To the Graduate Council:

I am submitting herewith a thesis written by Secil Oguz-Ozen entitled "Structural and Functional Analysis of the Interaction between Vitronectin and the Thrombin-Antithrombin Pair." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry, Cellular and Molecular Biology.

Cynthia B. Peterson

Cynthia B. Peterson, Major Professor

We have read this thesis
and recommend its acceptance:

Engin Serpersu

Elizabeth E Howell

Accepted for the Council:

Vice Provost and Dean of Graduate Studies

Structural and Functional Analysis of the Interaction Between Vitronectin and the Thrombin-Antithrombin Pair

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Secil Oguz-Ozen
December, 2001

DEDICATION

This thesis is dedicated to my mother, Nuriye Oguz and my sister, Serpil Oguz who always supported me throughout my education.

ACKNOWLEDGEMENTS

There are many people to whom I am thankful for making my time in Biochemistry department so rewarding. My "special thank you" goes to Dr. Cynthia Peterson for her guidance, support and encouragement. I am particularly grateful to my thesis committee members, Dr. Elizabeth Howell and Dr. Engin Serpersu for their insight and helpful comments about my thesis.

There are a number of other people whose contribution should be recognized. I would like to thank Nancy Horn, Kenneth Minor and Jodi Watson for their generous help and friendship which made working in this lab very enjoyable.

ABSTRACT

Vitronectin is a multifunctional glycoprotein found both in the circulation and in the extracellular matrix. Circulating vitronectin is in monomeric form while matrix and tissue associated vitronectin tend to form multimers. Interacts with a wide variety of ligands that are involved in control of diverse processes including coagulation and fibrinolysis. Some target macromolecules that interact with vitronectin are heparin, PAI-1, thrombin-antithrombin pair, collagen, integrin receptors (on the cell surfaces), and urokinase plasminogen activator receptor (uPAR).

Recently, a computational model of vitronectin was proposed using threading method. This model showed that vitronectin is made up of an N-terminal somatomedin-B domain, a central β-propeller hemopexin homology domain, and a C-terminal heparin-binding domain. β-propeller domain has been found in other proteins and it has been reported to be important in protein-protein interactions. Vitronectin is known to interact with the thrombin-antithrombin pair and mediates the removal of this inactive complex from the circulation. Although there has been extensive research performed concerning vitronectin interaction with the thrombin-antithrombin pair, questions about how this interaction takes place and what the consequences of this association have not been fully understood.

The purpose of this study was to use techniques of molecular biology and analytical chemistry to analyze hemopexin homology region of vitronectin and ultimately elucidate the functional contribution of this region to the whole protein. Also, preliminary work concerning association of vitronectin with the thrombin-

antithrombin pair has been performed using enzyme linked immunoabsorbant assays and fluorescence labeling.

Results of these studies indicate that vitronectin forms complexes with the thrombin-antithrombin pair through ionic interactions and the interaction of vitronectin with the thrombin-antithrombin pair results in multimerization.

TABLE OF CONTENTS

Chapter **Page**

1. VITRONECTIN: AN OVERVIEW OF STRUCTURAL AND FUNCTIONAL PROPERTIES 1

Introduction 1

Functional Properties 2

Domain Organization 5

- Somatomedin B Domain 7
- Integrin Binding Sequence 10
- Connecting Region 11
- Hemopexin Homology Repeats 12

Conformational Lability of Vitronectin 14

The Thrombin-Antithrombin Complex and Its Interaction with Vitronectin 15

Questions Addressed in This Work 16

2. EXPRESSION AND PURIFICATION OF HEMOPEXIN HOMOLOGY REPEAT SEQUENCES IN VITRONECTIN 17

Introduction 17

Experimental Procedures 23

- Subcloning of Hemopexin Repeats of Vitronectin 23
- Site-directed mutagenesis to selectively remove the cysteine residues 23
- Expression of the recombinant domains in *E. coli* 27
- Western blotting 27
- Solubilization and Purification of HPD 1-4(C196A) 28

Results 29

Generation of the hemopexin homology domains of vitronectin using molecular cloning techniques 29
Strategy for replacement of cysteine residues in the hemopexin domains by site-directed mutagenesis 32
Development of Optimized Purification Protocol for HPD 1-4 (C196A) 34
Refolding Strategies 38
Discussion 41
Concluding Remarks and Future Directions 45

3. CHARACTERIZATION OF COMPLEX FORMATION BETWEEN VITRONECTIN AND THE THROMBIN-ANTITHROMBIN PAIR 47

Introduction 47
Thrombin 48
Antithrombin 57
Interaction between Thrombin and Antithrombin 58
Binding of the Thrombin-Antithrombin complex to Vitronectin 61
Experimental Procedures 62
Direct Enzyme-Linked Immunosorbant Assay 62
Competitive Enzyme-Linked Immunosorbant Assay 64
Thrombin-Antithrombin Kinetics Assay 66
Immunoblotting 66
Fluorescence Labeling of Vitronectin 67
Fluorescence Labeling of Thrombin 67
Size Exclusion HPLC to monitor formation of complexes 68
Fluorescence Spectroscopy to monitor binding of the Thrombin-Antithrombin binding to Vitronectin 69
Results 70
Characterization of T-AT complex formation 70
Binding of Thrombin, Antithrombin and Thrombin-Antithrombin complex to vitronectin 73
Determination of Thrombin-Antithrombin complex with vitronectin formation on size-exclusion HPLC 73
NaCl and Iodoacetamide effect on complex formation 76
Competition between γ-thrombin and PAI-1 with Thrombin-Antithrombin complex for their binding to vitronectin 80
Evaluation of complex formation between T-AT and vitronectin by fluorescence studies 83
Discussion 85

Concluding Remarks and Future Directions 89
REFERENCES 91
VITA 118

LIST OF FIGURES

Figure	Page
Figure 1.1: Domain organization of vitronectin predicted by computational threading study	8
Figure 1.2: Proposed domain organization of vitronectin	9
Figure 2.1 Comparison of homology domains of vitronectin with hemopexin	19
Figure 2.2 Crystal Structure of C-terminal domain of rabbit serum hemopexin (1.8 A° resolution) and topology diagram of four bladed β-propeller structure	21
Figure 2.3: Synthetic oligonucleotides that were used for generation of hemopexin repeats	24
Figure 2.4: Cartoon showing the constructs made from the hemopexin homology domains (HPDs) of vitronectin	25
Figure 2.5: HPD 1-4 (C196A) solubilization and purification strategy	30
Figure 2.6: 2% agarose gel showing the four HDP constructs after PCR amplification	31
Figure 2.7: Cartoon representing the HPD 1-4 region of vitronectin	33
Figure 2.8: Western blot result of the induced and uninduced samples	35
Figure 2.9: Commassie stained 15% SDS-PAGE gel and western blot showing the purification of HPD 1-4	39
Figure 2.10: Refolding strategies that had been performed	42
Figure 3.1: Coagulation cascade	49
Figure 3.2: Interaction between thrombin and antithrombin	50
Figure 3.3: A cartoon showing the heparin and fibrinogen binding sites of thrombin	54
Figure 3.4: Pathway showing the formation of γ-thrombin from α-thrombin	56
Figure 3.5: Crystal structure of intact and cleaved antithrombin	59
Figure 3.6: Model of interaction between thrombin-antithrombin pair and vitronectin	63
Figure 3.7: 10-20% SDS-PAGE gradient gel showing the thrombin-antithrombin complex formation at different ratios	71
Figure 3.8: 10% SDS-PAGE showing stability of thrombin-antithrombin complex	72
Figure 3.9: HPLC chromatogram showing the complex formation between thrombin and antithrombin	74
Figure 3.10: Binding of Thrombin, Antithrombin and Thrombin-Antithrombin complex to vitronectin	75
Figure 3.11: Complex formation between thrombin antithrombin pair and vitronectin over a period of time analyzed on size exclusion HPLC	77
Figure 3.12: Solid phase assays analyzing NaCl effect on interaction between vitronectin and thrombin-antithrombin pair	78

Figure 3.13: Solid phase assays analyzing IAA effect on interaction between vitronectin and thrombin antithrombin pair _____79
Figure 3.14: gamma thrombin-AT/alpha thrombin competition _____81
Figure 3.15: PAI-1 competition with antithrombin-thrombin complex _____82
Figure 3.16: Separation of monomeric FITC labeled vitronectin on size-exclusion HPLC _____84
Figure 3.17: Percent fluorescence intensity increase of FITC-vitronectin upon addition of thrombin-antithrombin complex_____86

ABBREVIATIONS USED

Ab	Antibody
BME	β- mercaptoethanol
CNBr	Cyanogen Bromide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FITC	Fluorescein isothiocyanide
GdnHCl	Guanidine-HCl
HPD	Hemopexin homology domain
HXN	Hemopexin
IPTG	Isopropyl-β-D-thiogalactopyranoside
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPACK	D-phenylalanyl-prolyl-arginine chloromethyl ketone
SERPIN	Serine protease inhibitor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
T-AT	Thrombin antithrombin complex

Chapter 1

VITRONECTIN: AN OVERVIEW OF STRUCTURAL AND FUNCTIONAL PROPERTIES

Introduction

Vitronectin is a multifunctional glycoprotein present both in circulation and the extracellular matrix that was first identified as a serum spreading factor responsible for adhesion and spreading (Holmes, *et. al.*, 1967). It interacts with a wide array of ligands that are involved in various physiological processes including coagulation, fibrinolysis, tumor metastasis, cellular migration and the humoral immune response (Preissner, *et. al.*, 1991a and 1991b).

Vitronectin is primarily synthesized in the liver, and it is expressed at low levels in lung, kidney, skin and brain (Seiffert, *et. al.*, 1991a and 1991b). There are

two forms of vitronectin that exist *in vivo*. Most circulating vitronectin is in a monomeric form at a concentration of 200-400 μg/ml, whereas matrix and other tissue-associated forms appear to be predominantly multimeric (Preissner, *et. al.*, 1985, Barnes, *et. al.*, 1983).

Some of the molecular ligands with which vitronectin associates are heparin, plasminogen activator inhibitor-1 (PAI-1) (Mimuro, *et. al.,* 1989), plasminogen, collagen, integrin receptors (Ruoslahti, *et. al.* 1985) and serine protease inhibitor-protease complexes (Ill, *et. al.*, 1985). Vitronectin also interacts with the components of the fibrinolysis and coagulation cascades and with constituents of extracellular matrix. These activities suggest that vitronectin is also involved in wound healing, tissue remodeling, angiogenesis and metastasis.

Although diverse functionality of vitronectin has been suggested due to its interaction with a wide array of ligands, the structure of this protein has not been solved experimentally. One of the drawbacks is that the conformational liability of this protein disfavors crystallization (Suzuki *et. al.*, 1984, Morris, *et. al.*, 1994).

Functional Properties

Vitronectin plays diverse roles in the body. It is an important protein in the control of heamostasis. In the process of blood clotting, prothrombin is converted into active thrombin as a result of the proteolytic events which take place due to vessel injury. Thrombin, the terminal enzyme in a cascade of proteases, cleaves fibrinogen

into insoluble fibrin molecules to start blood clotting. Antithrombin inhibits this proteolytic activity by forming an inactive protease-inhibitor complex with thrombin. This complex formation is facilitated by proteoglycans such as heparin, which are present on endothelial surfaces (Rosenberg, *et. al.*, 1973). Vitronectin binds to heparin and neutralizes its anticoagulant activity. It competes with the thrombin-antithrombin complex for heparin binding to accomplish this neutralization activity (Zhuang, *et. al.*, 1997, Preissner, *et. al.*, 1986). Vitronectin is also known to bind to the thrombin-antithrombin complex and mediates the rapid clearance of this inactivated complex from the circulatory system (Ill, *et. al.*, 1985, de Boer, *et. al.*, 1993, Shifman, *et. al.*, 1982).

During fibrinolysis, vitronectin maintains the active conformations of the protease inhibitors that are required for the degradation of fibrin networks. Plasminogen activators cleave the precursor plasminogen and form active plasmin, which is the major protein involved in this degradation process. But this plasmin formation is limited due to inhibition of plasminogen activators. A potent inhibitor of plasminogen activators is a serine protease inhibitor known as plasminogen activator inhibitor-1 (PAI-1). PAI-1 binds to the active sites of plasminogen activators and forms an inactive protease-inhibitor complex that prevents plasmin formation (Heckman, *et. al.*, 1988, Huber, *et. al.*, 1989). PAI-1, however, is not a stable protein; it readily converts to a latent form which is unable to bind to plasminogen activators. Vitronectin binds to PAI-1 and stabilizes it in the active conformation, and, therefore, prevents fibrinolysis (Wun, *et. al.*, 1989).

The matrix form of vitronectin is important in cell-to-cell attachment and migration. It localizes PAI-1 and plasminogen to site of injury and thus regulates the tissue remodeling processes. Besides a function in tissue remodeling, proteases are important for tumor metastasis. The presence of protease inhibitors such as PAI-1 regulates extracellular proteolysis since it inhibits protease activity required for plasmin formation. PAI-1 is stabilized by vitronectin in its active form. Therefore the amount of vitronectin and PAI-1 at injury sites controls whether cells will attach or migrate freely. A recent study from this laboratory showed that interaction with PAI-1 alters vitronectin by changing it into a self-associated multimeric form that more readily associates with the extracellular matrix and assumes its adhesive role (Minor and Peterson, manuscript submitted).

During wound repair, cells at injury sites must acquire the ability to migrate and adhere to each other in order to proliferate. Integrins are the key proteins that function as an anchor to ensure cellular attachment. Vitronectin has an integrin-binding site and has been proposed to have a role in cell adhesion and migration. It has been shown to be important for cell migration processes due to its interaction with $\alpha_v\beta_3$ integrin, the so-called vitronectin receptor (Stefansson, *et. al.*, 1996). Besides $\alpha_v\beta_3$, there are three more integrin receptors have been detected to bind to vitronectin; these are $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ (Felding, *et. al.*, 1993).

Vitronectin plays a variety of roles due to its interaction with different ligands that control important physiological processes such as coagulation, fibrinolysis, cell-to-cell attachment, cellular migration, and tumor metastasis as described above. No

crystal structures are available for vitronectin, but there are experimental data that give insight into its structure from variety of sources. Two sulfydryl groups (cysteine 196 and 411) which are buried (Zhuang, *et. al.*, 1996a, Xu, et. al., 2001) and an exposed heparin binding site (Zhuang, *et. al.*, 1997) have been determined in this laboratory. Vitronectin has two regions that are particularly prone to proteolytic digestion. One is located between residues 44 and 90 at N-terminal region, and the other one is located between residues between 340 and 370 close to the C-terminus of the protein. Plasmin has been shown to cleave the protein at R361-S362, and also at K88-G89 if digested for a longer time period (Kost, *et. al.*, 1996, Chain, *et. al.*, 1991). A trypsin cleavage site was mapped at R45-G46 (Sigurdardottir, *et. al.*, 1994). Two thrombin-cleavage sites have been identified at R305-T306 and R370-N371, and also two elastase-cleavage sites at A330-M331 and L383-S384 were experimentally determined (Gechtman, *et. al.*, 1997).

Domain Organization

The complete open reading frame of vitronectin encodes for a mature polypeptide of 459 amino acids that is preceded by a 19-amino acid signal peptide. The apparent molecular weight of human vitronectin is 72kDa (Zhuang, *et. al.*, 1996a). A distant homology between vitronectin and hemopexin has been identified from sequence comparison quite a while ago (Jenne, *et. al.*, 1987). The crystal structure of the C-terminal domain of rabbit hemopexin has been solved. It has a β-

propeller domain organization, which is a disc-shaped arrangement with a funnel-like tunnel in the middle for ion binding (Faber, *et. al.*, 1995). Later on, crystal structures of collagenase and gelatinase were shown to contain domains similar to hemopexin (Gomis-Ruth, *et. al.*, 1996, Gohlke, *et. al.*, 1996). Almost all of the proteins with a β-propeller fold have a multi-domain organization (Fulop, *et. al.*, 1999). These hemopexin type domains are important in protein-protein interactions. Besides hemopexin itself, both collagenase and gelatinase have sequence similarity to vitronectin (Faber, *et. al.*, 1995). Because of the known crystal structures of these proteins with sequence homologies to vitronectin, it is possible that vitronectin also adopts the same fold in this homology region.

Recently, in the light of experimental results, a computational model of vitronectin was proposed using a threading algorithm (Xu, *et. al.*, 2001). Sequence-structure alignment and fold recognition of domains were performed using the threading program called PROSPECT (Protein Structure Prediction and Evaluation Computer Toolkit). The energy function used for PROSPECT contains a singleton term defined by solvent accessibility and secondary structure, a pair-wise interaction term and a gap penalty for unaligned sequences. The vitronectin sequence was "threaded" through two sets of known structure templates: protein chains (defined by FSSP non-redundant set) and compact domains (defined by the DALI non-redundant domain library). Based on the threading of the aligned sequences, the atomic structures were generated with the program called MODELLER. Using known constraints such as inter-domain disulfide bond formed between C274-C453 and an

intra-domain disulfide bond between C137-C161, the GRAMM program was run to carry out docking of the predicted domains to each other. This model showed that vitronectin is made up of an N-terminal somatomedin-B domain (1-53), a central β-propeller domain homologous to that of hemopexin (131-342), and a C-terminal heparin-binding domain (347-459). Figure 1.1 shows these predicted structural domains of vitronectin.

A domain organization of VN was proposed initially from sequencing human and rabbit cDNAs and is largely based on sequence homology shared with hemopexin (Suzuki, *et. al.*, 1984 and 1985). The proposed localization of the domains within the linear sequence vitronectin is represented in Figure 1.2. It is made up of three separate domains, an N-terminal somatomedin B domain, a central hemopexin domain and a C-terminal heparin-binding domain. These domains are discussed in detail in the following sections.

Somatomedin B domain: The amino terminal 44 amino acid segment of VN is identical to circulating somatomedin B. Somatomedin A (IGF-II) and somatomedin C (IGF-I) stimulate the growth of bone and muscle, and also influence calcium, phosphate, carbohydrate and lipid metabolism. Although somatomedin A and somatomedin C have known roles in the body, somatomedin B is a serum factor of uncertain function (Fryklund, *et. al.*, 1978).

Within this short amino acid stretch are 8 cysteines forming an intradomain disulfide bond network. PAI-1 binding has been localized to this domain (Seiffert, *et.*

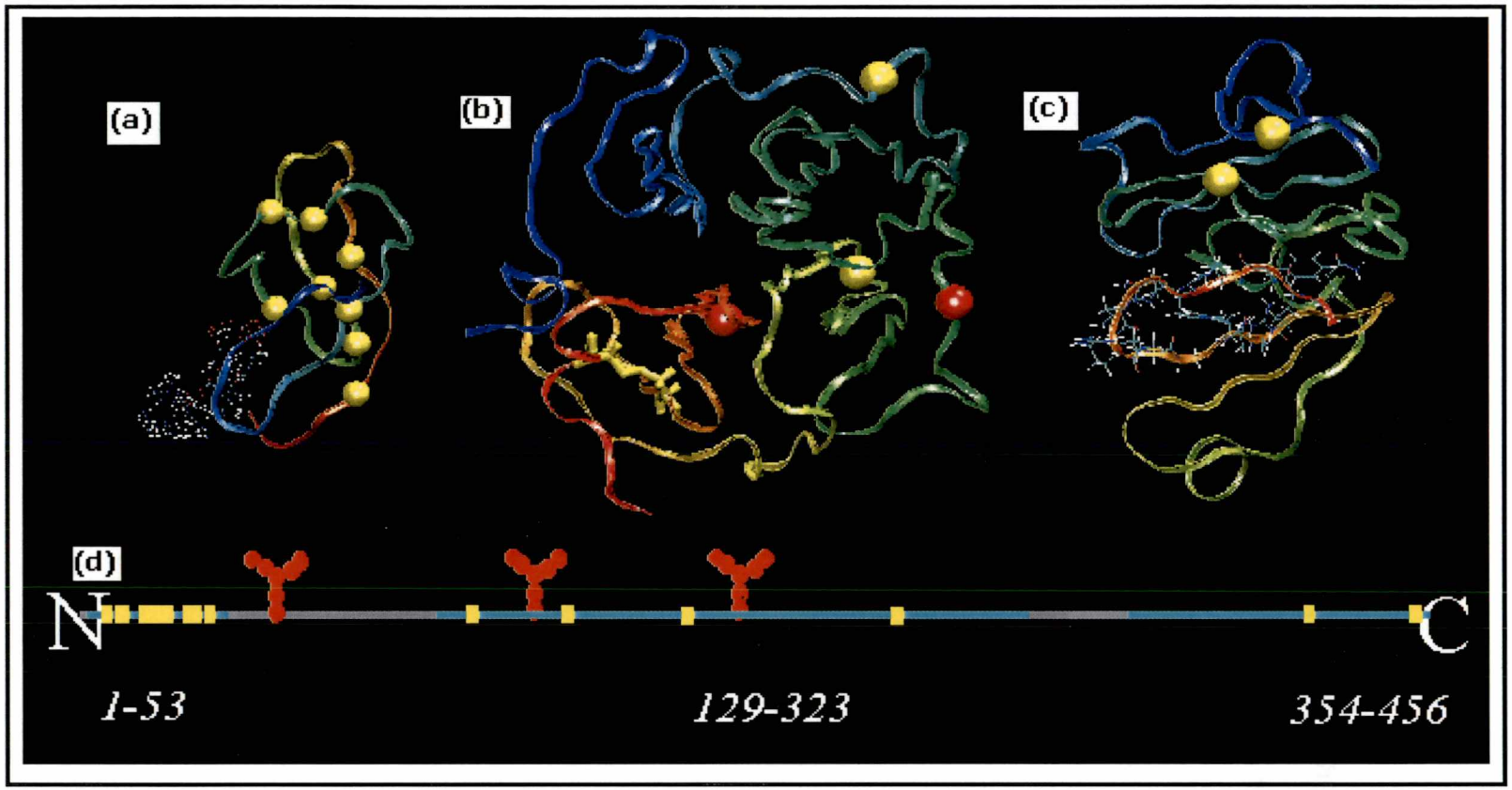


Figure 1.1: Domain organization of vitronectin predicted by computational threading study (Xu, *et. al.*, 2001). (a) The somatomedin B domain, (b) the central hemopexin homology domain forming four bladed beta propeller structure, (c) heparin binding domain, and (d) the linearized vitronectin showing the cysteines (yellow) and glycosylation sites (red) that are also indicated on three dimensional predicted folds.

