839-44 (2001).

209. Moore, J.H., Smolkin, M.E., Lamb, J.M., Brown, N.J. & Vaughan, D.E. The relationship between plasma t-PA and PAI-1 levels is dependent on epistatic effects of the ACE I/D and PAI-1 4G/5G polymorphisms. *Clin Genet* 62, 53-9 (2002).

210. Moreno, R., Fernandez, C., Sanchez-Recalde, A., Galeote, G., Calvo, L., Alfonso, F., Hernandez, R., Sanchez-Aquino, R., Angiolillo, D.J., Villarreal, S., Macaya, C. & Lopez-Sendon, J.L. Clinical impact of in-stent late loss after drug-eluting coronary stent implantation. *Eur Heart J* (2007).

211. Joner, M., Finn, A.V., Farb, A., Mont, E.K., Kolodgie, F.D., Ladich, E., Kutys, R., Skorija, K., Gold, H.K. & Virmani, R. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. *J Am Coll Cardiol* 48, 193-202 (2006).

212. Muldowney, J.A., 3rd, Stringham, J.R., Levy, S.E., Gleaves, L.A., Eren, M., Piana, R.N. & Vaughan, D.E. Antiproliferative agents alter vascular plasminogen activator inhibitor-1 expression: a potential prothrombotic mechanism of drug-eluting stents. *Arterioscler Thromb Vasc Biol* 27, 400-6 (2007).

213. Chambers, S.K., Ivins, C.M. & Carcangiu, M.L. Plasminogen activator inhibitor-1 is an independent poor prognostic factor for survival in advanced stage epithelial ovarian cancer patients. *Int J Cancer* 79, 449-54. (1998).

214. Costantini, V., Sidoni, A., Deveglia, R., Cazzato, O.A., Bellezza, G., Ferri, I., Bucciarelli, E. & Nenci, G.G. Combined overexpression of urokinase, urokinase receptor, and plasminogen activator inhibitor-1 is associated with breast cancer progression: an immunohistochemical comparison of normal, benign, and malignant breast tissues. *Cancer* 77, 1079-88. (1996).

215. Vaughan, D.E. PAI-1 and atherothrombosis. *J Thromb Haemost* 3, 1879-83 (2005).

216. Ma, J., Weisberg, A., Griffin, J.P., Vaughan, D.E., Fogo, A.B. & Brown, N.J. Plasminogen activator inhibitor-1 deficiency protects against aldosterone-induced glomerular injury. *Kidney Int* 69, 1064-72 (2006).

217. Gils, A. & Declerck, P.J. The structural basis for the pathophysiological relevance of PAI-I in cardiovascular diseases and the development of potential PAI-I inhibitors. *Thromb Haemost* 91, 425-37 (2004).

218. Xiang, G., Schuster, M.D., Seki, T., Witkowski, P., Eshghi, S. & Itescu, S. Downregulated expression of plasminogen activator inhibitor-1 augments myocardial neovascularization and reduces cardiomyocyte apoptosis after acute myocardial infarction. *J Am Coll Cardiol* 46, 536-41 (2005).

219. Bjorquist, P., Ehnebom, J., Inghardt, T., Hansson, L., Lindberg, M., Linschoten, M., Stromqvist, M. & Deinum, J. Identification of the binding site for a low-molecular-weight inhibitor of plasminogen activator inhibitor type 1 by site-directed mutagenesis. *Biochemistry* 37, 1227-34 (1998).

220. Einholm, A.P., Pedersen, K.E., Wind, T., Kulig, P., Overgaard, M.T., Jensen, J.K., Bodker, J.S., Christensen, A., Charlton, P. & Andreasen, P.A. Biochemical mechanism of action of a diketopiperazine inactivator of plasminogen activator inhibitor-1. *Biochem J* 373, 723-32 (2003).

221. Elokdah, H., Abou-Gharbia, M., Hennan, J.K., McFarlane, G., Mugford, C.P., Krishnamurthy, G. & Crandall, D.L. Tiplaxtinin, a novel, orally efficacious inhibitor of plasminogen activator inhibitor-1: design, synthesis, and preclinical characterization. *J Med Chem* 47, 3491-4 (2004).

222. Folkes, A., Roe, M.B., Sohal, S., Golec, J., Faint, R., Brooks, T. & Charlton, P. Synthesis and in vitro evaluation of a series of diketopiperazine inhibitors of plasminogen activator inhibitor-1. *Bioorg Med Chem Lett* 11, 2589-92 (2001).