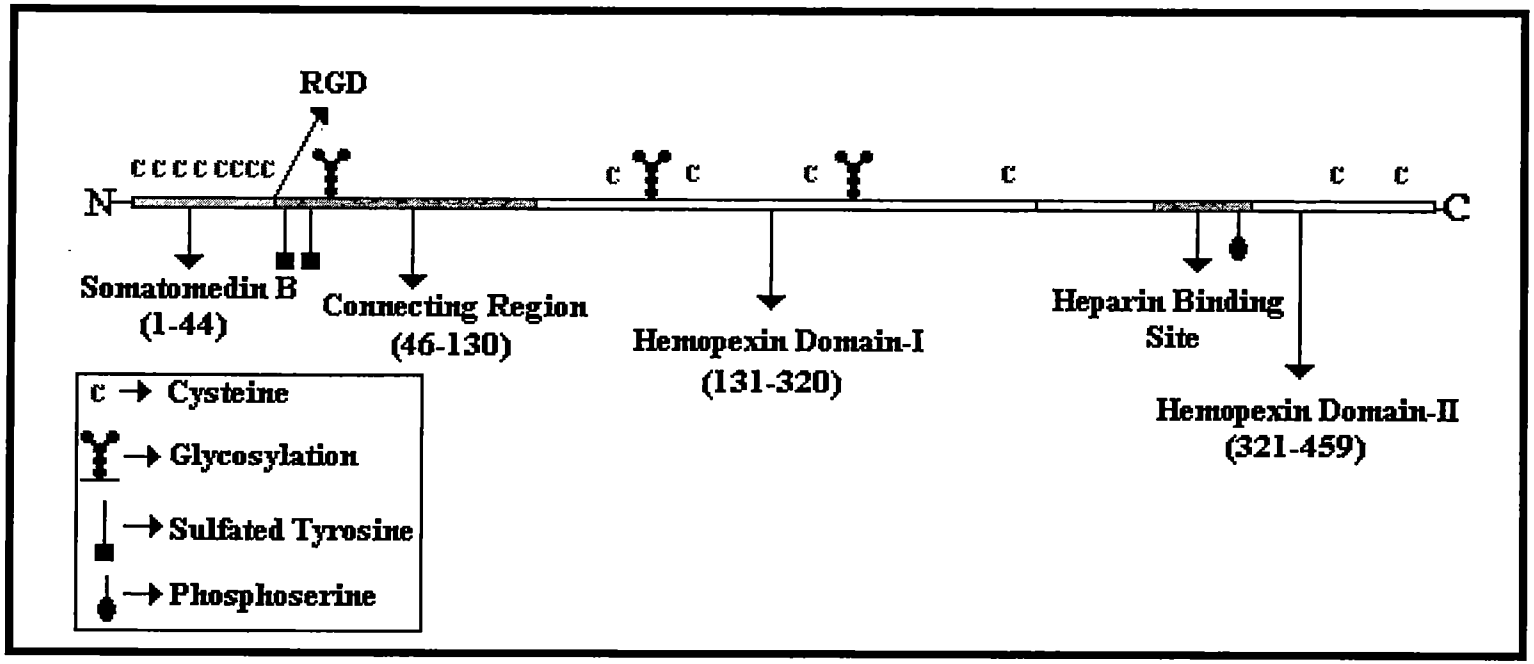


Figure 1.2: Proposed domain organization of vitronectin. It is made up of an N-terminal somatomedin-B domain, a central connecting region and two hemopexin homology domains. The second, incomplete hemopexin domain contains the positively charged heparin-binding site, which is also the site for proteolytic cleavage. Post-translational modification sites (glycosylation, sulfation and phosphorylation sites), the RGD cell-binding sequence and cysteine residues are indicated on the figure.

al., 1991b). Even after it is released from the protein by cleavage with trypsin, this N-terminal region of vitronectin remains intact and appears to bind and stabilize PAI-1 in its active form (Sigurdardottir, *et. al.*, 1994). PAI-1 binding to this region has also been verified with a competition experiment between PAI-1 and monoclonal antibody, mAb 153, which recognizes the somatomedin region of vitronectin (Seiffert, *et. al.*, 1994). Another study performed with chimeric pieces of the somatomedin B domain of vitronectin showed that residues between 12 and 30 constitute the PAI-1 binding region (Deng, *et. al.*, 1996a). The same study also showed the importance of cysteine residues for desired folding of somatomedin B domain of the protein.

Other than PAI-1, urokinase plasminogen activator receptor (uPAR) binding was located to the somatomedin B region of vitronectin both with competition assay performed with synthetic peptides from this domain and with monoclonal antibody (mAb 153) which binds to this region (Deng, *et. al.*, 1996b).

Integrin Binding Sequence: Following the somatomedin B domain, VN has an arginine-glycine-aspartic acid (RGD) sequence (residues 45-47) through which the integrin-mediated cell binding spreading to the extracellular matrix takes place (Ruoslahti, *et. al.*, 1987). Removal of the RGD sequence or selective mutations in the sequence resulted in the loss of cell binding activity of the protein (Cherny, *et. al.*, 1993, Kost, *et. al.*, 1996, Suzuki, *et. al.*, 1984).

The integrins that are known to bind to this region are $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_5$ of which $\alpha_v\beta_3$ is also refered to the vitronectin receptor (Felding, *et. al.*, 1993). Studies have suggested that vitronectin is a more adhesive protein compared to fibronectin and is essential for integrin-dependent cellular attachment (Hayman, *et. al.*, 1985).

Connecting Region: Flanking the RGD sequence is the connecting region, which is the site for a number of post-translational modifications. There are two tyrosine residues (56 and 59) modified by sulfation and there is an asparagine residue (67) for N-linked glycosylation (Jenne, *et. al.*, 1989).

In a competition experiment, using proteolytic fragments from vitronectin, another PAI-1 site has been identified which resides between residues 115 through 121 of the connecting region (Mimuro, *et. al.*, 1993). In kinetic studies, this proteolytic fragment also stabilized the PAI-1 activity. Heparin binding has also been localized to the connecting region of vitronectin with the help of phage display method; a synthetic peptide from this region was found to compete for heparin binding with immobilized vitronectin (Liang, *et. al.*, 1997). Polypeptides from this region that were obtained by a formic acid hydrolysis showed affinity towards collagen (Ishikawa, *et. al.*, 1993). This study was later verified with another study using monoclonal antibodies targeted to this region. These monoclonal antibodies displayed a competition with collagen for vitronectin that was immobilized on a surface (Morris, *et. al.*, 1994).

Hemopexin Homology Repeats: The remaining portion of vitronectin (residues 132-459) accommodates 7 hemopexin repeats that are organized into two homologous hemopexin domains (HPDs). This region of vitronectin has high homology to the heme transport protein, hemopexin (Jenne, *et. al.*, 1987). Hemopexin is composed of two identical domains folded into a four-bladed β-propeller arrangement. These two domains are connected to each other by a flexible hinge region. Unlike hemopexin, vitronectin has seven tandem repeats instead of eight, so that it forms two hemopexin domains one of which is not complete. From computational threading studies, the first four repeats have been shown to be the complete domain while the remaining three form an incomplete fold (Xu, *et. al.*, 2001). Vitronectin does not bind to heme as in the case of hemopexin, but these homology regions may be the potential interaction sites for protein-protein recognition.

There are two more N-linked glycosylation sites (asparagines 150 and 223). This portion of vitronectin contains 6 cysteines, two of which are reduced (cysteine 196 and 411; Xu, *et. al.*, 2001) and buried within the native fold of molecule (Zhuang, *et. al.*, 1996b). Cysteine-274 and Cysteine-453 form a disulfide bridge that links the endogenously cleaved 10kDa fragment to the rest of the molecule (Zhuang, *et. al.*, 1996a, Xu, *et. al.*, 2001).

Within the second HPD, there is a cluster of basic amino acids (residues 345-379) that contains the binding site for heparin (Kost, *et. al.*, 1992). In this region there

appear to be two consensus heparin-binding sequences found in heparin-binding proteins (Cardin, *et. al.*, 1989). A serine residue located at position 378 within the heparin-binding region is phosphorylated both *in vivo* and *in vitro* (McGuire, *et. al.*, 1988). Proteolytic cleavage of vitronectin occurs between arginine 379 and alanine 380 and generates two forms of vitronectin that exist in the circulation, a single-chain and a two-chain protein (Dahlback, *et. al.*, 1985).

Heparin-binding activity of this region was determined first with a CNBr digestion product of vitronectin that competed with full-length vitronectin for heparin binding and inhibited anticoagulant activity of heparin (Suzuki, *et. al.*, 1984). In another study, removal of sequences between residues 306-370 and 331-383 gave rise to loss of the heparin-binding ability of the protein (Gechtman, *et. al.*, 1997).

In this heparin-binding region, several other ligands have been implicated to interact with vitronectin. These include uPAR (Deng, *et. al.*, 1996b), collagen (Ishikawa, *et. al.*, 1993), the terminal attack complex of complement (Millis, *et. al.*, 1993), PAI-1 (Chain, *et. al.*, 1991, Preissner, *et. al.*, 1990a), plasminogen (Preissner, *et. al.*, 1990b) and the thrombin-antithrombin complex (Preissner, *et. al.*, 1987). Thrombin-antithrombin, uPAR, collagen and complement binding sites were identified using a series of synthetic peptides or CNBr cleaved fragments containing the heparin-binding region that inhibited the binding of these ligands to vitronectin. PAI-1 and heparin may share a binding determinant on vitronectin as suggested by several experiments performed with plasmin proteolyzed vitronectin (Chain, *et. al.*,

1991, Kost, *et. al.*, 1996). When cleaved with plasmin, vitronectin lost its binding ability to both heparin and PAI-1 (Sane, *et. al.*, 1991, Preissner, *et. al.*, 1990b).

Conformational Lability of Vitronectin

Vitronectin displays different binding activities depending on its specific fold. Early studies with urea treatment, pH or heat denaturation of vitronectin showed that the denatured protein tightly bound to a heparin column. This observation was ratonalized as "cryptic heparin-binding mode" in which the heparin-binding region of vitronectin was buried within the native molecule and became exposed (by denaturation *in vitro* and by unknown mechanisms *in vivo*) with denaturation so that heparin binding can take place (Preissner, *et. al.*, 1991b). Several groups agreed on this model that is depicted as a "closed" structure which becomes "open" when denatured and exposes the otherwise encrypted heparin-binding site (Preissner, *et. al.*, 1987b, Tomasini, *et. al.*, 1988).

Some time after, extensive studies on heparin binding carried out in this laboratory showed that under most physiological conditions, vitronectin has a tendency to refold into multimeric form (Zhuang, *et. al.*, 1996a and 1996b). When the salt amount is increased, electrostatic interactions are disturbed and the monomeric form of vitronectin is favored (Zhuang, *et. al.*, 1997, Zhuang, *et al.*, 1996a and 1996b). Studies on heparin binding to both monomeric (native) and multimeric (denatured, renatured) vitronectin indicated that the heparin binding activities at

individual heparin-binding sites of both are the same. Therefore, the heparin-binding site appears to be fully exposed, not buried (Zhuang, *et. al.*, 1997).

The Thrombin-Antithrombin Complex and Its Interactions with Vitronectin

One of the many important roles that vitronectin plays is its regulatory role in the coagulation process where it forms complexes with the thrombin-antithrombin pair. Vitronectin binds to the inactivated thrombin-antithrombin pair and mediates the clearance of this complex from the circulation (deBoer, *et. al.*, 1993, Ill, *et. al.*, 1985).

How this complex formation between vitronectin and the thrombin-antithrombin pair takes place and what types of complexes are formed are still not well understood. Some groups hypothesize that a unique basic region in thrombin is the site where interaction between vitronectin and thrombin antithrombin complex takes place (de Boer, *et. al.*, 1993), while some groups believe that the thrombin-antithrombin complex binds to vitronectin through a site in antithrombin (Ill, *et. al.*, 1985). Although there is some evidence showing that thrombin can bind to vitronectin by itself, some groups believe that complex formation happens only when thrombin and antithrombin interact and form a stable complex (Essex, *et. al.*, 1999).

Research groups that have accepted the cryptic heparin-binding model explain the removal of the thrombin-antithrombin complex from the circulation as follows; when vitronectin binds to the thrombin-antithrombin pair, the cryptic heparin-binding site becomes exposed. As a result of this, vitronectin binds to heparin and the

thrombin-antithrombin pair is removed from the circulation rapidly (de Boer, *et. al.*, 1992, Hogasen, *et. al.*, 1994). Alternatively, in the light of the former experiment performed in this laboratory, it is possible that when vitronectin binds to the thrombin-antithrombin pair, it becomes multimerized. This multimerization could result in increased heparin binding affinity and in turn, the removal of thrombin-antithrombin pair happens rapidly.

Questions addressed in this work

This thesis was designed to answer several questions concerning structural and functional properties of vitronectin. This thesis involves two separate projects that are discussed in detail in chapter 2 and chapter 3 of this document. The first project was to generate recombinant domains from the hemopexin homology region of vitronectin and then study the structural and functional contribution of this region to the whole protein. The second project is to analyze the interaction between vitronectin and the thrombin-antithrombin complex using solid-phase and solution-phase techniques to elucidate the mode of interaction and the size and composition of these complexes.

Chapter 2

EXPRESSION AND PURIFICATION OF HEMOPEXIN HOMOLOGY REPEAT SEQUENCES IN VITRONECTIN

Introduction

Vitronectin is a multi-domain protein that interacts with variety of ligands such as glycosaminoglycans, serpin-protease complexes, several cell surface integrins and other receptors. Since it has been shown to interact with a wide variety of macromolecules, several different functions of vitronectin have been proposed. These include adhesive properties and regulation of coagulation and thrombolysis. Studies on structure/function relationships over the years have resulted in a multi-domain model by which binding to diverse ligands at various loci was proposed to regulate humoral defense mechanisms. A domain organization was originally proposed based on sequence homology to other proteins, with support from limited

proteolysis and chemical hydrolysis studies (Jenne *et. al.*, 1987, Sigurdardottir, *et. al.*, 1994, Gechtman, *et.al.*, 1997, Kost, *et. al.*, 1996). Using Edman degradation, the sequence of the N-terminus of the protein was determined (Suzuki, *et. al.*, 1984). PAI-1 binding was localized near the N-terminus of vitronectin with proteolytic studies and epitope mapping using monoclonal antibodies (Sheiffert, *et. al.*, 1991b and 1991c). Also, PAI-1 and uPAR were shown to compete with each other for vitronectin binding, indicating a shared binding site (Waltz, *et. al.*, 1997). The heparin-binding site was identified with the help of synthetic peptides and monoclonal antibodies mapping to the C-terminus of the protein. Also proteolytic deletion of sequences between 306-370 and 331-383 disrupted the heparin binding activity of vitronectin. (Kost, *et. al.*, 1992, Gechtman, *et.al.*, 1997). A sequence corresponding to the connecting region was derived from formic acid hydrolysis and this region displayed collagen-binding activity (Ishikawa-Sakurai, *et. al.*, 1993).

Vitronectin has sequence repeats sharing homology with hemopexin that are correspond to the blades of the propeller structure; each of the repeats units consists of approximately 38 amino acid residues (Stanley, *et. al.*, 1986, Jenne, *et. al.*, 1987). Vitronectin has seven repeats in this homology region instead of the eight observed in hemopexin. Thus, one of the domains has an incomplete number of repeats compared to hemopexin. Figure 2.1 shows the comparison of the domain organization between hemopexin and vitronectin. There is a deletion of repeat 4 (or 5) in the first (or in the second) hemopexin homology domain. The most probable reason for this uneven number of subdomains

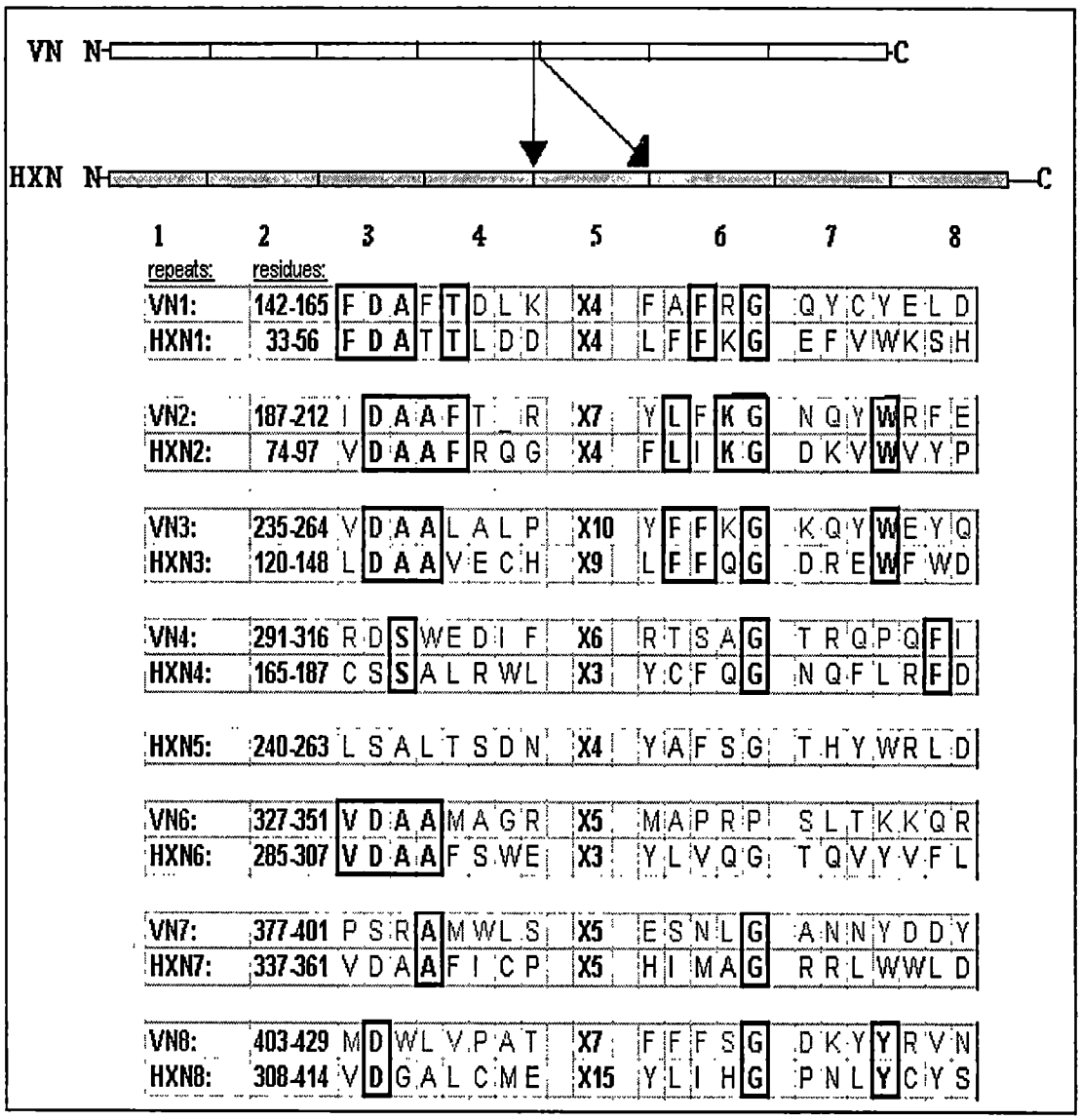


Figure 2.1 Comparison of homology domains of vitronectin (VN) with hemopexin (HXN). Seven homology repeats of vitronectin are aligned with hemopexin repeats excluding the repeat 5 of hemopexin. Conserved residues are indicated in frames.

is that an incomplete duplication event occurred during evolution (Preissner, *et. al.*, 1991b).

Hemopexin is a heme-binding plasma protein. It has a symmetrical two-domain structure. The two domains are tethered with a flexible hinge. This, in turn, holds the two domains in close proximity (Faber, *et. al.*, 1995, Preissner, *et. al.*, 1991a). Figure 2.2 shows the β-propeller assembly of one of the two domains, the C-terminal domain of rabbit hemopexin that has been determined by x-ray crystallography. Each of the domains contains four tandem repeats that correspond to the separate blades on the β-propeller structure. These four repeats are arranged in a pseudo-four-fold axis where a disulfide bond connects the first and the last repeating units. This disulfide linkage is a conserved feature in all hemopexin type domains characterized to date. Other proteins having hemopexin homology domains are collagenase-3 (Gomis-Ruth, *et. al.*, 1996) and gelatinase-A (Gohlke, *et. al.*, 1996).

A common functional similarity of proteins with hemopexin-like domains is suggested to be their protein-protein interaction property (Faber, *et. al.*, 1995). Also in this family proteins, including hemopexin, collagenases and gelatinases, these hemopexin domains are combined with other domains of different functionality specific for the protein activity. For example, in matrix metalloproteins, these homology domains are usually adjacent to the catalytic domain and contribute to ligand binding activities (Hunt *et. al.,* 1987). Vitronectin contains two hemopexin homology domains (Jenne, *et. al.*, 1987) which may be critical for protein-protein interaction properties.

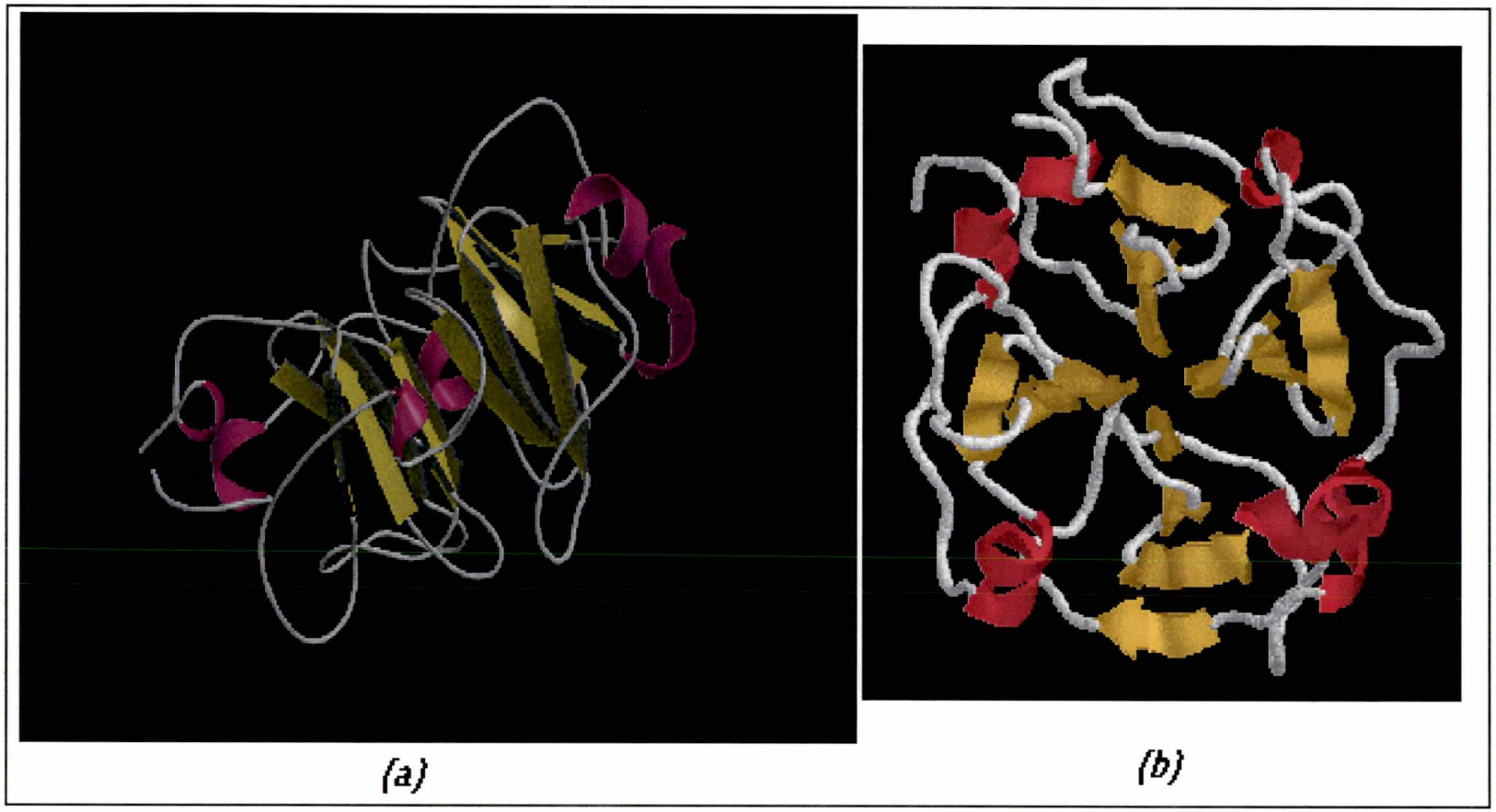


Figure 2.2 (a) Crystal Structure of C-terminal domain of rabbit serum hemopexin (1.8 A° resolution) (Faber, *et. al.*, 1995). (b) Topology diagram of four bladed β-propeller structure.

In this part of the thesis, we intended to determine the structural organization of the hemopexin homology region of vitronectin and to address the functional role and structural contributions of this region to the whole molecule. For this purpose, we engineered this hemopexin homology region for expression in a heterologous system for determination of the three-dimensional structure experimentally. Ultimately our goal is characterization of functional properties of this region that will lend further understanding of the diverse molecular interactions that vitronectin exhibits.

As described above, several proteins having hemopexin homology regions have solved crystal structures. The C-terminal domains of collagenase-3 and gelatinase-A have been expressed recombinantly, for their crystallization (Gomis-Ruth, *et. al.*, 1996, Gohlke, *et. al.*, 1996). In light of these successful uses of recombinant proteins, we designed strategies to clone and express constructs from the hemopexin homology region of vitronectin. For the PCR-based amplification of the desired segments of the protein, we engineered the oligonucleotides in a way to allow cloning and expression in both eukaryotic and prokaryotic systems. This chapter describes the initial studies on optimization of the expression, lysis, solubilization, purification and refolding of these recombinant constructs from the hemopexin homology region of vitronectin.

Experimental Procedures

Subcloning of Hemopexin Repeats of Vitronectin: Synthetic oligonucleotides were designed to generate recombinant constructs from the hemopexin homology region of vitronectin and these were ordered from GIBCO-BRL (Figure 2.3).

The cloning vector pT7-7 containing full length vitronectin (pCP36) was used as a template to perform PCR reactions to amplify different HPD constructs. Five different constructs were made containing a different number of consecutive hemopexin repeats; HPD1-4, HPD1-3, HPD4-7, HPD5-7 and HPD1-7 (Figure 2.4). These fragments were all ligated into the pCR2.1 vector (TA Cloning Kit, Invitrogen). After checking the orientation of the inserts with restriction digestion and sequencing, these fragments were subcloned into the pET16b (Novagen) expression vector using *XhoI* and *BamHI* sites of the vector. All HPD constructs had a His-tag at their N-terminus.

Site-directed mutagenesis to selectively remove the cysteine residues: Cysteine residues were mutated to alanine using a PCR-based site-directed mutagenesis kit following the product instructions (QuikChange Site-Directed Mutagenesis Kit, Stratagene). Synthetic oligonucleotides were designed and ordered from GIBCO-BRL and site-directed mutagenesis was performed to modify the

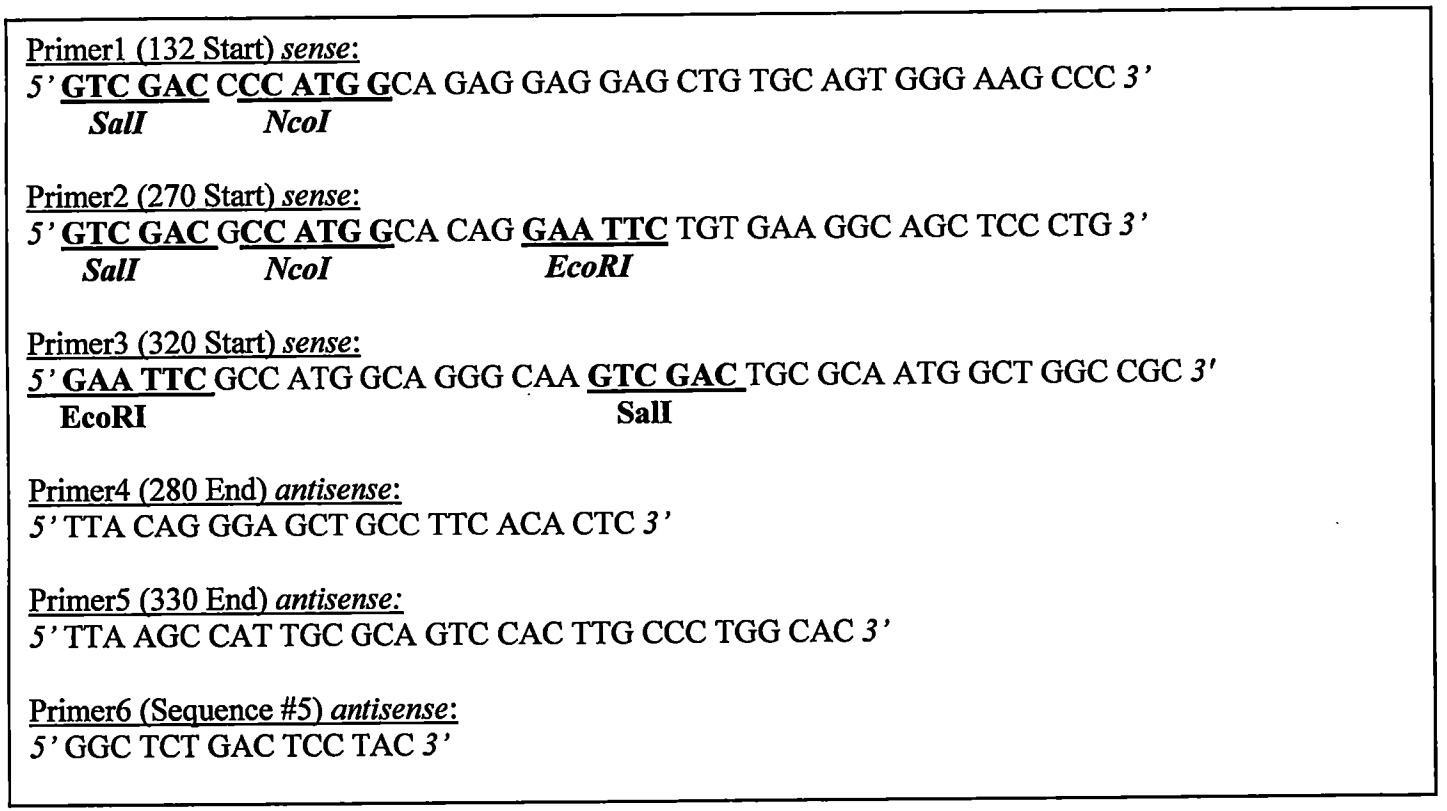


Figure 2.3: Synthetic oligonucleotides that were used for generation of hemopexin repeats. Restriction sites (RE) that were introduced for cloning purposes are indicated in bold and also underlined. Primer 4, 5, and 6 do not have specific RE sites since the specific sites on pCR2.1 vector were used. These primers were designed in a way that different expression systems (other than bacteria) can be used such as baculovirus and yeast.

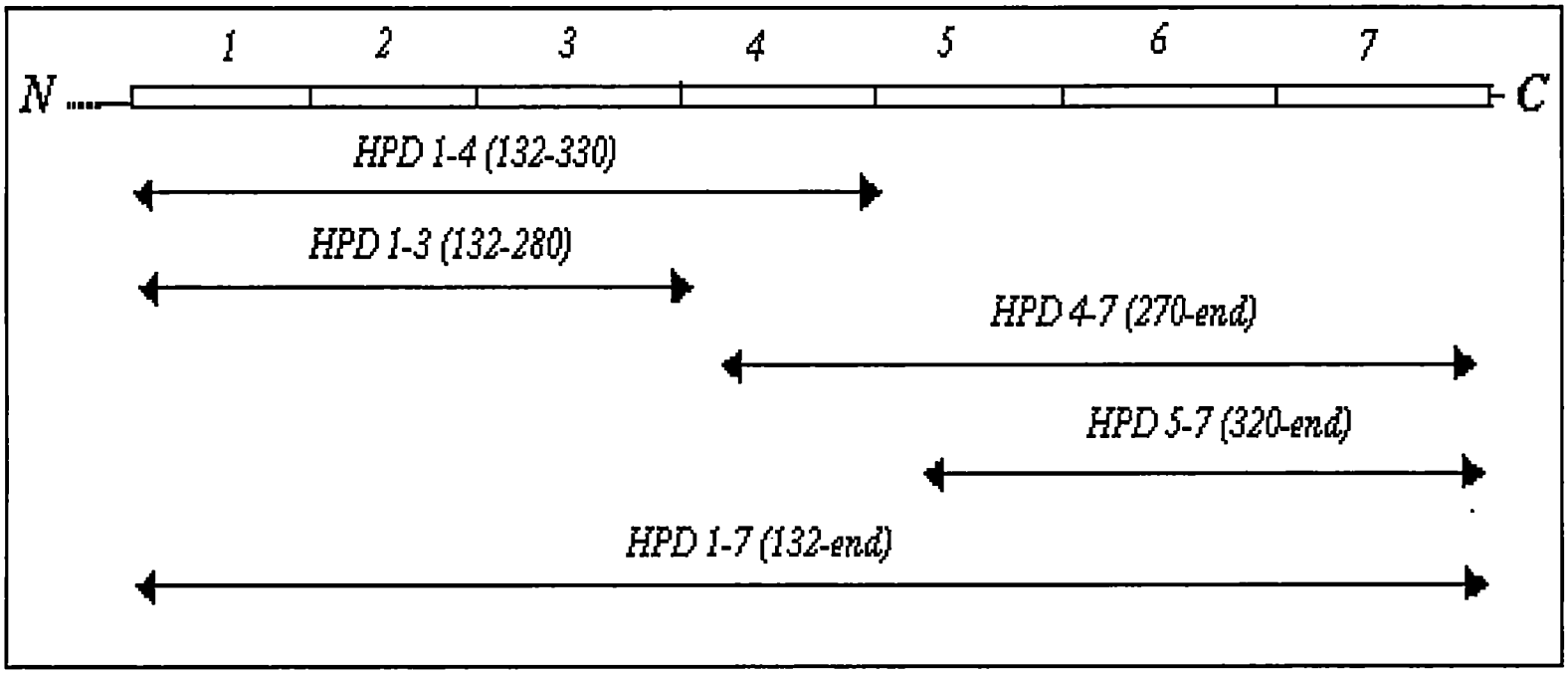


Figure 2.4: Cartoon showing the five constructs made from the hemopexin homology domains (HPDs) of vitronectin; HPD1-4, HPD1-3, HPD4-7, HPD5-7 and HPD 1-7. Oligonucleotides for these constructs were designed in a way that formed construct can be studied in different cloning and expression systems (e.g. Baculovirus, yeast systems besides bacteria). Also the introduced restriction sites were used for orientation check. (HPD 1-4 is formed with oligonucleotides 132 Start-*sense* and 330 End-*antisense*. HPD 1-3 is formed with 132 Start-*sense* and 270 End- *antisense* HPD 4-7 is formed with 270 Start-*sense* and Sequence #5-*antisense*. HPD 5-7 is formed with 320 Start-*sense* and Sequence #5-*antisense*. HPD1-7 is formed with 132 Start-*sense* and Sequence #5 –*antisense*).

room temperature, and then was washed with PBS/0.05% Tween three times. Primary antibody (anti-polyhistidine) was diluted 1:2000 in 1% nonfat-dry milk in PBS and incubated with the membrane for 1 hr at room temperature. The membrane was washed with PBS/ 0.05% Tween three times. Secondary antibody (anti-mouse IgG peroxidase labeled, Vector Labs) was added in 1% non-fat dry milk solution with a dilution of 1:5000. The membrane was incubated at room temperature for 1 hr and then washed three times as described above. Protein bands were visualized using developing solution (30 ml PBS, 50 mg/ml 4-chloro-napthol, 12μl 30% H_2O_2). The reaction was stopped by washing the membrane with water.

Solubilization and Purification of HPD 1-4 (C196A): Thirty grams of cell paste was washed with 300ml H_2O containing protease inhibitors (50ug/ml PMSF, 2ug/ml Leupeptin and 25ug/ml Pepstatin A). Sonication was followed by dounce homogenization. Centrifuged at 5000rpm for 20min at 4°C. Pellet was resuspended in 300ml of solubilization buffer (6M Urea, 50mM TrisHCl, 2mM BME, 0.15M NaCl, pH 8.5 and protease inhibitors). Stirred overnight at 4°C and then centrifuged at 13,000rpm for 2.5 hours at 4°C.

A nickel column was used as a first step on purification. All steps were performed at 4°C. Chelating Sepharose-Fast Flow (Pharmacia-Amersham) was used to pack a 3cm diameter, 20cm tall column. The column was charged with 100mM $NiSO_4.6H_2O$ solution and equilibrated with the solubilization buffer. After sample loading, the column was washed with solubilization buffer, and the bound protein was

eluted with elution buffer containing 0.5M Imidazole. The eluted protein sample was dialyzed overnight at 4°C against EDTA containing buffer (6M Urea, 50mM TrisHCl, 2mM BME, 10mM EDTA, protease inhibitors, pH 6.0). Centrifuged at 4°C for 30minutes at 4000rpm before running on an ion exchange column.

A Toyo-Pearl SP-650C fast-flow chromatography resin (TSK-GEL Toyopearl, Supelco) was packed into a 3cm diameter, 30cm tall column and equilibrated with the same EDTA containing buffer. After the sample was loaded, the column was washed with EDTA buffer, and absorbance at 280nm was monitored to establish a baseline. Elution was performed with a NaCl gradient (0- 0.53M) at a flow rate of 2ml/min. Fractions were analyzed by Coommassie staining and Western blotting. Fractions containing the protein were pooled and concentrated by ultrafiltration (Amicon, YM3, MWCO 3,000). Figure 2.5 summarizes the purification strategy that was applied on HPD 1-4 (C196A).

Results

Generation of the hemopexin homology region of vitronectin using molecular cloning techniques. Five different HPD constructs were generated from full-length vitronectin using PCR. Samples containing low salt buffer exhibited better amplifications (Figure 2.6). These fragments were later cloned into the pCRII vector. These HPD constructs were all digested with *BamHI* and *SalI* and then gel purified.