223. Friederich, P.W., Levi, M., Biemond, B.J., Charlton, P., Templeton, D., van Zonneveld, A.J., Bevan, P., Pannekoek, H. & ten Cate, J.W. Novel low-molecular-weight inhibitor of PAI-1 (XR5118) promotes endogenous fibrinolysis and reduces postthrombolysis thrombus growth in rabbits. *Circulation* 96, 916-21 (1997).

224. Gils, A., Stassen, J.M., Nar, H., Kley, J.T., Wienen, W., Ries, U.J. & Declerck, P.J. Characterization and comparative evaluation of a novel PAI-1 inhibitor. *Thromb Haemost* 88, 137-43 (2002).

225. Leik, C.E., Su, E.J., Nambi, P., Crandall, D.L. & Lawrence, D.A. Effect of pharmacologic plasminogen activator inhibitor-1 inhibition on cell motility and tumor angiogenesis. *J Thromb Haemost* 4, 2710-5 (2006).

226. Liang, A., Wu, F., Tran, K., Jones, S.W., Deng, G., Ye, B., Zhao, Z., Snider, R.M., Dole, W.P., Morser, J. & Wu, Q. Characterization of a small molecule PAI-1 inhibitor, ZK4044. *Thromb Res* 115, 341-50 (2005).

227. Pedersen, K.E., Einholm, A.P., Christensen, A., Schack, L., Wind, T., Kenney, J.M. & Andreasen, P.A. Plasminogen activator inhibitor-1 polymers, induced by inactivating amphipathic organochemical ligands. *Biochem J* 372, 747-55 (2003).

228. Church, F.C., Noyes, C.M. & Griffith, M.J. Inhibition of chymotrypsin by heparin cofactor II. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6431-6434 (1985).

229. Parker, K.A. & Tollefsen, D.M. The protease specificity of heparin cofactor II. Inhibition of thrombin generated during coagulation. *J. Biol. Chem.* 260, 3501-3505 (1985).

230. Toulon, P., Chadeuf, G., Bouillot, J.L., Amiral, J., Cambillau, M., Sultan, Y. & Aiach, M. Involvement of heparin cofactor II in chymotrypsin neutralization and in the pancreatic proteinase-antiproteinase interaction during acute pancreatitis in man. *Eur. J. Clin. Invest.* 21, 303-309 (1991).

231. Patston, P.A., Church, F.C. & Olson, S.T. Serpin-ligand interactions. *Methods* 32, 93-109 (2004).

232. Kresse, H., Hausser, H. & Schonherr, E. Small proteoglycans. *Exs* (1994).

233. Tollefsen, D.M. The interaction of glycosaminoglycans with heparin cofactor II: structure and activity of a high-affinity dermatan sulfate hexasaccharide. *Adv. Exp. Med. Biol.* 313, 167-176 (1992).

234. Cardin, A.D., Demeter, D.A., Weintraub, H.J.R. & Jackson, R.L. Molecular design and modeling of protein-heparin interactions. *Meth Enzymol* 203, 556-583 (1991).

235. Casu, B. Structure and biological activity of heparin and other glycosaminoglycans. *Pharmacol Res Commun* 11, 1-18 (1979).

236. Höök, M., Kjellén, L., Johansson, S. & Robinson, J. Cell-Surface Glycosaminoglycans. *Ann. Rev. Biochem.* 53, 847-869 (1984).

237. Liu, L., Dewar, L., Song, Y., Kulczycky, M., Blajchman, M.A., II, J.W.F., Andrew, M., Delorme, M., Ginsberg, J., Preissner, K.T. & Ofosu, F.A. Inhibition of thrombin by antithrombin III and heparin cofactor II in vivo. *Thromb Haemost* 73, 405-412 (1995).

238. Villa, P., Aznar, J., Espana, F., Ferrando, F., Mira, Y. & Estelles, A. Hereditary homozygous heparin cofactor II deficiency and the risk of developing venous thrombosis. *Thromb. Haemost.* 82, 1011-1014 (1999).

239. Corral, J., Aznar, J., Gonzalez-Conejero, R., Villa, P., Minano, A., Vaya, A., Carrell, R.W., Huntington, J.A. & Vicente, V. Homozygous deficiency of heparin cofactor II: relevance of P17 glutamate residue in serpins, relationship with conformational diseases, and role in thrombosis. *Circulation* 110, 1303-7 (2004).