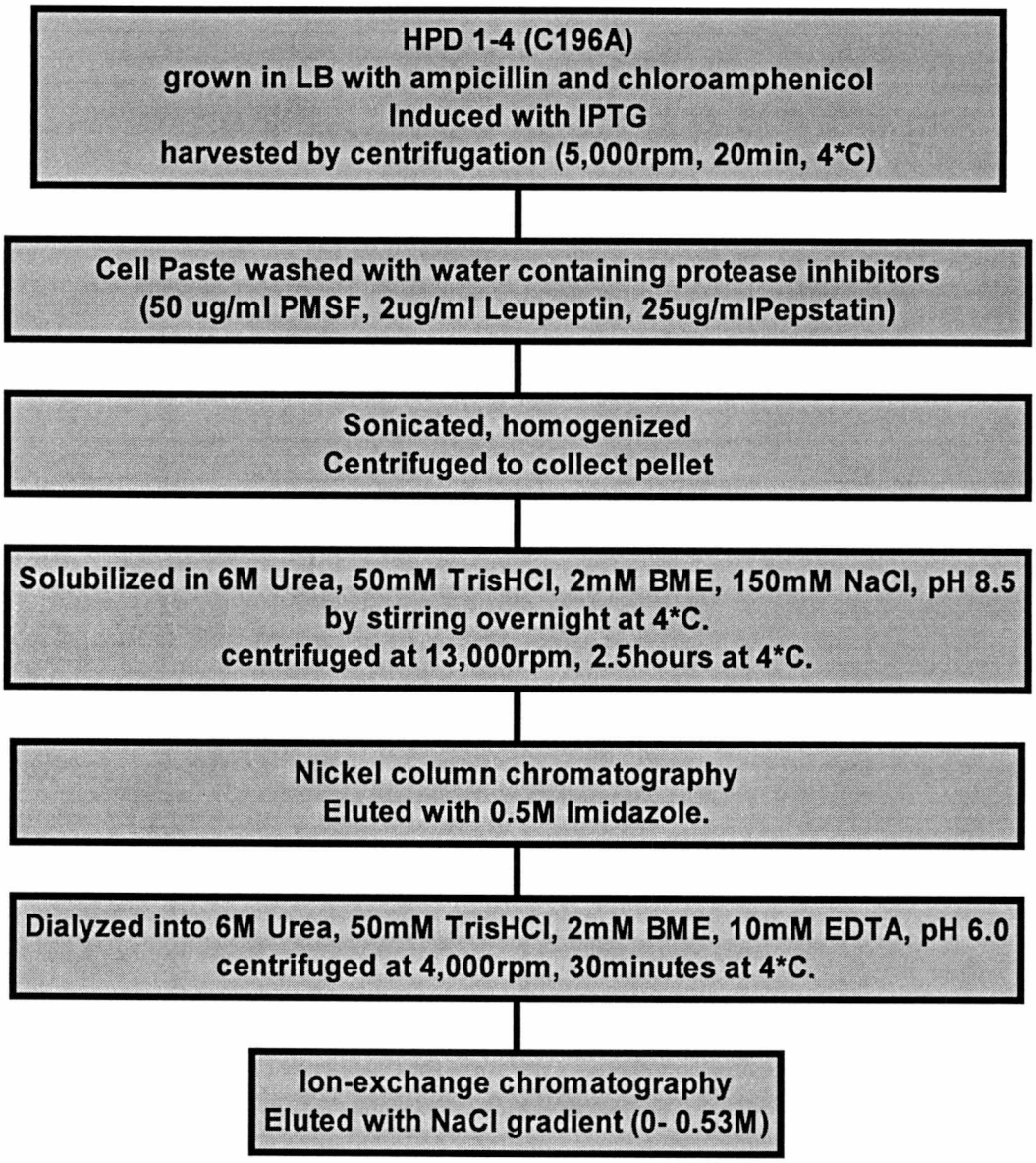


Figure 2.5: HPD 1-4 (C196A) solubilization and purification strategy. Solubilization was achieved in 6M Urea and 2mM βME. Purification was achieved after two chromatographic steps. A nickel chelating column used, and protein was eluted with 0.5M Imidazole. Then a cation exchange chromatography was employed, and final purification was accomplished with a NaCl gradient. See Experimental Procedures for details.

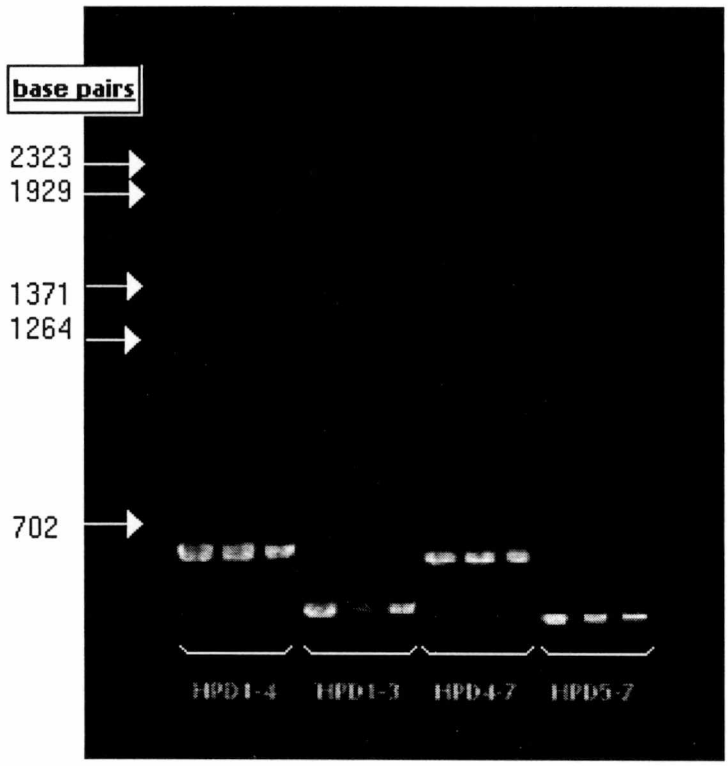


Figure 2.6: 2% agarose gel showing four of the constructs after PCR amplification. Lane1is blank, lane2 is λ-BstEII digest. Lanes 3, 4, and 5 are HPD1-4; lanes 6, 7, and 8 are HPD1-3; lanes 9, 10 and 11 are HPD 4-7; lanes 12, 13, and 14 are HPD5-7. The PCR reaction was set with three different $MgCl_2$ buffer for each construct: low (1mM), medium (2mM) and high (5mM), respectively. These samples are loaded in the low, medium and high order in each triplicate set.

These constructs were cloned into the pET16b expression vector which was excised by BamHI and XhoI restriction. In this way constructs were generated had a histidine tag at the N-termini for use in purification. After transformation into competent BL21(DE3)pLysS cells, protein expression was induced by IPTG addition. Both induced and uninduced samples, as well as untransformed the BL21(DE)pLysS, were run on SDS-PAGE and western blotting.

Strategy for replacement of cysteine residues in the hemopexin domains by site-directed mutagenesis: There are five cysteine residues in the engineered hemopexin domain containing vitronectin repeats 1-4 (Figure 2.7). Two of these cysteines, C137 and C161, appear to form a disulfide bond in the native structure of the protein (Skorstengaard, *et al.*, 1990). C196 is one of the two free sulfydryls in vitronectin (Zhuang, *et. al.*, 1996b). C274 has been shown to form a disulfide bond with C453, which is located in the second hemopexin domain of vitronectin. We generated three different HPD 1-4 mutants in which cysteine residues were selectively mutated by site-directed mutagenesis. Since we know that C196 is free in the native protein, we mutated this residue into alanine first. This way we eliminated the possibility of this free cysteine residue to be involved in disulfide bond formation. For the second HPD construct we left only C161 and C137 which form the disulfide bridge, and we mutated other three cysteines (C196, C274 and C329) to alanine. In all propeller structures there are usually two cysteine residues, one at the beginning one at the end, to tether this fold. For this reason, for a third construct, we left C137 and

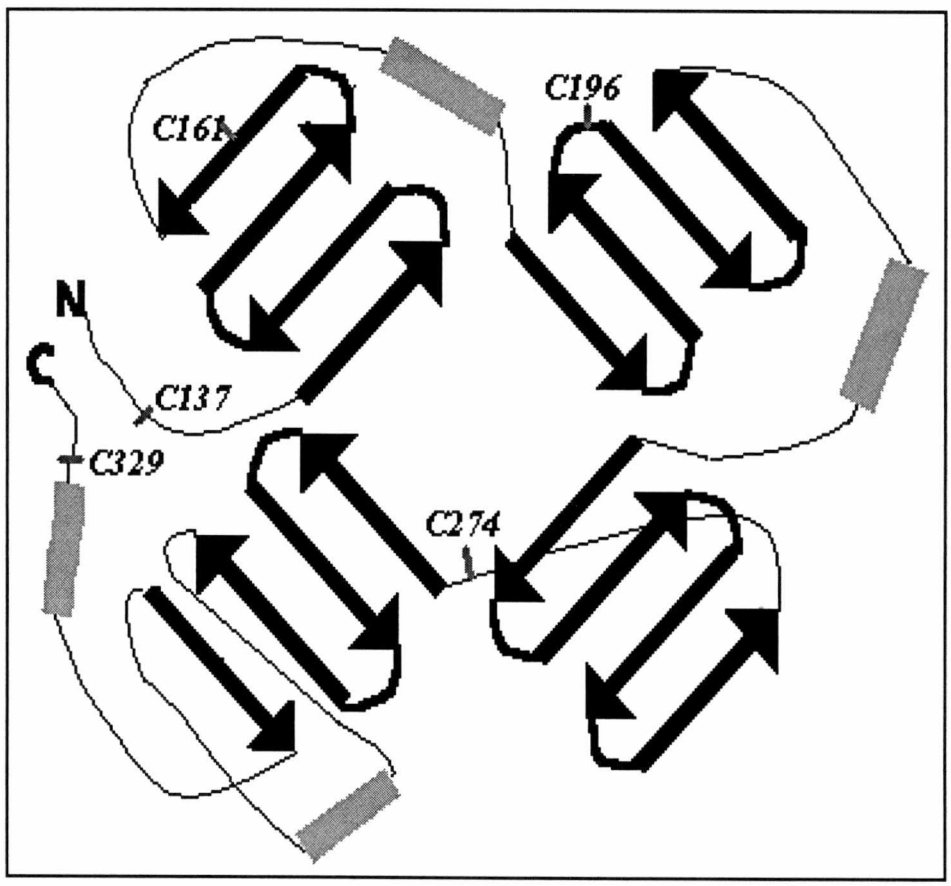


Figure 2.7: Cartoon representing the HPD 1-4 region of vitronectin. Five cysteine residues are shown in red. C137- C161 is disulfide bonded and C196 is free. C274 is forming disulfide bond with C453 that is located in the second hemopexin domain of vitronectin. C329 was introduced to HPD1-4 on purpose to substitute C453 (which makes a disulfide bond with C274). Mutations performed in HPD 1-4 region are; **a)** C196-A, **b)** C196-A, C274-A, C329-A and **c)** C196-A, C274-A, C161-A.

C329 to satisfy this criterion and mutated the other three cysteines (C161, C196 and C274). These mutation experiments were designed prior to our molecular modeling and the assignment of two of the six disulfides in vitronectin (Xu, *et. al.*, 2001). Therefore this mutant disturbs a native disulfide bond

For solubilization and purification, we focused first on HPD 1-4 with the C196-A mutation. Cysteine 196 is free in the native fold of vitronectin. Purification of HPD 1-4 (C196A) is discussed in the following section.

Development of Optimized Purification Protocol for HPD 1-4 (C196A):

Several strategies were pursued to express the recombinant fragments and purify them as soluble proteins. Since the computational studies suggest that the HPD1-4 adopts a four bladed β-propeller structure (Xu, *et. al.*, 2001), this recombinant piece was studied for solubilization and purification before the other HPD constructs. In all of the samples, expression of HPD 1-4 was observed at a size corresponding to 27,000 Dalton, when SDS-PAGE and Western blotting were performed (Figure 2.8).

Both supernatant and the cell pellet of the crude extract were analyzed by Coomassie staining and Western blotting, but the protein was found to be insoluble. The general strategy used to recover proteins from insoluble aggregates involves three main steps: lysis and isolation of insoluble fraction, solubilization of the aggregated protein, and refolding. We followed several different experimental strategies to solubilize and purify the expressed protein:

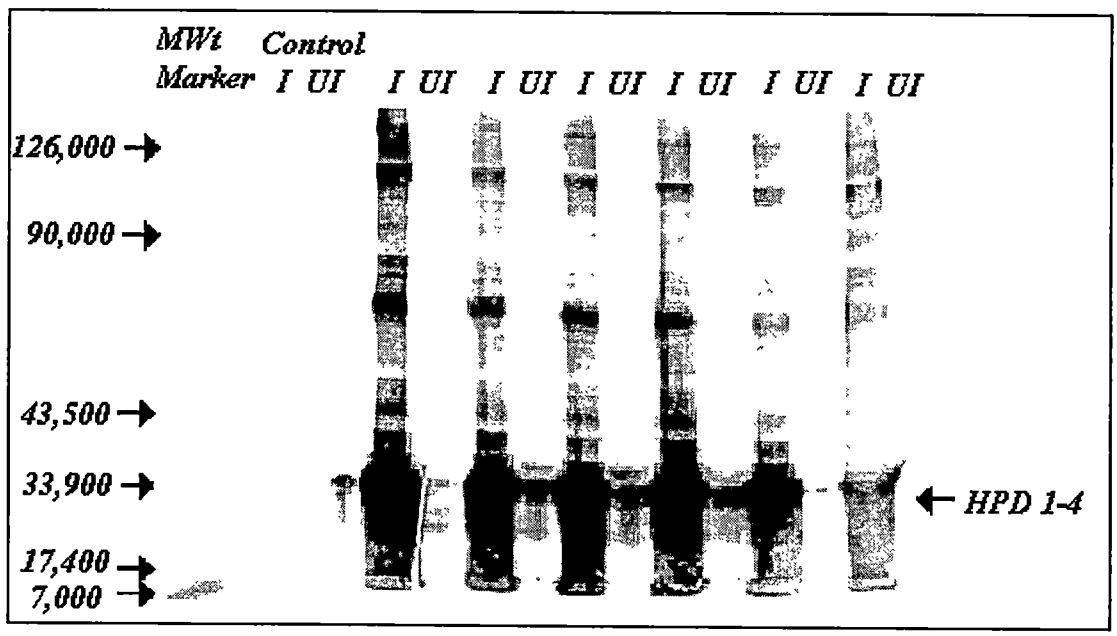


Figure 2.8: Western blot result of the induced and uninduced samples. Lane 1 is kaleidescope molecular weight marker; lane 2 induced and lane 3 is uninduced control plasmid. Lanes 4 through 15 are HPD1-4 samples alternating induced and uninduced. 1°Ab was mouse-monoclonal anti-polyhistidine (1:2000 dil.), and 2°Ab was peroxidase labeled anti-mouse IgG (H+L) (1:4000 dil.). Arrow indicates the HPD 1-4 expression.

Optimization of lysis conditions: It is important that cell lysis is complete due to the fact that intact cells sediment together with the insoluble aggregates. Therefore, ineffective cell lysis gives rise to contamination with host proteins. There are several ways to disrupt cells, such as freeze-thaw, sonication, and homogenization (Xie, *et. al.*, 1998, Hlavac, *et. al.*, 1997, Gustafson, *et. al.*, 1994). Following one or combination of these methods, the suspension is centrifuged to remove the soluble fraction. This resulting pellet containing insoluble protein is usually washed to remove contaminants such as proteins and lipids that may affect the refolding yield. For example, low concentrations of chaotropic agents such as urea or guanidium chloride (Li, *et. al.*, 1999, Rajan, *et. al.*, 1998, Xie, *et. al.*, 1998) or detergents such as Triton X-100 (Dabrowski, *et. al.*, 1999, Wetlaufer, *et. al.*, 1995) have been used to wash the pellet to remove contaminants. We used several different lysis approaches that have been applied for lysis of cells containing other insoluble proteins. After evaluating several methods, we chose to use sonication in combination with dounce homogenization, in which the cells were washed with water containing protease inhibitors, resulting complete cell lysis and isolation of the insoluble protein.

Optimization of Solubilization: After washing the pellet, the next step is solubilization in which strong denaturants such as guanidium chloride or urea are used (Rajan, *et. al.*, 1998, Xie, *et. al.*, 1998, Li, *et. al.*, 1999, Betemps, *et. al.*, 1999). Also, DTT, βME or cysteine can be added to the solubilization buffer to keep all cysteines in reduced states and cleave disulfide bonds (Seddon, et. al., 1994, Cowley,

et. al., 1997, Paduel, *et. al.*, 1999). Chelating agents such as EDTA or EGTA are sometimes used in the solubilization buffer in order to scavenge metal ions that may cause unwanted oxidation reactions (Seddon, *et. al.*, 1994). Also, addition of acids, such as formic acid, has been used to achieve solubilization (Cowley, *et. al.*, 1997). In light of solubilization studies available from the literature, we varied both amounts and types of denaturants, reducing agents, temperature, and pH of the solubilization buffer. At the end, successful solubilization was achieved when lysed cells were stirred overnight at 4°C in the presence of 6M Urea, 50mM TrisHCl, 150mM NaCl, 2mM βME pH 8.5.

Optimization of Purification: Purification can be performed either before or after the refolding process. Solubilized protein solutions may contain host proteins, nucleic acids, and cell membrane components that can cause aggregation during refolding and the overall yield is reduced. In order to overcome this problem, a purification step prior to refolding was used. The purification step usually contains several chromatographic steps such as ion exchange, size exclusion or metal affinity chromatography that operate with buffers that keep the protein denatured and reduced during the process (Cardamone, *et. al.*, 1995, Ahn, *et. al.*, 1997, Simmons, *et. al.*, 1997). All of our constructs have an N-terminal histidine tag. It has been documented that proteins fused to a histidine tag bind to a nickel-chelate affinity resin under denaturing conditions (Li, *et. al.*, 1999, Dabrowski, *et. al.*, 1999, Rajan, *et. al.*, 1998).

We used nickel charged chelating affinity resin as a first step of our purification process and we achieved elution of the protein with 0.5M Imidazole. However, Nickel column chromatography by itself was not enough to achieve adequate purification. Since many contaminating proteins were observed SDS-PAGE, a subsequent cation exchange chromatography step successfully purified the HPD 1-4 (C196A) from contaminants, with a NaCl gradient in the presence of urea and βME. The purification of HPD 1-4 (C196) is shown in figure 2.9.

Refolding Strategies: After successful solubilization and purification of HPD 1-4 (C196A), refolding studies were performed. There are several different methods that have been employed to remove or reduce excess denaturing and reducing agents. These include large dilution or dialysis using a refolding buffer that allows proteins to renature. When the dialysis method was attempted, aggregation was encountered presumably due to the exposure of hydrophobic surfaces in the protein at intermediate concentrations of the denaturant.