240. Tanaka, K.A., Szlam, F., Vinten-Johansen, J., Cardin, A.D. & Levy, J.H. Effects of antithrombin and heparin cofactor II levels on anticoagulation with Intimatan. *Thromb Haemost* 94, 808-13 (2005).

241. Giri, T.K. & Tollefsen, D.M. Placental dermatan sulfate: isolation, anticoagulant activity, and association with heparin cofactor II. *Blood* 107, 2753-8 (2006).

242. Massouh, M., Jatoi, A., Gordon, E.M. & Ratnoff, O.D. Heparin cofactor II activity in plasma during pregnancy and oral contraceptive use. *J Lab Clin Med* 114, 697-9 (1989).

243. Andersson, T., Lorentzen, B., Hogdahl, H., Clausen, T., Mowinckel, M.C. & Abildgaard, U. Thrombin-inhibitor complexes in the blood during and after delivery. *Thromb. Res.* 82, 109-117 (1996).

244. Andrew, M., Mitchell, L., Berry, L., Paes, B., Delorme, M., Ofosu, F., Burrows, R. & Khambalia, B. An anticoagulant dermatan sulfate proteoglycan circulates in the pregnant woman and her fetus. *J Clin Invest* 89, 321-6 (1992).

245. Bellart, J., Gilabert, R., Cabero, L., Fontcuberta, J., Monasterio, J. & Miralles, R.M. Heparin cofactor II: a new marker for pre-eclampsia. *Blood Coagul Fibrinolysis* 9, 205-8 (1998).

246. He, I. & Tollefsen, D.M. Heparin cofactor II-deficient mice are viable and fertile. *Blood*, 45a (2000).

247. Huang, P.H., Leu, H.B., Chen, J.W., Wu, T.C., Lu, T.M., Yu-An Ding, P. & Lin, S.J. Decreased heparin cofactor II activity is associated with impaired endothelial function determined by brachial ultrasonography and predicts cardiovascular events. *Int J Cardiol* 114, 152-8 (2007).

248. Giri, T.K., Ahn, C.W., Wu, K.K. & Tollefsen, D.M. Heparin cofactor II levels do not predict the development of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *Arterioscler Thromb Vasc Biol* 25, 2689-90 (2005).

249. Strong, J.P., Malcom, G.T., McMahan, C.A., Tracy, R.E., Newman, W.P., Herderick, E.E. & Cornhill, J.F. Prevalence and extent of atherosclerosis in adolescents and young adults. Implications for prevention from the pathobiological determinants of atherosclerosis in youth study. *JAMA* 281, 727-735 (1999).

250. McGill, H.C., Jr. & McMahan, C.A. Determinants of atherosclerosis in the young. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *Am J Cardiol* 82, 30T-36T (1998).

251. Strong, J.P., Malcom, G.T., Oalmann, M.C. & Wissler, R.W. The PDAY Study: natural history, risk factors, and pathobiology. Pathobiological Determinants of Atherosclerosis in Youth. *Ann N Y Acad Sci* 811, 226-35; discussion 235-7 (1997).

252. Cornhill, J.F., Herderick, E.E. & Vince, D.G. The clinical morphology of human atherosclerotic lesions. Lessons from the PDAY Study. Pathobiological Determinants of Atherosclerosis in Youth. *Wien Klin Wochenschr* 107, 540-3 (1995).

253. Wissler, R.W. New insights into the pathogenesis of atherosclerosis as revealed by PDAY. Pathobiological Determinants of Atherosclerosis in Youth. *Atherosclerosis* 108 Suppl, S3-20 (1994).

254. Griffith, M.J., Noyes, C.M. & Church, F.C. Reactive site peptide structural similarity between heparin cofactor II and antithrombin III. *J. Biol. Chem.* 260, 2218-2225 (1985).

255. Church, F.C. & Whinna, H.C. Rapid sulfopropyl-disk chromatographic purification of bovine and human thrombin. *Anal. Biochem.* 157, 77-83 (1986).

256. Shirk, R.A., Church, F.C. & Wagner, W.D. Arterial smooth muscle cell heparan sulfate proteoglycans accelerate thrombin inhibition by heparin cofactor II. *Arterioscler. Thromb. Vasc. Biol.* 16, 1138-1146 (1996).

257. Feletou, M. & Vanhoutte, P.M. Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol* 291, H985-1002 (2006).

258. O'Brien, K.D., Olin, K.L., Alpers, C.E., Chiu, W., Ferguson, M., Hudkins, K., Wight, T.N. & Chait, A. Comparison of apolipoprotein and proteoglycan deposits in human coronary atherosclerotic plaques: colocalization of biglycan with apolipoproteins. *Circulation* 98, 519-27 (1998).

259. Wyler von Ballmoos, M., Dubler, D., Mirlacher, M., Cathomas, G., Muser, J. & Biedermann, B.C. Increased apolipoprotein deposits in early atherosclerotic lesions distinguish symptomatic from asymptomatic patients. *Arterioscler Thromb Vasc Biol* 26, 359-64 (2006).

260. Sun, H., Koike, T., Ichikawa, T., Hatakeyama, K., Shiomi, M., Zhang, B., Kitajima, S., Morimoto, M., Watanabe, T., Asada, Y., Chen, Y.E. & Fan, J. C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. *Am J Pathol* 167, 1139-48 (2005).

261. Burnett, M.S., Lee, C.W., Kinnaird, T.D., Stabile, E., Durrani, S., Dullum, M.K., Devaney, J.M., Fishman, C., Stamou, S., Canos, D., Zbinden, S., Clavijo, L.C., Jang, G.J., Andrews, J.A., Zhu, J. & Epstein, S.E. The potential role of resistin in atherogenesis. *Atherosclerosis* 182, 241-8 (2005).

262. Kaikita, K., Takeya, M., Ogawa, H., Suefuji, H., Yasue, H. & Takahashi, K. Co-localization of tissue factor and tissue factor pathway inhibitor in coronary atherosclerosis. *J Pathol* 188, 180-8 (1999).

263. Hunt, L.T. & Dayhoff, M.O. A surprising new protein superfamily containing ovalbumin. *Biochem Biophys Res Commun* 95, 864-871 (1980).

264. Huber, R. & Carrell, R.W. Implications of the three-dimensional structure of alpha1-antitrypsin for structure and function of serpins. *Biochem* 28, 8951-8966 (1989).

265. Irving, J.A., Pike, R.N., Lesk, A.M. & Whisstock, J.C. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* 10, 1845-64 (2000).

266. Huntington, J.A., Olson, S.T., Fan, B. & Gettins, P.G. Mechanism of heparin activation of antithrombin. Evidence for reactive center loop preinsertion with expulsion upon heparin binding. *Biochemistry* 35, 8495-503 (1996).

267. Huntington, J.A., McCoy, A., Belzar, K.J., Pei, X.Y., Gettins, P.G.W. & Carrell, R.W. The conformational activation of antithrombin. *JBC* 275, 15377-15383 (2000).

268. Izaguirre, G., Swanson, R., Raja, S.M., Rezaie, A.R. & Olson, S.T. Mechanism by which exosites promote the inhibition of blood coagulation proteases by heparin-activated antithrombin. *J Biol Chem* 282, 33609-22 (2007).

269. Tollefsen, D.M. & Blank, M.K. Detection of a new heparin-dependent inhibitor of thrombin in human plasma. *J. Clin. Invest.* 68, 589-596 (1981).

270. Griffith, M.J., Carraway, T., White, G.C. & Dombrose, F.A. Heparin cofactor activities in a family with hereditary antithrombin III deficiency: Evidence for a second heparin cofactor in human plasma. *Blood* 61, 111-118 (1983).

271. Ragg, H. A new member of the plasma protease inhibitor gene family. *Nuc. Acids Res.* 14, 1073-1087 (1986).

272. Blinder, M.A., Marasa, J.C., Reynolds, C.H., Deaven, L.L. & Tollefsen, D.M. Heparin cofactor II: cDNA sequence, chromosome localization, restriction fragment length polymorphism, and expression in *Escherichia coli*. *Biochem* 27, 752-759 (1988).

273. Griffith, M.J., Noyes, C.M., Tyndall, J.A. & Church, F.C. Structural evidence for leucine at the reactive site of heparin cofactor II. *Biochemistry* 24, 6777-6782 (1985).

274. Tollefsen, D.M., Pestka, C.A. & Monafo, W.J. Activation of heparin cofactor II by dermatan sulfate. *J. Biol. Chem.* 258, 6713-6716 (1983).