When refolding proteins that have disulfide bonds in the native structure, the buffer conditions should promote oxidation so that disulfide bond formation can occur. Air oxidation, using mixed disulfides or using an oxido-shuffling system are the most common methods that have been used for this purpose. We were not successful when we used air oxidation for refolding. Air oxidation is an inexpensive

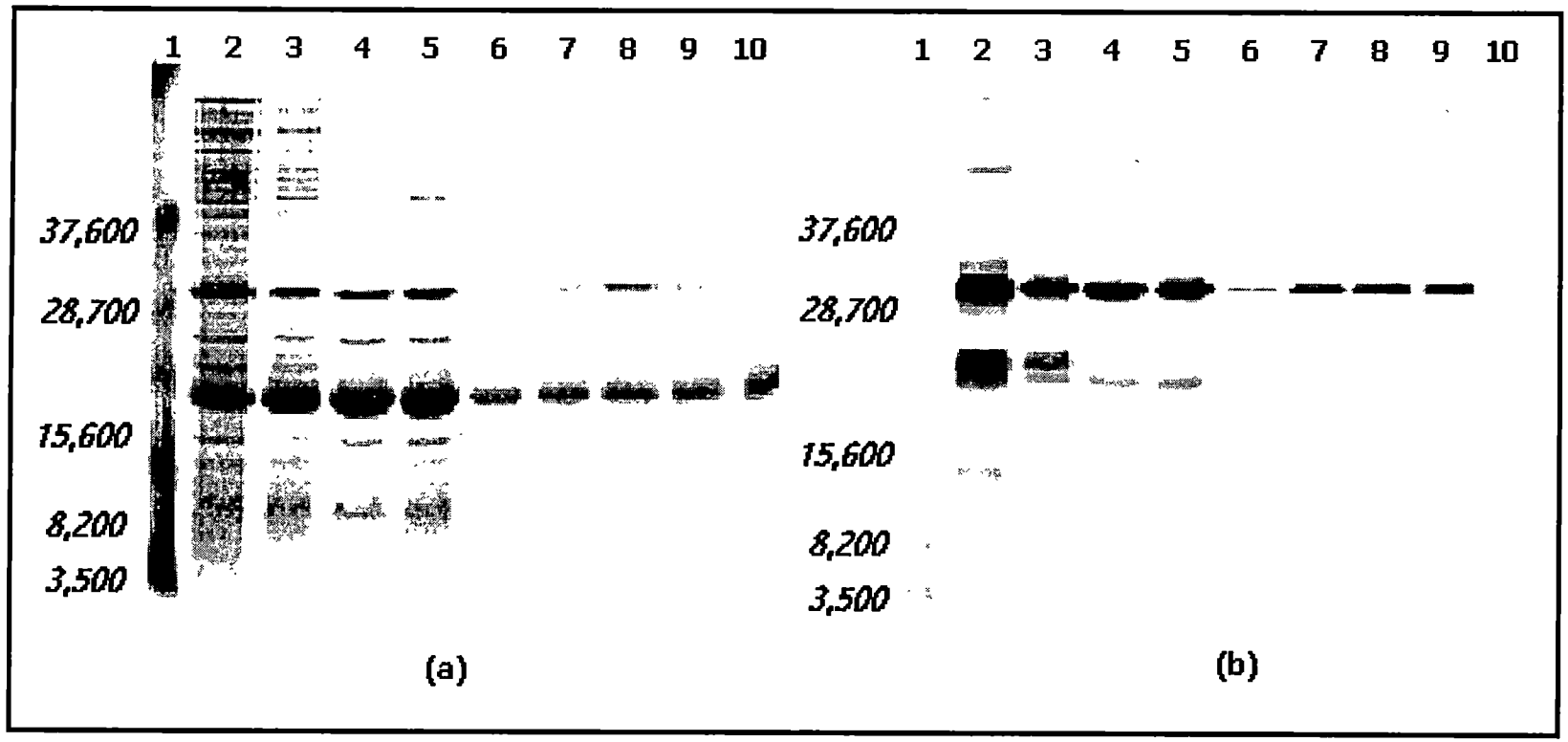


Figure 2.9: (a) Commassie stained 15% SDS-PAGE gel and **(b)** western blot showing the purification of HPD 1-4 before and after each column. Lanes 1: Kaleidescope protein standards, 2: crude extract, 3: before nickel column, 4: 0.5M Imidazole elution from Nickel column, 5: Before ion-exchange column, 6-9: ion exchange salt gradient elutions. 10: blank (the band that showed up on Commassie stain at around 16,000 dalton, indicates a contaminant in the running buffer).

method, but it has been reported that the renaturation rates and yields can be low (Rudolph, *et. al.*, 1996). When low molecular weight thiols in reduced and oxidized forms are used, it has been observed that higher oxidation rates and yields were obtained (Rajan, *et. al.*, 1998, Wetlaufer, *et. al.*, 1987, Moroder, *et al.*, 1996). The most common oxido-shuffling reagents are reduced and oxidized glutathione and cysteine/cystine pair. Disadvantages of these systems are the high cost of some of these reagents. Even though we performed several different reduced to oxidized thiol ratios to promote proper disulfide bond formation, we observed aggregation, presumably due to the low solubility of folding intermediates. Thus, oxidative refolding was not effective. A simpler strategy to prevent aggregation is to use additives that can interfere with intermolecular hydrophobic interactions and can be easily removed from the medium after the refolding process is complete. There are different additives that their ability to prevent aggregation has been studied such as glycerol, ethylene glycol, DMSO and Triton X-100 (Wetlaufer, *et. al.*, 1995, Maeda, *et al.*, 1996, Ahn, *et al.*, 1997, Kurucz, *et al.*, 1995).

For refolding of HPD 1-4 (C196A), we pursued following conditions. 10% DMSO, 0.1% Triton-X or 10%Glycerol/5% Ethylene Glycol added to a buffer containing 50mM TrisHCl, 50mM NaCl, 2mM BME, 10mM EDTA and protease inhibitors. The pH of these buffers were set to either 5 or 9. The purified protein sample was diluted 1:100 with one of these six buffers and left at room temperature. Samples were taken at different time points and centrifuged at 4000rpm for 30min. All of these largely diluted samples were concentrated by ultrafiltration and analyzed

on a Western blot. Ones with positive Western results were subjected to gradual BME removal by two-step dialysis (2mM to 1mM BME then 1mM to no BME buffer) at room temperature. Samples at each dialysis step were analyzed by Western blotting.

Although our attempts at disulfide oxidation have not yet been successful, some of these trial methods gave promising results those have to be further studied. The flow chart presented on figure 2.10 summarizes our refolding strategy.

Discussion

Vitronectin is a multifunctional protein, which interacts with a wide array of ligands. It has a multi-domain structure that allows it to bind to different ligands and carry out a variety of physiologically relevant processes. Unfortunately, there is no crystal structure available for vitronectin. The protein is conformationally labile. Different conformations adopt different oligomeric forms which exhibit different ligand-binding activities. This conformational lability creates an obstacle in the formation of regularly arrayed crystalline formation which is required for x-ray studies. Also, there are many post-transcriptional modifications, including sulfation, analytical and computational methods (Xu, *et. al.*, 2001) to explain the structure-function relationships of vitronectin.

It is known that vitronectin binds to variety of cells and molecules. It contains two hemopexin homology domains, which most probably mediate binding to different ligands. Because of this reason, we aimed to use recombinant techniques to

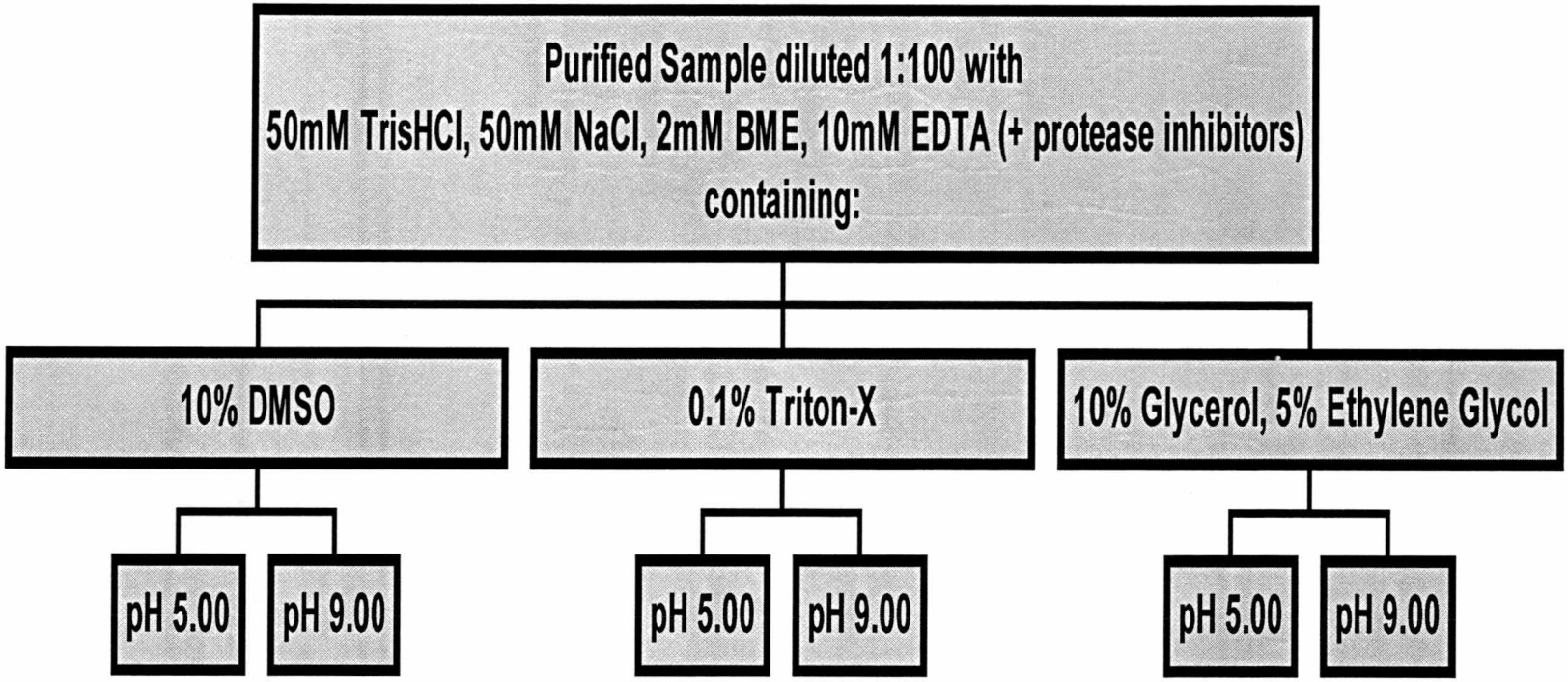


Figure 2.10: Refolding strategies that had been performed. Samples were incubated at room temperature under these six different conditions and analyzed on Western blots at different time intervals. Ones with positive Western results were subjected to two-step dialysis in order to remove βME (2mM → 1mM→ no βME).

express and purify the hemopexin homology domains of vitronectin and to study structure-function properties of these regions. The first homology domain, HPD 1-4, is thought to mediate collagen binding and also interaction with complement factors. We mainly focused on expression, purification and in turn experimental structural determination of HPD 1-4 since we know from the computational studies that this first four repeat is suppose to fold into a so called four bladed β-propeller structure.

Expression of HPD 1-4 was successful, as detected by Coomassie staining, and Western blotting with monoclonal antibodies against the polyhistidine tag. Unfortunately, HPD1-4 was detected mostly in the insoluble fraction of the bacteria. Several different conditions were explored, and a scheme for solubilization and purification of this domain was optimized for one of the HPD1-4 constructs. The challenge now remains in optimizing refolding conditions for the protein.

For the formation of disulfide bridges, the denaturant of the medium should be removed. There are several ways to accomplish this. For example, redox conditions of the system can be set to achieve disulfide exchange. Although we pursued numerous refolding strategies, we were able to solubilize protein in significant quantities only in the presence of a reducing agent. Stepwise dialysis, large dilution and oxidation were some of the strategies that we followed. Even the addition of detergents organic stabilizers (such as DMSO, Triton-X) to the refolding buffer was not helpful to remove the denaturant or the reducing agent without getting aggregation.

Proteins containing hemopexin homology domains share common functional properties such as the ability to participate in protein-protein interactions and specific binding to small molecules (Hunt, *et. al.*, 1987). There are many proteins in which the interaction between domains are essential to obtain correct folding of the protein that in turn, indispensable for proper functioning (Zabin, *et. al.*, 1982). The proteins with hemopexin homology domains, such as matrix metalloproteinases, these homology domains are usually attached to the catalytic domain and modulate the substrate binding properties of these proteins. Therefore, there is a strong possibility that for the proper functioning of the hemopexin domain(s) of vitronectin, the presence of other domain(s) may be required to stabilize the structure of the protein. This may be the reason that we were unable to get correctly refolded hemopexin domain since other essential domain(s) are missing.

Concluding Remarks and Future Directions

Solving the structure of this hemopexin repeats of vitronectin will be helpful to fill the gaps concerning ligand binding specificity and functionality of the protein. Since we have successfully accomplished the purification of insoluble protein from bacterial extracts, we must now pursue other refolding strategies. The work described in this thesis provides logical starting points for this future work.

Now that we have more information concerning the structure of vitronectin from computational modeling (Xu, *et. al.*, 2001), we can further work on HPD 1-4

containing only C137 and C161 that form the disulfide bond. Also, we can attempt to purify HPD1-7, which contains the whole hemopexin homology region. In this way, we can test whether or not HPD1-4 needs the remaining three repeats (even though those forming an incomplete fold) as in the case of hemopexin, in which interaction of both domains are required for stabilization of the structure and proper functioning of the protein.

As a future goal, we plan to optimize the refolding strategy of HPD 1-4. We also have HPD 1-7 and HPD 1-4 with different cysteine contents, particularly one with C137 and C161, that we will express and purify and work on refolding. After achievement of purification and refolding of the recombinant domains, next step will be the structural analysis of hemopexin domains with the NMR analysis.

Further characterization of the hemopexin homology region of vitronectin will be interesting to evaluate ligand binding specificity and structural properties of the protein.

Chapter 3

CHARACTERIZATION OF COMPLEX FORMATION BETWEEN VITRONECTIN AND THE THROMBIN-ANTITHROMBIN PAIR

Introduction

Vitronectin is a multifunctional adhesive plasma glycoprotein that mediates attachment of cells to surfaces, stabilizes plasminogen activator inhibitor (PAI-1), and inhibits the complement system (Preissner, *et. al.*, 1988; Mimuro, *et. al.*, 1989; Bhakdi, *et. al.*, 1988). It also has an anticoagulant activity by which it binds to heparin and neutralizes its coagulant effect. Vitronectin binds to the inactivated thrombin-antithrombin pair and mediates the clearance of this complex from the circulation (deBoer, *et. al.*, 1993, Ill, *et. al.*, 1985).

Blood coagulation is a complex process in which specific enzymes activate zymogens through proteolytic cleavage, and each activated enzyme activates another zymogen in the same way in a chain reaction leaading to clot formation. The blood coagulation cascade is depicted in figure 3.1. The final enzymatic product of this cascade is thrombin, which forms insoluble fibrin by cleaving fibrinogen in order to form a thrombus of fibrin fibrils and attached platelets. Thrombin also activates two other cofactors (Factor Va and factor VIIIa) that are essential cofactors for thrombin production (Davie, *et. al.*, 1991, Hsieh, *et. al.*, 1997). There must be a balance between anticoagulant and procoagulant control mechanisms in order to maintain hemostasis. Inhibition of the coagulant activity of thrombin is the first control mechanism which is achieved through serine protease inhibitors (serpins) circulating in the blood, the most important being antithrombin III. Heparin increases the interaction rate between thrombin and antithrombin by 1000-10,000 fold (Bjork, *et. al.*, 1982, Travis, *et. al.*, 1983). Figure 3.2 shows the interaction between thrombin and antithrombin and the role of heparin in increasing the rate of this interaction.

Detailed information about thrombin and antithrombin, how they interact with each other, and their functionality will be reviewed in the following sections.

Thrombin: Although the biological roles of thrombin in the body are extremely diverse, the protease is mostly known for its critical role in blood coagulation. Following injury, clotting occurs in an enzymatic cascade with the activated form of one 'clotting factor' catalyzing the activation of the next. Thrombin,

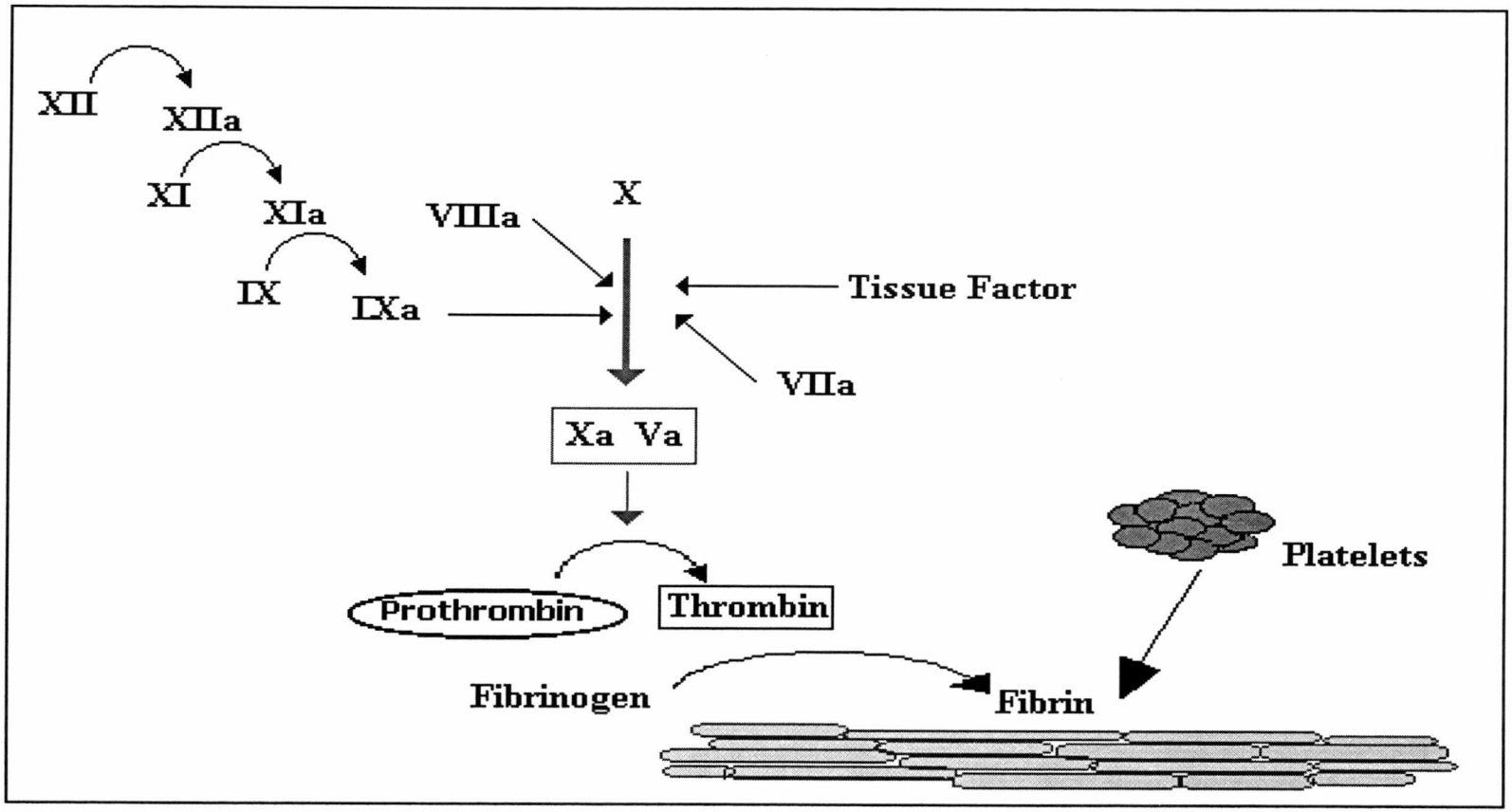


Figure 3.1: Coagulation cascade involving several steps of proteolytic cleavage of zymogens into active forms. Final products Factor Xa and Factor Va initiate the formation of active thrombin from prothrombin. Then thrombin proteolytically cleaves soluble fibrinogen and forms fibrin clot (Adapted form Eitzman, *et. al.*, 1997).