275. Blinder, M.A. & Tollefsen, D.M. Site-directed mutagenesis of arginine 103 and lysine 185 in the proposed glycosaminoglycan-binding site of heparin cofactor II. *J. Biol. Chem.* 265, 286-291 (1990).

276. Ragg, H., Ulshofer, T. & Gerewitz, J. Glycosaminoglycan-mediated leuserpin-2/thrombin interaction: Structure-function relationships. *J. Biol. Chem.* 265, 22386-22391 (1990).

277. Ragg, H., Ulshofer, T. & Gerewitz, J. On the activation of human leuserpin-2, a thrombin inhibitor, by glycosaminoglycans. *J. Biol. Chem.* 265, 5211-5218 (1990).

278. Blinder, M.A., Andersson, T.R., Abildgaard, U. & Tollefsen, D.M. Heparin cofactor II Oslo. Mutation of Arg-189 to His decreases the affinity for dermatan sulfate. *J. Biol. Chem.* 264, 5128-5133 (1989).

279. Whinna, H.C., Blinder, M.A., Szewczyk, M., Tollefsen, D.M. & Church, F.C. Role of lysine 173 in heparin binding to heparin cofactor II. *J. Biol. Chem.* 266, 8129-8135 (1991).

280. Verhamme, I.M., Bock, P.E. & Jackson, C.M. The preferred pathway of glycosaminoglycan-accelerated inactivation of thrombin by heparin cofactor II. *J Biol Chem* 279, 9785-95 (2004).

281. Grasberger, H., Buettner, C. & Janssen, O.E. Modularity of Serpins. *JBC* 274, 15046-15051 (1999).

282. Colwell, N.S., Grupe, M.J. & Tollesfen, D.M. Amino acid residues of heparin cofactor II required for stimulation of thrombin inhibition by sulphated polyanions. *Biochimica at Biophysia Acta* 1431, 148-156 (1999).

283. Lee, K.N., Im, H., Kang, S.W. & Yu, M.H. Characterization of a human alpha1-antitrypsin variant that is as stable as ovalbumin. *J Biol Chem* 273, 2509-16 (1998).

284. Peterson, F.C., Gordon, N.C. & Gettins, P.G. Formation of a noncovalent serpin-proteinase complex involves no conformational change in the serpin. Use of 1H-15N HSQC NMR as a sensitive nonperturbing monitor of conformation. *Biochemistry* 39, 11884-92 (2000).

285. Kim, S., Woo, J., Seo, E.J., Yu, M. & Ryu, S. A 2.1 A resolution structure of an uncleaved alpha(1)-antitrypsin shows variability of the reactive center and other loops. *J Mol Biol* 306, 109-19 (2001).

286. Dementiev, A., Simonovic, M., Volz, K. & Gettins, P.G. Canonical inhibitor-like interactions explain reactivity of alpha1-proteinase inhibitor Pittsburgh and antithrombin with proteinases. *J Biol Chem* 278, 37881-7 (2003).

287. Bauman, S.J. & Church, F.C. Enhancement of heparin cofactor II anticoagulant activity. *J Biol Chem* 274, 34556-34565 (1999).

288. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S.R. & Hofsteenge, J. The refined 1.9 Å structure of human a-thrombin: interaction with D-Phe-Pro-Arg

chloromethyl ketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* 8, 3467-3475 (1989).

289. Leung, L.L. & Hall, S.W. Dissociation of thrombin's substrate interactions using site-directed mutagenesis. *Trends Cardiovasc Med* 10, 89-92 (2000).

290. Stefansson, S., Yepes, M., Gorlatova, N., Day, D.E., Moore, E.G., Zabaleta, A., McMahon, G.A. & Lawrence, D.A. Mutants of plasminogen activator inhibitor-1 designed to inhibit neutrophil elastase and cathepsin G are more effective in vivo than their endogenous inhibitors. *J Biol Chem* 279, 29981-7 (2004).

291. Ciaccia, A.V., Cunningham, E.L. & Church, F.C. Characterization of recombinant heparin cofactor II expressed in insect cells. *Protein Express. Purific.* 6, 806-812 (1995).

292. Olson, S.T., Srinivasan, K.R., Bjork, I. & Shore, J.D. Binding of high affinity heparin to antithrombin-III. *J. Biol. Chem.* 256, 11073-11079 (1981).