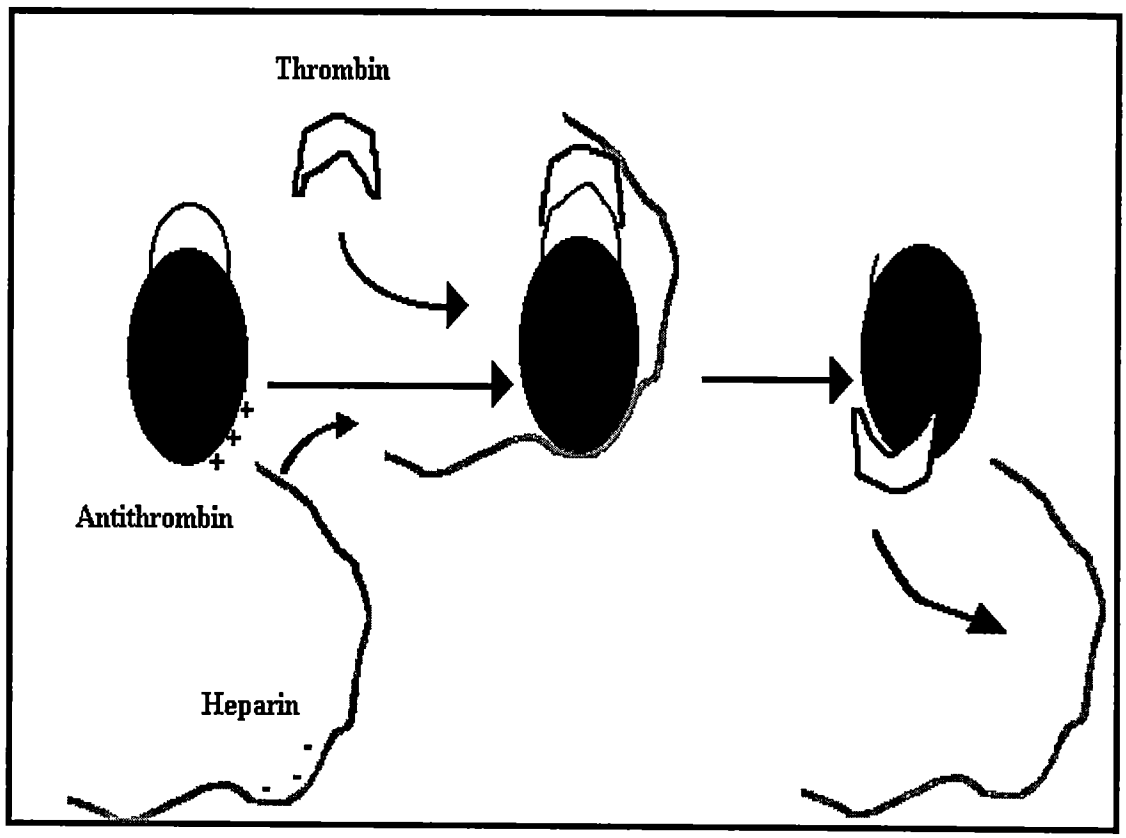


Figure 3.2: Interaction between thrombin and antithrombin. Heparin binds to a specific site on antithrombin. This binding results in conformational change in antithrombin and makes it much reactive to thrombin. Then thrombin binds and cleaves this loop and forms a stable intermediate. Heparin circulates in the system and binds to another thrombin-antithrombin pair.

a trypsin-like serine protease, acts in the final step of the clotting process and converts fibrinogen to fibrin. Once thrombin is formed, it plays diverse roles, as a catalyst for the conversion of fibrinogen to fibrin to form a clot and, importantly, as a regulator of the coagulation system by providing stimulatory and inhibitory feedback. In order to control coagulation, it is important to control the functioning of the key enzyme in this process. Since thrombin has a critical role in this pathway, it has become a very suitable enzyme to be a target for anticoagulant drug therapy (Fenton, *et. al.*, 1991).

Thrombin is a highly specific proteolytic enzyme that catalyses the hydrolysis of specific arginine-glycine peptide bonds in fibrinogen to form fibrin monomers that assemble into a fibrous blood clot (Blomback, *et. al.*, 1967, Mosesson, *et. al.*, 1998). During this process, the monomers are left with a specific surface charge pattern that causes them to aggregate. Besides its role in fibrin formation from fibrinogen, it has also been shown to trigger platelet aggregation and catalyze the activation of other blood clotting factors V, VIII and XIII (Davie, *et. al.*, 1991).

Thrombin must be present at a critical concentration in the blood plasma for proper blood clotting. Human plasma contains approximately 40-50 mg of thrombin per liter. Thrombin acts at the site of the wound and is generated by proteolytic activation of a circulating zymogen, prothrombin, by the prothrombinase complex comprising factors Xa and Va. Several inactivators negatively regulate thrombin. Antithrombin III and hirudin are such inactivators that prevent the functioning of thrombin by binding to its active site.

Thrombin has structural homology with trypsin and chymotrypsin, serine protease prototypes. Thrombin is a glycoprotein formed by two peptides chains of 36 and 259 amino acids linked by disulfide bonds. There are three important sites that have been identified. The catalytic site that confers to the molecule its serine protease activity is negatively charged. The catalytic triad residues of thrombin are histidine 43, aspartic acid 99 and serine 205 (Church, *et. al.*, 1989).

Two other positively charged domains have drawn interest from researchers over the years. One of these electropositive domains, exosite 1, borders the catalytic site and is responsible for the binding of the substrate (fibrinogen or thrombin receptor). The other domain, exosite 2, is in near opposition on the enzyme surface and is responsible for the binding of antithrombin III and inactivation of thrombin (Fenton, *et. al.*, 1988, Stubbs, *et. al.*, 1995, Bode, *et. al.*, 1992). Exosite 1 bears the binding sites for fibrinogen, Factor V and Factor VIII. Exosite 2 has the binding site for heparin and it is also thought to be involved in interaction with Factor V and Factor VIII (Stubbs, *et. al.*, 1993, Esmon, *et. al.*, 1996). When thrombin binds to fibrin, it still remains active since it interacts with fibrin through a site that is distinct from its catalytic center (Vali, *et. al.*, 1988, Kaminski, *et. al.*, 1983, Berliner, *et. al.*, 1985). If heparin is not present in the environment, the fibrin binding by thrombin takes place through exosite 1. If heparin is present in the environment, this binding process becomes more complicated. Both the mode of interaction and the amount of thrombin that interacts with fibrin change, depending on the amount of heparin present. In the absence of heparin, thrombin binds to fibrin through exosite 1, whereas

it has been shown to bind to fibrin through exosite 2 in the presence of heparin. Figure 3.3 depicts the domains of thrombin where fibrin and heparin binding occur. It has been shown that the interaction between thrombin and fibrin increases with heparin concentrations up to 250nM; however concentrations higher than this value showed decreasing fibrin binding affinity for thrombin (Hogg, *et. al.*, 1990).

As well as inhibiting fibrinolysis, thrombin also has indirect effects that are stimulatory for this phenomenon. Thrombin is a chemoattractant for neutrophils that take a role in degradation of the fibrin clot (Yamaguchi, *et. al.*, 2000). It also releases plasminogen activators from endothelial cells that generate plasmin that initiates fibrinolysis and inactivates prothrombin (Fenton, *et. al.,* 1998). The way that thrombin plays a role both in enhancement and inhibition of fibrinolysis is an example of the high degree of regulation that maintains the fine balance of haemostasis. The extreme ends of this process are either an excessive hemorrhage or an excessive coagulation. Thrombin plays an important role in balancing this system.

Thrombin is also involved in inflammation. It is a chemoattractant for neutrophils and monocytes. In this way, it induces the cells to move down a chemical gradient to the site of injury where the thrombin is most concentrated. Therefore, the neutrophils and monocytes carry out their phagocytic role during a potential bacterial invasion (Esmon, *et. al.*, 2000, Becker, *et. al.*, 1998). Thrombin acts as an anticoagulant agent when it activates protein C or when it is bound to thrombomodulin which is an endothelial cell receptor. When it interacts with thrombomodulin, it shows increased reactivity towards protein C, which is the agent

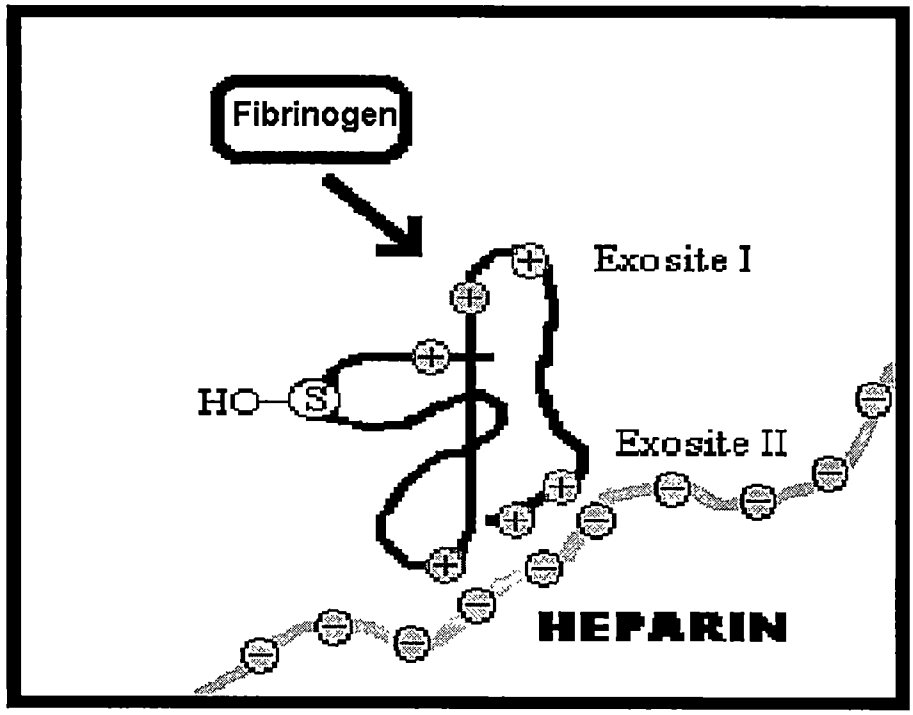


Figure 3.3: A cartoon showing the heparin and fibrinogen binding sites of thrombin. Both exosite 1 and exosite 2 are positively charged. Fibrinogen binding takes place on exosite 1 while heparin binding takes place through exosite 2. The active site of thrombin is indicated with the serine residue which reacts with the protease inhibitor.

responsible for inactivation of coagulation factor Va and factor VIIIa (Rydel, *et. al.*, 1994, Esmon, *et. al.*, 1986 and 2000).

Human α-thrombin is composed of two amino-acid chains, a 36 amino acid A chain, and a 259 amino acid B chain that are linked to each other through a disulfide bond (Degen, *et. al.*, 1983). When α-thrombin is treated with trypsin, limited proteolysis of the B chain results in β- and γ-thrombin. Figure 3.4 depicts the autolysis pathway of α-thrombin yielding γ-thrombin formation. γ-thrombin is comprised of three non-covalently associated polypeptide segments (Fenton, *et. al.*, 1977). It has serine protease catalytic triad residues (aspartate-histidine-serine) that are located separately on each three domains. Aspartate 99 is located on the A-B_3 chain, histidine 43 is located on the B_1 chain and serine 205 is located on the B_4 chain (Chang, *et. al.*, 1980). Both β-thrombin and γ-thrombin have been isolated after proteolytic degradation of α-thrombin (Chang, et. al., 1986). β-thrombin is formed by cleavage at arginine 75-tyrosine 76 and/or arginine 77-asparagine 78, whereas γ-thrombin has an additional cleavage at lysine 149-glycine 150 (Hofsteenge, *et. al.*, 1988). The most significant structural difference between α- and γ-thrombin is contained within the fibrinogen binding exosite due to proteolytic cleavages that occur. When converted from α-thrombin to γ-thrombin, the clotting activity was either lost or very low compared to α-thrombin, while the esterase activity was retained (Fenton, *et. al.*, 1977, Chang, *et. al.*, 1980). γ-thrombin has been shown to catalyze fibrinogen cleavage with approximately 2400-fold lower k_{cat}/K_m value

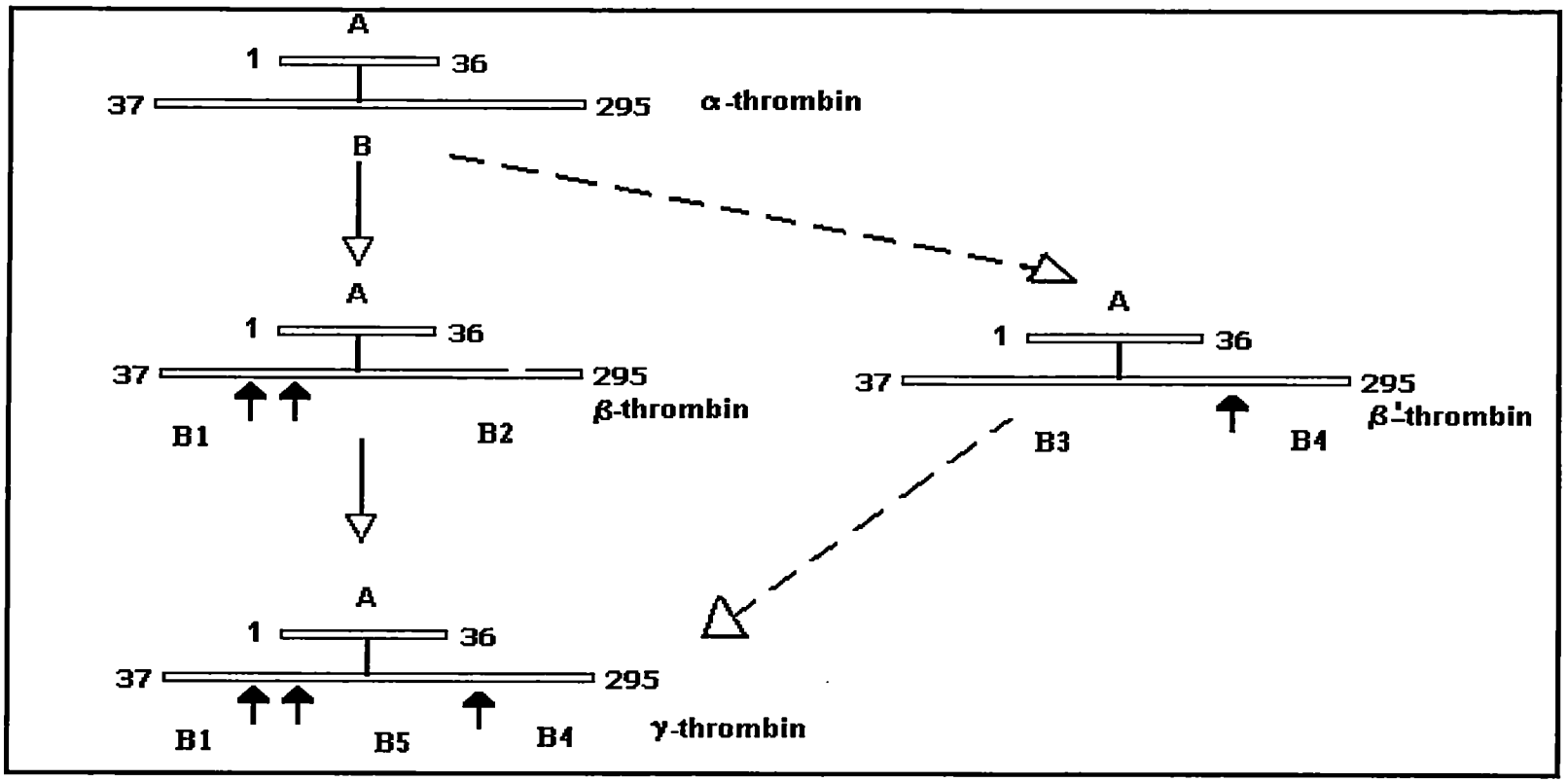


Figure 3.4: Pathway showing the formation of γ-thrombin from α-thrombin. When analyzed, dissolved crystals contain mostly γ-thrombin with some minor amounts of β-thrombin (Rydel, *et. al.*, 1994). Alternative pathway is shown with broken arrows. Three cleavage sites were indicated with arrows. The line connecting A and B chains of thrombin is representing the disulfide bond.

($5X10^3$ $M^{-1}.s^{-1}$) compared to that of α-thrombin. This is thought to result from a decreased access of the active site of γ-thrombin to polypeptide substrates and from a disruption of the fibrin(ogen) recognition site (Lewis, *et. al.*, 1987). It has been shown that γ-thrombin also binds to antithrombin but with a slower rate compared to α-thrombin (Bezeaud, *et. al.*, 1985). Electron spin resonance studies showed that γ-thrombin has an altered active site containing a hydrophobic region which is not found in α-thrombin (Berliner, *et. al.*, 1986).

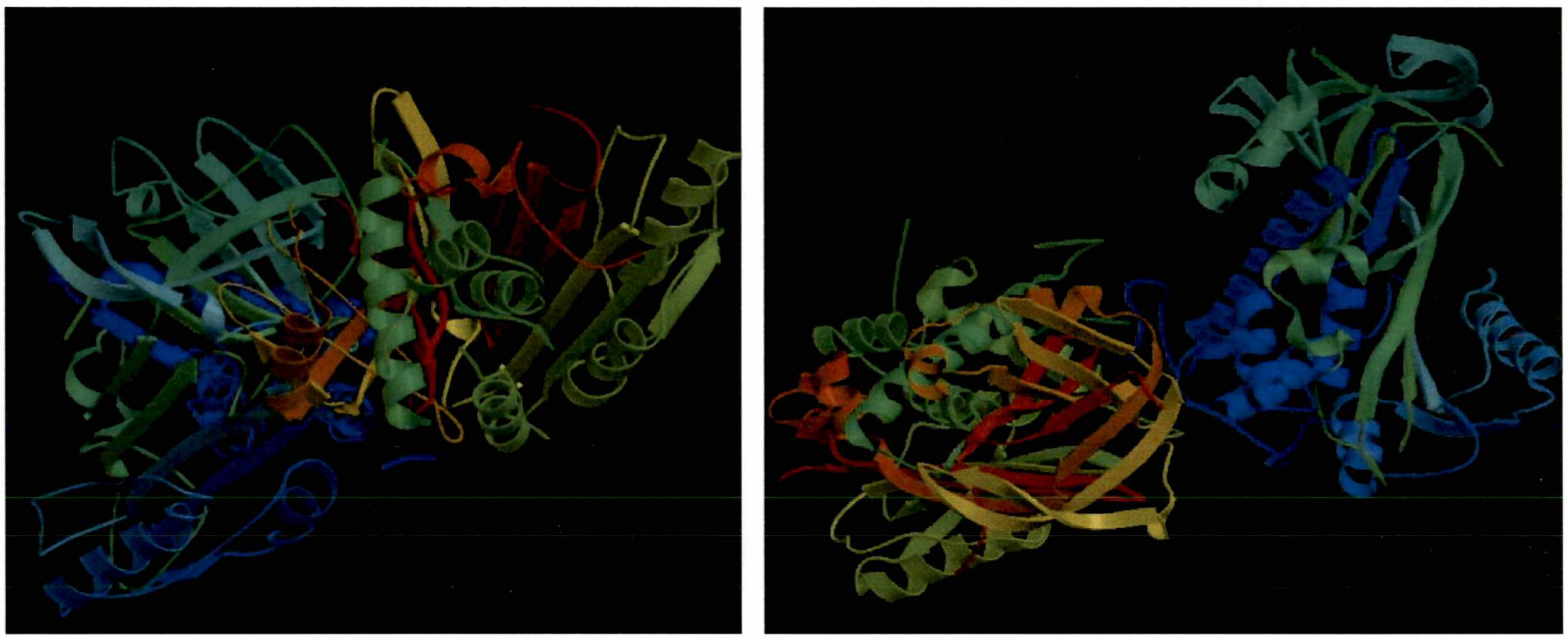
Antithrombin: Clot formation is controlled by numerous physiological mechanisms. In the plasma, there are several serine protease inhibitors (serpins) that prevent spreading of clots beyond the vicinity of the injury site. Antithrombin is a serpin, that circulates in the blood and inactivates coagulation proteinases, such as thrombin, factors IXa, Xa and XIa, plasmin, urokinase, and trypsin. This inhibition takes place via stoichiometric complex formation between the active site of the protease and the scissile bond (Arg 385-Ser 386) of ATIII (Longas, *et. al.*, 1980).

Antithrombin is a 432 amino acid glycoprotein that has homology with other proteinase inhibitors such as α_1-proteinase inhibitor, plasminogen activator inhibitor-1, heparin cofactor II, α_1-antichymotrypsin (Hunt, et. al., 1980, Huber, et. al., 1989). It differs from most serpins since its inhibitory activity is limited, due to the fact that its reactive site loop is partly inserted into the molecule (Carrell, *et. al.*, 1994, Schreuder, *et. al.*, 1994). Antithrombin contains a protease-binding site on this reactive loop where thrombin and other proteases bind and become inactivated. There

is also another site for heparin binding that is separate from the protease-binding site. The interaction between antithrombin and heparin involves a unique pentasaccaride unit on heparin, and critical lysine residues on antithrombin (Jin, *et. al.*, 1997).