293. Bode, W., Turk, D. & Karshikov, A. The refined 1.9-Å X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human a-thrombin: Structural analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. *Protein Sci.* 1, 426-471 (1992).

294. Church, F.C., Pratt, C.W., Noyes, C.M., Kalayanamit, T., Sherrill, G.B., Tobin, R.B. & Meade, J.B. Structural and functional properties of human alpha-thrombin, phosphopyridoxylated alpha-thrombin, and gamma-thrombin: identification of lysyl residues in alpha-thrombin that are critical for heparin and fibrin(ogen) interactions. *J Biol Chem* 264, 18419-18425 (1989).

295. Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. & Fenton, J.W., II. The structure of a complex of recombinant hirudin and human a-thrombin. *Science* 249, 277-280 (1990).

296. Sheehan, J.P. & Sadler, J.E. Molecular mapping of the heparin-binding exosite of thrombin. *Proc. Natl. Acad. Sci., U.S.A.* 91, 5518-5522 (1994).

297. van Deerlin, V.M.D. & Tollefsen, D.M. The N-terminal acidic domain of heparin cofactor II mediates the inhibition of a-thrombin in the presence of glycosaminoglycans. *J. Biol. Chem.* 266, 20223-20231 (1991).

298. Sheehan, J.P., Tollefsen, D.M. & Sadler, J.E. Heparin cofactor II is regulated allosterically and not primarily by template effects. Studies with mutant thrombins and glycosaminoglycans. *J. Biol. Chem.* 269, 32747-32751 (1994).

299. Fortenberry, Y.M., Whinna, H.C., Gentry, H.R., Myles, T., Leung, L.L. & Church, F.C. Molecular mapping of the thrombin-heparin cofactor II complex. *J Biol Chem* 279, 43237-44 (2004).

300. Pratt, C.W., Whinna, H.C., Meade, J.M., Treanor, R.E. & Church, F.C. Physicochemical aspects of heparin cofactor II. *Ann. N.Y. Acad. Sci.* 556, 104-114 (1989).

301. McCarthy, B.J. & Worrall, D.M. Analysis of serpin inhibitory function by mutagenesis of ovalbumin and generation of chimeric ovalbumin/PAI-2 fusion proteins. *J Mol Biol* 267, 561-9 (1997).

302. Sutherland, J.S., Bhakta, V., Filion, M.L. & Sheffield, W.P. The transferable tail: fusion of the N-terminal acidic extension of heparin cofactor II to alpha1-proteinase inhibitor M358R specifically increases the rate of thrombin inhibition. *Biochemistry* 45, 11444-52 (2006).

303. Liaw, P.C.Y., Austin, R.C., Fredenburgh, J.C., Stafford, A.R. & Weitz, J.I. Comparison of heparin- and dermatan sulfate-mediated catalysis of thrombin inactivation by heparin cofactor II. *J. Biol. Chem.* 274, 27597-27604 (1999).

304. Whinna, H.C. & Church, F.C. Interaction of thrombin with antithrombin, heparin cofactor II and protein C inhibitor. *J. Prot. Chem.* 12, 677-688 (1993).

305. Fredenburgh, J.C., Stafford, A.R. & Weitz, J.I. Evidence for allosteric linkage between exosites 1 and 2 of thrombin. *J. Biol. Chem.* 272, 25493-25499 (1997).

306. Verhamme, I.M., Olson, S.T., Tollefsen, D.M. & Bock, P.E. Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II. *J Biol Chem* 277, 6788-98 (2002).

307. Jin, L., Abrahams, J.P., Skinner, R., Petitou, M. & Pike, R.N. The anticoagulant activation of antithrombin by heparin. *Proc. Natl. Acad. Sci. USA* 94, 14683-14688 (1997).

308. van Boeckel, C.A.A., Grootenhuis, P.D.J. & Visser, A. A mechanism for heparin-induced potentiation of antithrombin III. *Nature Struct. Biol.* 1, 423-425 (1994).

309. Belzar, K.J., Zhou, A., Carrell, R.W., Gettins, P.G. & Huntington, J.A. Helix D elongation and allosteric activation of antithrombin. *J Biol Chem* 277, 8551-8 (2002).

310. Albers, G.W., Easton, J.D., Sacco, R.L. & Teal, P. Antithrombotic and thrombolytic therapy for ischemic stroke. *Chest* 114, 683S-698S (1998).

311. Sacco, R.L., Adams, R., Albers, G., Alberts, M.J., Benavente, O., Furie, K., Goldstein, L.B., Gorelick, P., Halperin, J., Harbaugh, R., Johnston, S.C., Katzan, I., Kelly-Hayes, M., Kenton, E.J., Marks, M., Schwamm, L.H. & Tomsick, T. Guidelines for prevention of stroke in patients with ischemic stroke or transient ischemic attack: a statement for healthcare professionals from the American Heart Association/American Stroke Association Council on Stroke: co-sponsored by the Council on Cardiovascular Radiology and Intervention: the American Academy of Neurology affirms the value of this guideline. *Stroke* 37, 577-617 (2006).

312. Braunwald, E., Antman, E.M., Beasley, J.W., Califf, R.M., Cheitlin, M.D., Hochman, J.S., Jones, R.H., Kereiakes, D., Kupersmith, J., Levin, T.N., Pepine, C.J., Schaeffer, J.W., Smith, E.E., 3rd, Steward, D.E., Theroux, P., Gibbons, R.J., Alpert, J.S., Faxon, D.P., Fuster, V., Gregoratos, G., Hiratzka, L.F., Jacobs, A.K. & Smith, S.C., Jr. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction--summary article: a report of the American College of Cardiology/American Heart Association task force on practice guidelines (Committee on the Management of Patients With Unstable Angina). *J Am Coll Cardiol* 40, 1366-74 (2002).

313. Buller, H.R., Agnelli, G., Hull, R.D., Hyers, T.M., Prins, M.H. & Raskob, G.E. Antithrombotic therapy for venous thromboembolic disease: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest* 126, 401S-428S (2004).

314. Ng, H.J. & Crowther, M.A. New anti-thrombotic agents: emphasis on hemorrhagic complications and their management. *Semin Hematol* 43, S77-83 (2006).

315. Seligsohn, U., Berger, A., Abend, M., Rubin, L., Attias, D., Zivelin, A. & Rapaport, S.I. Homozygous protein C deficiency manifested by massive venous thrombosis in the newborn. *New Eng. J. Med.* 310, 559-562 (1984).

316. Broekmans, A.W., Veltkamp, J.J. & Bertina, R.M. Congenital protein C deficiency and venous thromboembolism. A study of three Dutch families. *N Engl J Med* 309, 340-4 (1983).

317. Seligsohn, U., Berger, A., Abend, M., Rubin, L., Attias, D., Zivelin, A. & Rapaport, S.I. Homozygous protein C deficiency manifested by massive venous thrombosis in the newborn. *N Engl J Med* 310, 559-62 (1984).

318. Berg, D.T., Gerlitz, B., Shang, J., Smith, T., Santa, P., Richardson, M.A., Kurz, K.D., Grinnell, B.W., Mace, K. & Jones, B.E. Engineering the proteolytic specificity of activated protein C improves its pharmacological properties. *Proc Natl Acad Sci U S A* 100, 4423-8 (2003).

319. Liu, D., Cheng, T., Guo, H., Fernandez, J.A., Griffin, J.H., Song, X. & Zlokovic, B.V. Tissue plasminogen activator neurovascular toxicity is controlled by activated protein C. *Nat Med* 10, 1379-83 (2004).

320. Lo, E.H. Combination stroke therapy: easy as APC? *Nat Med* 10, 1295-6 (2004).

321. Fenton, J.W., II, Olson, T.A., Zabinski, M.P. & Wilner, G.D. Anion-binding exosite of human a-thrombin and fibrin(ogen) recognition. *Biochemistry* 27, 7106-7112 (1988).

322. Rezaie, A.R. Exosite-dependent regulation of the protein C anticoagulant pathway. *Trends Cardiovasc Med* 13, 8-15 (2003).

323. Heeb, M.J. & Griffin, J.H. Physiologic inhibition of human activated protein C by a1-antitrypsin. *J. Biol. Chem.* 263, 11613-11616 (1988).

324. Chuang, Y.J., Swanson, R., Raja, S.M. & Olson, S.T. Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin. *J Biol Chem* 276, 14961-71 (2001).

325. Desai, U.R., Petitou, M., Bjork, I. & Olson, S.T. Mechanism of heparin activation of antithrombin. Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. *J Biol Chem* 273, 7478-87 (1998).