Within its structure, antithrombin contains three disulfide bonds and four glycosylation sites (Bock, *et. al.*, 1982, Chandra, *et. al.*, 1983). Figure 3.5 represents the crystal structures of both intact and cleaved antithrombins that have been solved (Schreuder, *et. al.*, 1994, Carrell, *et. al.*, 1994). The intact form has a five-stranded β-sheet, the A-sheet, and an exposed loop containing the reactive-bond between arginine 393-serine 394. This loop structure is a common feature of serine proteases (Wei, *et. al.*, 1994). The structure of inactive antithrombin is cleaved near the reactive bond which gives rise to a conformational change that stabilizes the protein in an altered folded form (Mast, *et. al.*, 1991), in which residues from the cleaved active-site loop region become incorporated as an additional β-strand in the A-sheet.

Interaction between Thrombin and Antithrombin: In biological systems, many potent proteolytic enzymes like proteases are essential for critical reactions. Thrombin is such an enzyme required for blood coagulation. However, antithrombin is equally important in regulating this process. Abnormal antithrombin activity resulting in defective proteinase binding may increase the risk of thrombosis development (Shifman, *et. al.*, 1982).



(a) **(b)**

Figure 3.5: Crystal structure of (a) intact and (b) cleaved antithrombin. Both structures were retrieved from Protein Data Base (PDB).

Antithrombin is required for regulation of active thrombin levels. It achieves this by inhibiting the enzyme. Inactivation of thrombin by antithrombin is initiated when the protease recognizes the reactive bond of antithrombin located in the loop region (Bjork, *et. al.*, 1981). In the loop region of antithrombin, the reactive bond which is between P1-P1' (arginine 393 and serine 394) is assumed to be fully exposed in order for thrombin to bind (Lane, *et. al.*, 1989, Stephens, *et. al.*, 1988). As with all serine proteases, the catalytic mechanism of thrombin interaction with antithrombin is through an acyl-enzyme intermediate formation. This reaction is initiated by the nucleophilic attack of active site Ser residue of thrombin on reactive bond between P-P' of antithrombin to form the tetrahedral intermediate transition state. This process is facilitated by the formation of a low barrier hydrogen bond between Asp and His residues. The tetrahedral intermediate decomposes to the acyl-enzyme intermediate through general acid catalysis by an active site Asp and polarized His residue. The amine leaving group is released from the enzyme and replaced by H_2O (Frey, *et. al.*, 1994, Hedstrom, *et al.*, 1992).

The inhibitory propensity of antithrombin is enhanced by the glycosaminoglycan heparin. The reaction rate of thrombin inactivation increases up to 2000-4000 fold with the involvement of heparin, or heparin-like molecules that are present on the cell surface of blood vessels (Griffith, *et. al.*, 1982, Olson, *et. al.*, 1991, Olson, *et. al.*, 1992). Heparin is not consumed, but is rather released from antithrombin immediately after inactivation of thrombin by formation of the inactive protease-serpin complex. Heparin interacts with target enzymes in the coagulation

cascade in addition to the serpin; however this binding to the proteases is not as strong as binding to antithrombin (Griffith, *et. al.*, 1982).

Binding of the Thrombin-Antithrombin pair to Vitronectin: What is known about the interaction between vitronectin and the thrombin-antithrombin pair? What kind of complexes do they form? Is there a specific binding site on vitronectin for the thrombin-antithrombin complex? This part of the project is focused on some of these questions.

Vitronectin has been proposed to be the protein responsible for clearance of the inactive thrombin-antithrombin complexes from circulation. Preliminary work performed in early 80's demonstrated that vitronectin forms complexes with the thrombin-antithrombin couple (Jenne, *et. al.*, 1985, Preissner, *et. al.*, 1985b). It was proposed that ternary complexes formed between vitronectin and the thrombin-antithrombin pair induce endothelial cell attachment and spreading in a similar manner to free vitronectin (Preissner, *et. al.*, 1988). A study performed by Tomasini and Mosher showed that γ-thrombin binds to vitronectin more tightly compared to α-thrombin, and it was not dependent on the cleavage of vitronectin by the protease. They concluded from this work that the α-thrombin component of the thrombin-antithrombin pair binds to vitronectin, presumably with a region that is exposed on γ-thrombin (Tomasini, *et. al.*, 1989). In this work, the complexes formed between vitronectin and the thrombin-antithrombin pair is proposed to form via a covalent interaction which is formed by a disulfide bond. Since neither thrombin nor

antithrombin has free sulfhydryl groups, this interaction has been thought be formed through a thiol-disulfide exchange with the free thiol coming form vitronectin (Tomasini, *et. al.*, 1989, Dahlback, *et. al.*, 1985). However, the stiochiometric formation of disulfide-bridged complexes was not evident from this work. An alternative hypothesis that should be tested is that, after thrombin and antithrombin form an acyl complex, they bind to vitronectin through a non-covalent interaction. Furthermore, we propose that this interaction gives rise to multimerization of vitronectin and may therefore altered adhesion activity of vitronectin. Figure 3.6 shows contrasts between the conventional model in the literature and new aspects of a model that we are interested in testing. In order to test our hypotheses, we used both enzyme-linked immunosorbant assays and analytical techniques and explained some of the unfulfilled questions about thrombin-antithrombin interaction with vitronectin

Experimental Procedures

Direct Enzyme-Linked Immunosorbant Assay: Interactions of vitronectin with thrombin, antithrombin or the thrombin-antithrombin complex were measured on 96-well microtiter plates (polystyrene, CoStar). Plates were washed two times with PBS and then coated with 50 µl of 1 µg/ml vitronectin overnight at 4°C. Wells were blocked with 3% BSA in PBS for an hour at 37°C. After washing three times with

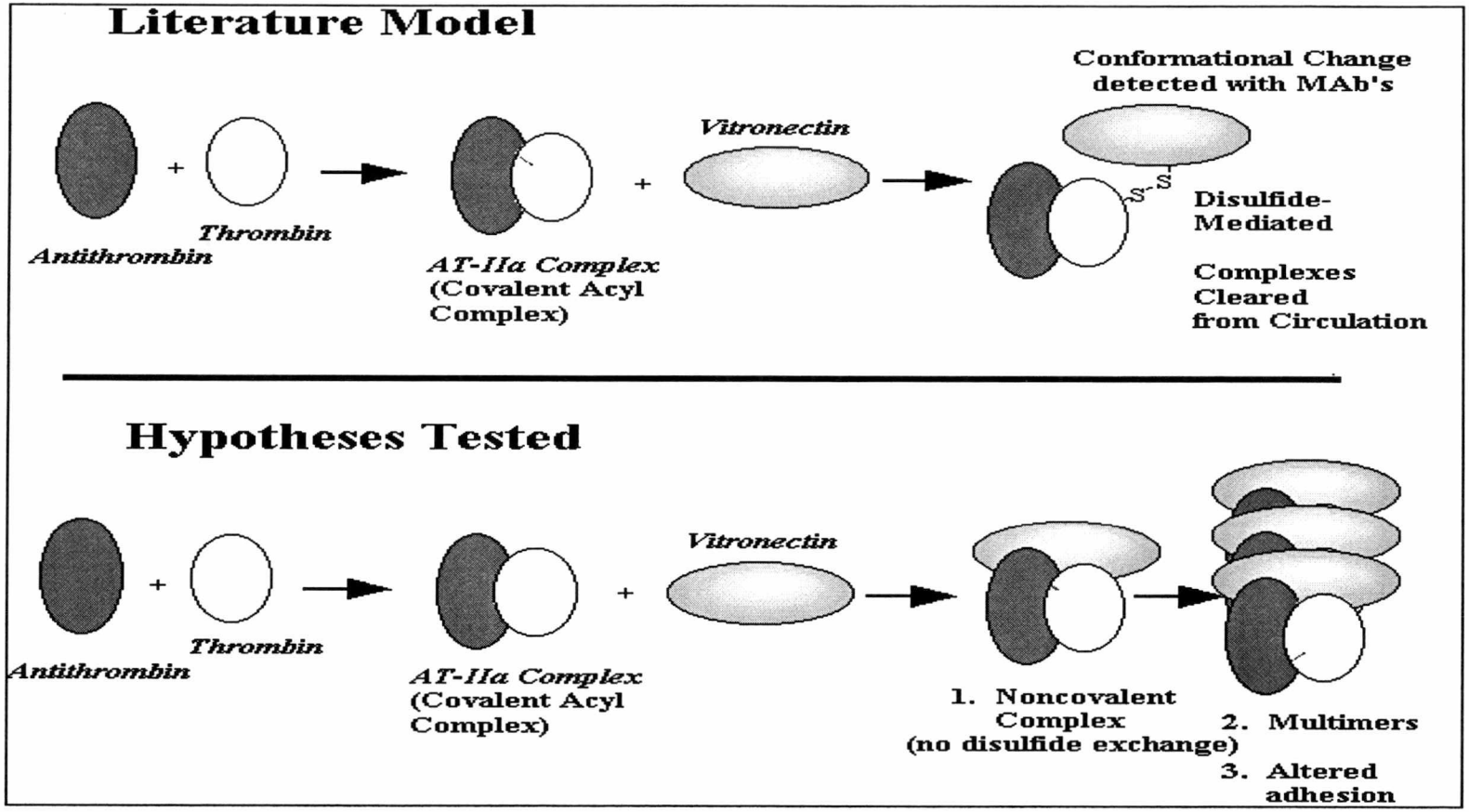


Figure 3.6: Model of interaction between thrombin-antithrombin pair and vitronectin through disulfide bridge formation (a) and the hypothesized model where the interaction is non-covalent (b).

PBS/0.1% BSA/0.1% Tween-20, either thrombin (Haematologic Technologies), antithrombin (a generous gift from Mike Blackburn, Cascade Therapeutics, King of Prussia, PA) or the thrombin-antithrombin complex (starting concentrations of 500nM, 1000nM or 500nM/1000nM, respectively) was serially diluted in the microtiters wells using a final volume of 50 µl per well. Thrombin-antithrombin complexes were prepared as concentrated solutions, incubated at room temperature for an hour, and then diluted to the final concentration on the plate. Plates were incubated at 37°C for 2 hours and washed three times with PBS/0.1% BSA/0.1% Tween-20. Either goat anti-antithrombin or sheep anti-thrombin (Haematologic Technologies) antibodies were diluted 1:1000 and 60 µl was added to each well. The plates were incubated at 37°C for an hour then washed with PBS/0.1% BSA/0.1% Tween-20 three times. Sixty µl of 1:1000 diluted HRP-labeled anti-goat or anti-sheep antibodies were added to each well as appropriate and incubated at 37°C for an hour. After washing the plates as described above, 100 µl of freshly prepared developer solution (0.2mg/ml solution of 2,2'azino-bis-(3 ethylbenzthiazoline-6-sulfonic acid) in 50mM sodium citrate, pH 5.5, with 1:2000 diluted 30% H_2O_2) was added to each well. Absorbance readings were recorded at 405 nm in a Wallac microtiter plate reader.

Competitive Enzyme-Linked Immunosorbent Assay: Competition between T-AT and PAI-1, or γ-thrombin (with or without PPACK blocking the active site), was

analyzed on vitronectin-coated microtiter plates. Briefly, plates were coated with 50μl of 1μg/ml vitronectin overnight at 4°C. Plates were washed with PBS and blocked with 3% BSA at 37°C for an hour. PAI-1 (100nM), or γ-thrombin (100nM, 250nM, 400nM or 500nM), was serially diluted in the microtiter plate in PBS/0.1% BSA/0.1% Tween-20 with a final volume of 50μl in each well. Then 50μl of thrombin-antithrombin complex (a constant concentration of 5nM/10nM) was added and incubated at 37°C for 2 hours. Again, the thrombin-antithrombin complex was prepared as a concentrated solution and incubated at room temperature for an hour and then diluted to the 5nM/10nM final concentration before adding to the plate. Plates were washed with PBS/0.1% BSA/0.1% Tween-20 three times before and after incubation with the primary antibody (either anti-antithrombin or anti-thrombin in 1:1000 dilution) for an hour at 37°C. Then 1:1000 diluted HRP linked anti-goat or anti-sheep antibodies were added and incubated for an hour at 37°C. Plates were washed and absorbances at 405nM were measured. In one experiment, the amount of PAI-1 was fixed (2nM) on the plate and AT-T (250nM-125nM) was serially diluted. In another experiment, the amount of γ-thrombin was fixed (150nM) and AT-T (50nM-50nM) was serially diluted on the plate. Either anti-PAI-1 (1:500 diluted) or anti-thrombin was used as the primary antibody and anti-mouse HRP or anti-sheep HRP was used as secondary antibody, respectively.

NaCl and iodoacetamide effects on the binding of thrombin-antithrombin to vitronectin were analyzed on vitronectin (1μg/ml)-coated plates in the same way as described above. The plates were incubated with a constant amount of the thrombin-

antithrombin complex (5nM/10nM) with serially diluted NaCl or iodoacetamide (starting concentrations of 2M, 100mM, respectively) in the microtiter plate.

Thrombin-Antithrombin Kinetics Assay: Thrombin inactivation was measured by incubating a fixed amount of thrombin (final concentration of 20nM) with varying concentrations of antithrombin (0-40nM) at 37°C for an hour in PBS/0.1% PEG-8000. Then the residual thrombin activity was determined by adding the chromogenic substance, 0.1mg/ml (from a 1mg/ml stock solution prepared in 1mM HCl) Chromozym-TH (Roche). 1 minute after the addition of the chromogenic substance, 50% acetic acid added to quench the reaction. The inactivation was determined by change in absorbance at 405 nm (Peterson, *et. al.*, 1987).

Heparin cofactor activity for antithrombin inhibition of thrombin was measured using a similar assay as described above but reacting thrombin and antithrombin for only one minute with the addition of heparin.

Immunoblotting: Thrombin and antithrombin together or alone were analyzed on 8% SDS-PAGE under non-reducing conditions. After electrophoresis, both gels and a nitrocellulose membrane were equilibrated in transfer buffer (0.35M Glycine, 25mM Tris, 20% Methanol) and then transferred at 20V for 20minutes in a Semi-Dry Blotter (BioRad). After transferring, the membrane was blocked in 10% milk for an hour at room temperature and then washed three times with PBS/0.05% Tween-20. The primary antibody (either anti-thrombin or anti-antithrombin, 1:1000 diluted) and

the secondary antibody (HRP linked anti-sheep or anti-goat, respectively, 1:2000 diluted) solutions were prepared in 1% milk in PBS and incubated with the membrane for an hour at room temperature. The membrane was washed extensively in PBS-Tween between each antibody step. In the final step, immunostained protein bands were visualized by using a freshly prepared developer solution (30ml PBS containing 50mg/ml 4-chloro-napthol and 1:3000 diluted 30% H_2O_2).

Fluorescent Labeling of Vitronectin: Vitronectin was labeled with an amine-reactive fluorescent dye, fluorescein isothiocyanate, according to the method recommended by the supplier (FITC protein labeling kit, Molecular Probes). Briefly, a total of 0.3mg vitronectin in 0.1M $NaHCO_3$, pH 9 was mixed with 1:40 molar ratio of reactive dye (prepared in DMSO) and stirred at room temperature protected from light. Reaction was stopped by adding 1.5M Hydroxylamine, pH 8.5 with 1:50 dilution, and stirred at room temperature, protected from light 30 minutes.

Fluorescent Labeling of Thrombin: Thrombin labeling was performed with two different fluorescent dyes: First FITC (fluorescein isothiocyanate) was used to label vitronectin as described above. Secondly Texas Red-X, which has a reactive succinimidyl ester moiety reacting with primary amines of the proteins was used in these experiments. The method recommended by the supplier was followed (Molecular Probes).

Size-exclusion HPLC to monitor formation of complexes: First, thrombin was mixed in a 1:2 ratio with antithrombin and incubated at room temperature for an hour and then the sample was injected onto a Phenomenex Biosep-SEC-2000 column. The column was equilibrated with PBS containing 0.005% sodium azide. Thrombin and antithrombin were injected onto the same column before the complex. Elution data at 280nm were collected.

Second, thrombin was mixed in a 1:1.5 ratio with antithrombin and incubated at room temperature for an hour. The sample was injected onto a Phenomenex Biosep-SEC-3000 column (300 x 7.8 mm) that was equilibrated with PBS containing 0.005% sodium azide. Vitronectin (at an equal molar ratio with thrombin) was added and allowed to incubate at room temperature for various times. Two hundred µl (approximately 50µg total protein per injection) sample injections were performed. Elution data (absorbance at 280 nm)were recorded over time at a flow rate of 0.5 ml/min.

A Phenomenex Biosep SEC-S3000 (300 x 7.8 mm) column attached to a Hewlett-Packard 1100 series HPLC system was used to purify FITC labeled monomeric vitronectin. PBS containing 0.005% sodium azide was used to equilibrate the column. The labeled vitronectin sample was injected onto the pre-equilibrated size-exclusion column (30µl injections) and chromatographed at a flow rate of 0.5 ml/min. Absorbance was detected directly in the flow cell at 494nm (excitation wave length of fluorocein) as well as at 280 and 220nm. Fractions were analyzed by Western blotting using polyclonal anti-vitronectin antibody, and the fractions

containing labeled monomeric vitronectin were pooled and dialyzed against PBS overnight at 4°C. Protein concentration was determined using BSA protein assay (BioRad). Labeled and purified protein was kept at 4°C protected from light.

Fluorescence Spectroscopy to Monitor Binding of the Thrombin-Antithrombin Complex to Vitronectin: Changes in the fluorescence emission of FITC-VN were measured using a Perkin Elmer LS50B Luminescence Spectrometer, with the excitation monochromator set at 494 nm and the emission wavelength set for 518 nm. Emission scans were performed over a wavelength range of 500-600 nm using an excitation wavelength of 494 nm. Excitation and emission slits were set at 5 and 17 mm, respectively. FITC-VN, thrombin and antithrombin were dialyzed against PBS then clarified by centrifugation. Buffer used for preparation of the samples was filtered before use. Solutions of FITC-VN (15nM) in PBS/0.1% PEG-8000, pH 7.4 were mixed with different concentrations of thrombin-antithrombin complexes (final concentrations of 1000nM, 500nM, 300nM, 250nM, 100nM, 50nM and 25nM). Samples were mixed in a total volume of 2.0 ml in acrylic cuvettes (Sarstedt), and intensity changes were recorded at different time intervals (1 minute to overnight). These experiments were performed in duplicates, and all data were corrected mathematically for dilution. Percent intensity changes were calculated using the equation [(F-Fo)/Fo]*100, where F is the emission intensity and Fo is the emission of FITC-VN in the absence of thrombin-antithrombin complex and then percent changes were plotted as function of time.

Results

Characterization of T-AT complex formation: Thrombin and antithrombin were mixed at different molar ratios, incubated for either an hour at room temperature or half an hour at 37°C and analyzed on 10% SDS-PAGE (Figure 3.7). Commassie staining showed that thrombin and antithrombin form a complex at a 1:1 molar ratio. Although the complex formation became much prominent at 1:2 ratio, there was also free antithrombin apparent on the gel.

The stability of complex formation over time was analyzed with or without PPACK (phenylalanyl propylarginine chloromethyl ketone) addition. PPACK is a peptide that blocks the active site of thrombin and therefore inhibits its protease activity. Thrombin and antithrombin were mixed at a 1:2 ratio (5μM: 10μM), incubated for an hour at room temperature, and then PPACK was added (10μM final concentration). At different time intervals, the complex was mixed with non-reducing running gel buffer, boiled and loaded on a 10% SDS-PAGE. Figure 3.8 is the result of Commassie staining. These findings show that the thrombin antithrombin complex is stable once formed, even after overnight incubation at room temperature.