326. Hopkins, P.C., Pike, R.N. & Stone, S.R. Evolution of serpin specificity: cooperative interactions in the reactive-site loop sequence of antithrombin specifically restrict the inhibition of activated protein C. *J Mol Evol* 51, 507-15 (2000).

327. Okwusidi, J.I., Anvari, N., Kulczycky, M., Blajchman, M.A., Buchanan, M.R. & Ofosu, F.A. Fibrin moderates the catalytic action of heparin but not that of dermatan sulfate on thrombin inhibition in human plasma. *J Lab Clin Med* 117, 359-64 (1991).

328. Hong, T.T., Van Gorp, C.L., Cardin, A.D. & Lucchesi, B.R. Intimatan (dermatan 4,6-O-disulfate) prevents rethrombosis after successful thrombolysis in the canine model of deep vessel wall injury. *Thromb Res* 117, 333-42 (2006).

329. Bendayan, P., Boccalon, H., Dupouy, D. & Boneu, B. Dermatan sulfate is a more potent inhibitor of clot-bound thrombin than unfractionated and low molecular weight heparins. *Thromb Haemost* 71, 576-80 (1994).

330. Tovar, A.M., de Mattos, D.A., Stelling, M.P., Sarcinelli-Luz, B.S., Nazareth, R.A. & Mourao, P.A. Dermatan sulfate is the predominant antithrombotic glycosaminoglycan in vessel walls: implications for a possible physiological function of heparin cofactor II. *Biochim Biophys Acta* 1740, 45-53 (2005).

331. Carrell, R.W. & Travis, J. a1-Antitrypsin and the serpins: variations and countervariations. *Trends Biochem. Sci.* 10, 20-24 (1985).

332. Bottomley, S.P. & Stone, S.R. Protein engineering of chimeric Serpins: an investigation into effects of the serpin scaffold and reactive centre loop length. *Protein Eng* 11, 1243-7 (1998).

333. Filion, M.L., Bhakta, V., Nguyen, L.H., Liaw, P.S. & Sheffield, W.P. Full or partial substitution of the reactive center loop of alpha-1-proteinase inhibitor by that of heparin cofactor II: P1 Arg is required for maximal thrombin inhibition. *Biochemistry* 43, 14864-72 (2004).

334. Rezaie, A.R. Insight into the molecular basis of coagulation proteinase specificity by mutagenesis of the serpin antithrombin. *Biochemistry* 41, 12179-85 (2002).

335. Ryu, S.E., Choi, H.J., Kwon, K.S., Lee, K.N. & Yu, M.H. The native strains in the hydrophobic core and flexible reactive loop of a serine protease inhibitor: crystal structure of an uncleaved alpha1-antitrypsin at 2.7 A. *Structure* 4, 1181-92 (1996).

336. Gill, S.C. & von Hippel, P.H. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182, 319-26 (1989).

337. Hayakawa, Y., Hirashima, Y., Kurimoto, M., Hayashi, N., Hamada, H., Kuwayama, N. & Endo, S. Contribution of basic residues of the A helix of heparin cofactor II to heparin- or dermatan sulfate-mediated thrombin inhibition. *FEBS Lett* 522, 147-50 (2002).

338. Zhang, M., Sheng, S., Maass, N. & Sager, R. mMaspin: the mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. *Mol Med* 3, 49-59 (1997).

339. Stein, P.E. & Carrell, R.W. What do dysfunctional serpins tell us about molecular mobility and disease? *Struct. Biol.* 2, 96-113 (1995).

340. Hjemel, L.M. & Chrambach, A. Electrophoresis and electrofocusing in detergent containing media: A discussion of basic concepts. *Electrophoresis* 2, 1-11 (1981).

341. Sallantin, M., Huet, J.C., Demarteau, C. & Pernollet, J.C. Reassessment of commercially available molecular weight standards for peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis using electroblotting and microsequencing. *Electrophoresis* 11, 34-6 (1990).

342. Hermans, J.M. & Stone, S.R. Interaction of activated protein C with serpins. *Biochem. J.* 295, 239-245 (1993).

343. Sutherland, J.S., Bhakta, V. & Sheffield, W.P. The appended tail region of heparin cofactor II and additional reactive centre loop mutations combine to increase the reactivity and specificity of alpha1-proteinase inhibitor M358R for thrombin. *Thromb Haemost* 98, 1014-23 (2007).