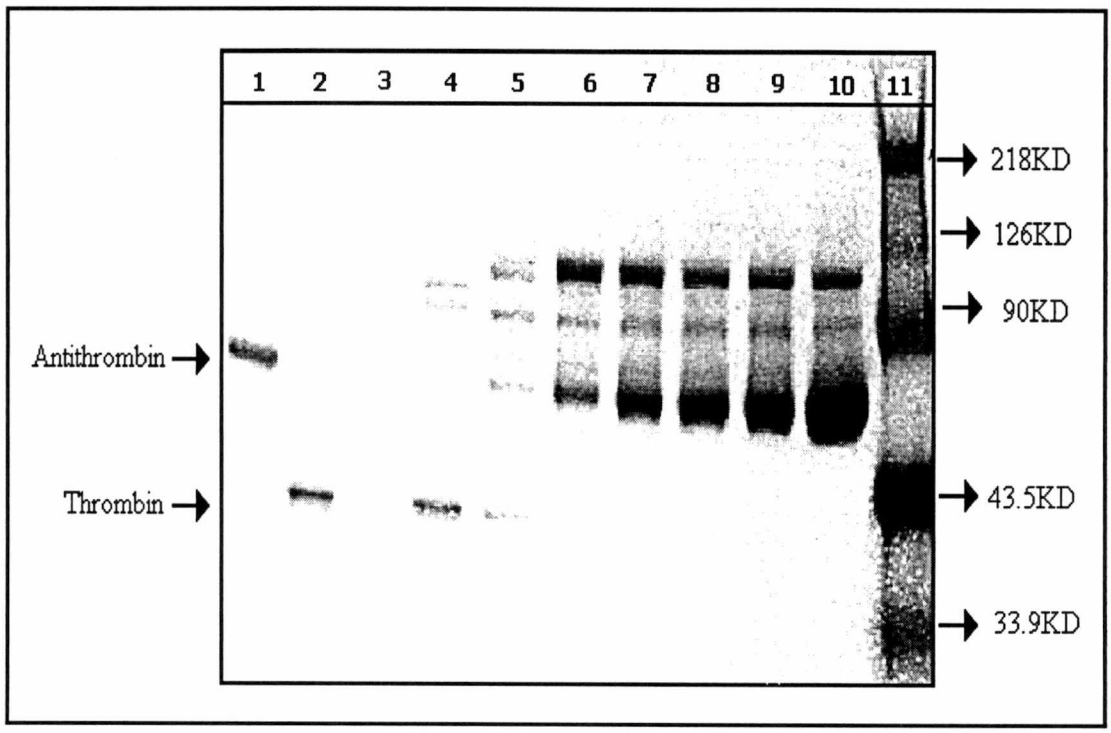


Figure 3.7: 10-20% SDS-PAGE gradient gel showing the thrombin-antithrombin complex formation at different ratios run under nonreducing conditions at 160V for an hour and then commassie stained. Lane 1 and lane 2 are antithrombin and thrombin at equimolar concentrations, respectively. Thrombin concentration was kept constant (final concentration of 1.5 μM) and antithrombin was varied. Starting form lane 4 through lane 10, thrombin antithrombin molar ratios on the gel are as follows, 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:10. Lane 11 is Kaleidoscope prestained protein standards.

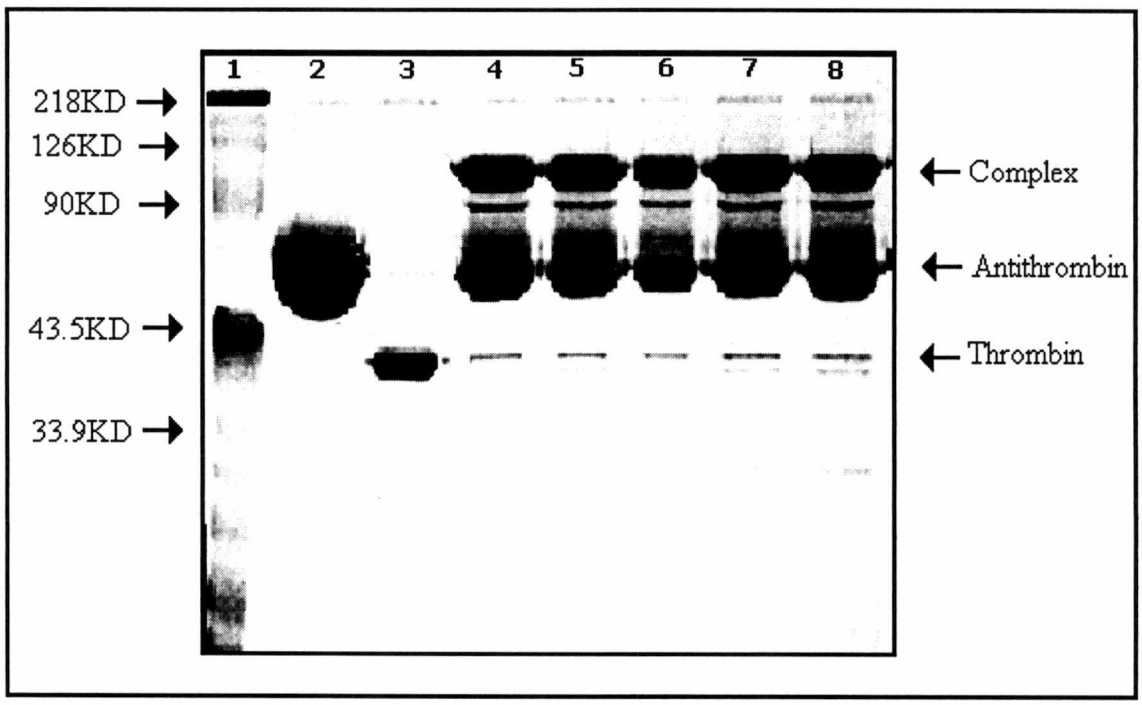


Figure 3.8: Antithrombin and thrombin were mixed at 2:1 ratio, incubated at room temperature for an hour before stopping it with PPACK. Non reducing running buffer added at different time points and boiled. All these samples were run on 10% SDS-PAGE at 160V for one hour and commassie stained afterwards. First lane is the kaleidoscope prestained protein standards. Lane 2 is antithrombin, lane 3 is thrombin loaded on the wells alone. Starting from lane 4 through lane 8 thrombin-antithrombin complexes were loaded after 1hour, 2 hours, 3 hours, 5hours and overnight incubations at room temperature.

Complex formation between thrombin and antithrombin was also studied with the help of high-pressure liquid chromatography. Figure 3.9 shows the complex formation between thrombin and antithrombin by gel filtration using an HPLC system (details are in the experimental methods section). Thrombin was mixed with antithrombin with 1:2 molar ratio, and incubated at room temperature for an hour. The sample was then analyzed by HPLC. In the chromatogram, excess antithrombin was evident, and essentially all the thrombin is present in the serpin-protease complex.

Binding of Thrombin, Antithrombin and Thrombin-Antithrombin complex to vitronectin: A solid phase ELISA experiment showed that thrombin can bind to vitronectin by itself or in complex with antithrombin, while antithrombin can only bind to vitronectin if it is complexed with thrombin (Figure.3.10). This shows that thrombin interacts with vitronectin; however these data alone are not conclusive regarding what protein(s) in T-AT complex interact with vitronectin. Also, it is not surprising that thrombin would recognize and bind to vitronectin since it is known to cleave vitronectin under some conditions.

Determination of Thrombin-Antithrombin complex with vitronectin formation on size-exclusion HPLC: Size exclusion HPLC was used to study the formation of complexes resulting from thrombin-antithrombin binding to vitronectin. A similar study carried out in this laboratory has demonstrated that the interaction of

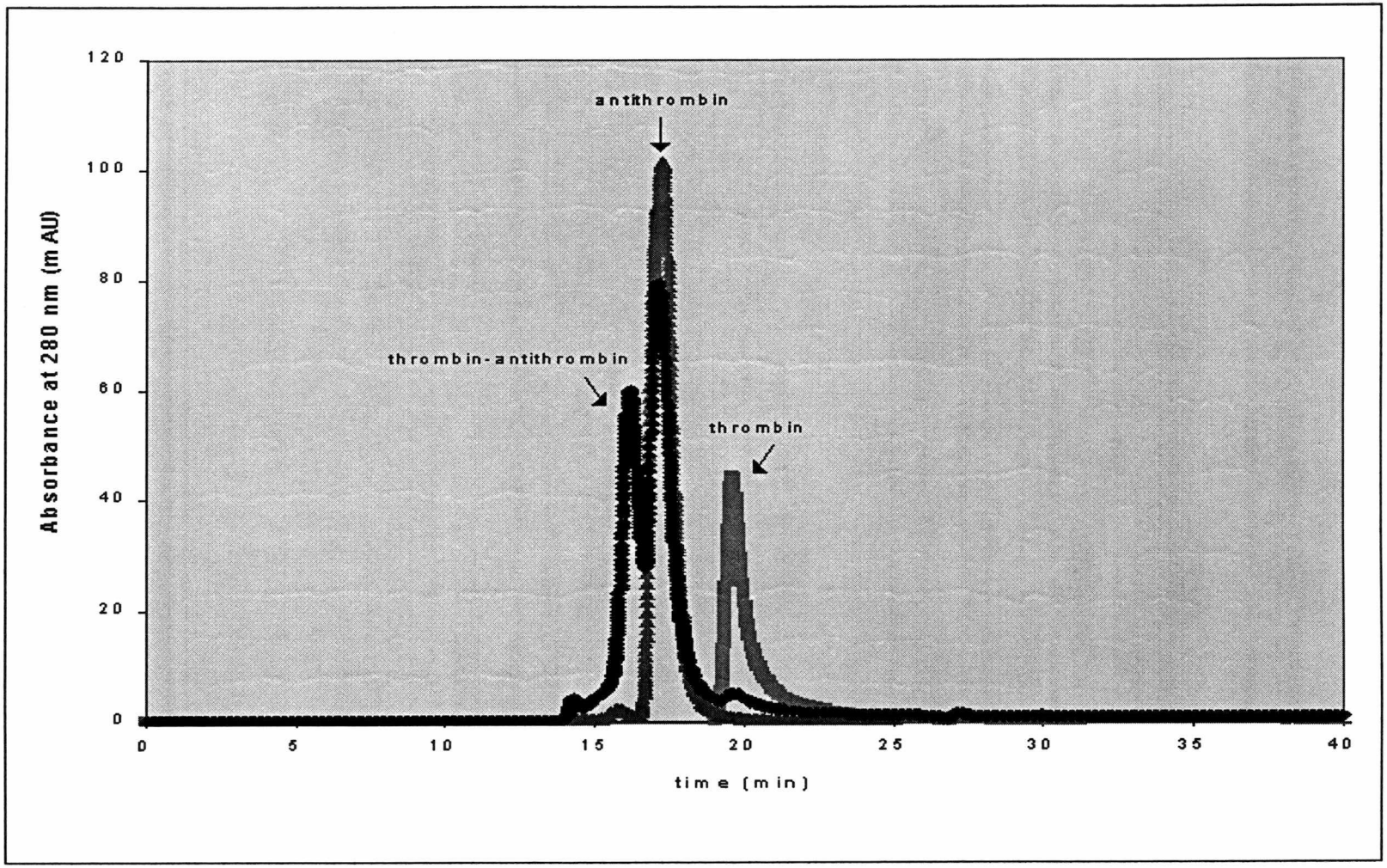


Figure 3.9: HPLC chromatogram showing the complex formation between thrombin and antithrombin. Thrombin was eluted at 20 minutes, while antithrombin was at 17 minutes. Complex formation after one hour incubation of thrombin and antithrombin at 1:2 molar ratio at room temperature was eluted at 16 minutes. See experimental procedures for details.

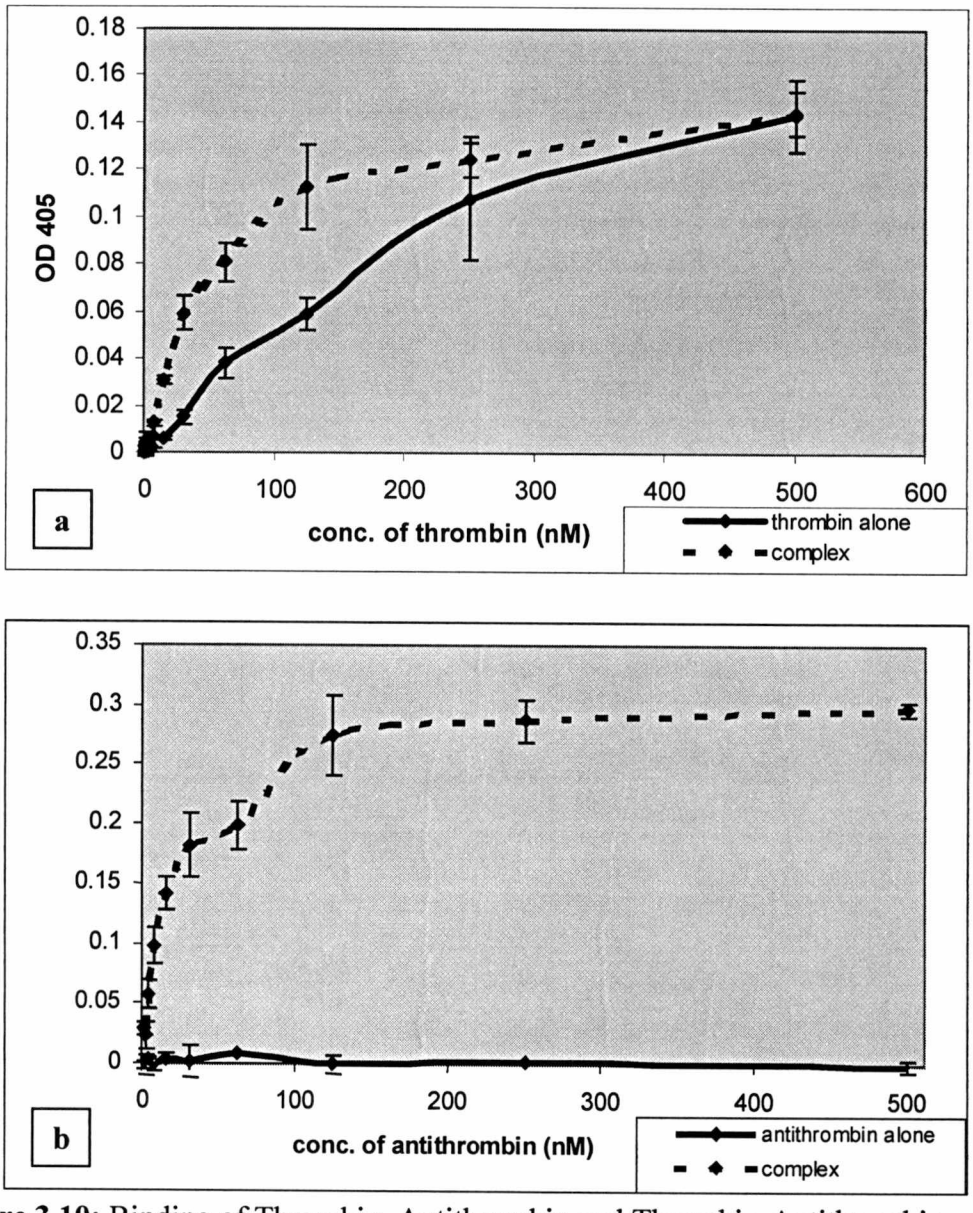


Figure 3.10: Binding of Thrombin, Antithrombin and Thrombin-Antithrombin complex to vitronectin. (a) Thrombin alone or in complex with antithrombin diluted out on an ELISA plate, and detected with sheep anti-thrombin antibody. (b) Antithrombin alone or in complex with thrombin diluted out on the plate and goat anti-antithrombin antibody was used for detection. Thrombin antithrombin complexes were prepared at 1:2 ratio by incubating at room temperature. Absorbances were read at 405nm.

vitronectin with PAI-1 results in the formation of large complexes (Minor and Peterson, in press). Binding of vitronectin to ligands in the circulation has been suggested to lead to enhanced binding of other potential targets. In order to study the possibility that vitronectin multimerization may be induced upon binding to the T-AT complex, the sizes of complexes were directly evaluated using size-exclusion chromatography on an HPLC system. The thrombin antithrombin complex was prepared by incubating the proteins at room temperature for an hour prior to mixing with vitronectin. The molar ratio of thrombin, antithrombin and vitronectin was 1: 1.5: 1, respectively. Chromatograms were recorded at different time points and are shown in figure 3.11. These HPLC results indicate that vitronectin makes complexes with thrombin-antithrombin couple. Over time, higher molecular weight complexes are observed suggests that complex formation with T-AT gives rise to multimerization of the complexes.

NaCl and Iodoacetamide effect on complex formation: ELISA assays with vitronectin (1μg/ml)-coated plates were performed in which the thrombin-antithrombin complex was incubated at different NaCl or iodoacetamide amounts. These studies indicated the interaction between thrombin-antithrombin with vitronectin is decreased at increasing salt concentrations (figure 3.12). However, iodoacetamide had no effect at all even at very high concentrations (figure 3.13). These experiments were also performed to analyze the effect of NaCl or Iodoacetamide on interaction of thrombin or antithrombin alone. Iodoacetamide

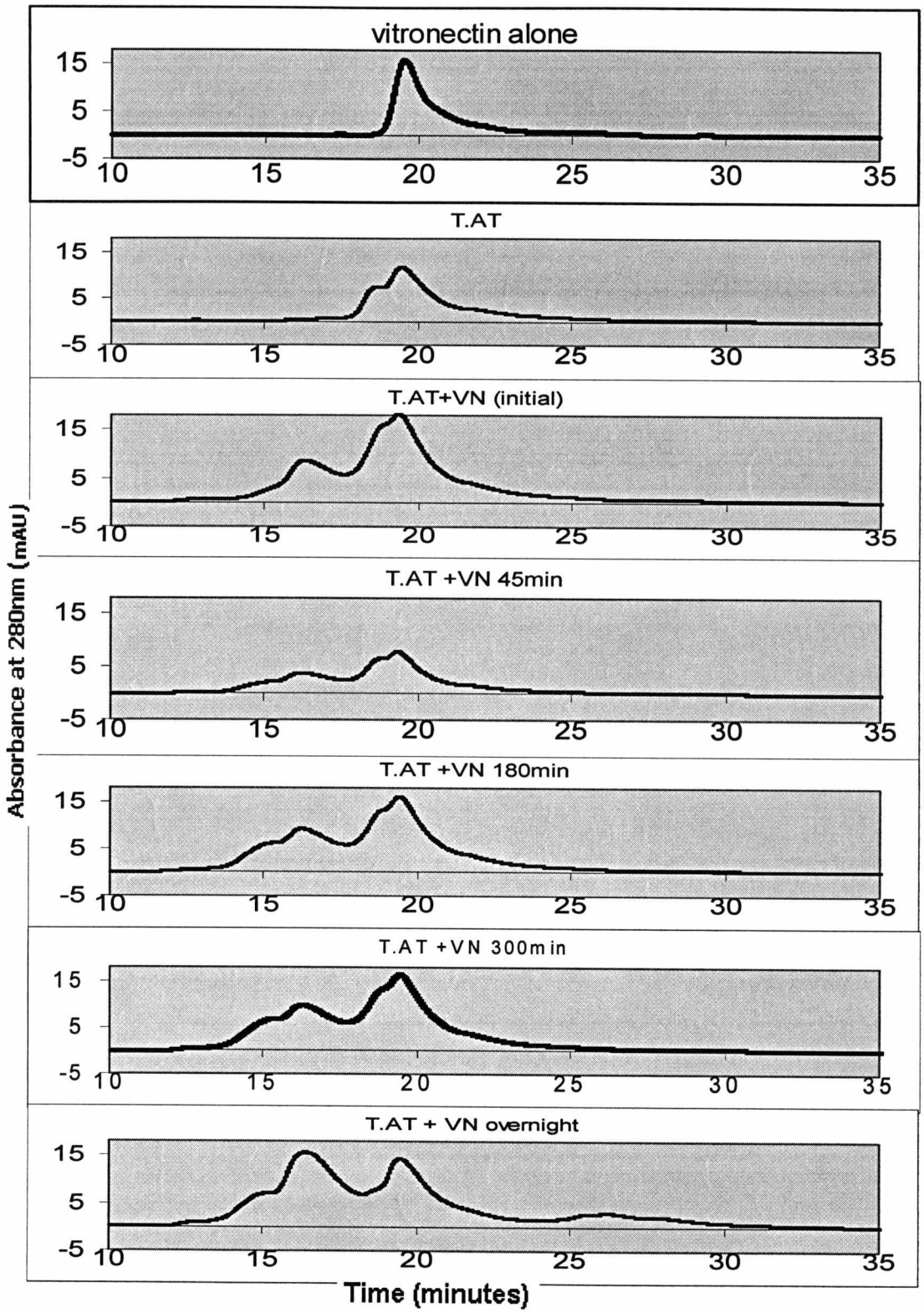


Figure 3.11: Complex formation between thrombin antithrombin pair and vitronectin over a period of time analyzed on size exclusion HPLC. T.AT complex is eluted around 18th minute. After VN addition, complexes formed between T.AT.VN eluted around 16th minute. Overtime, multimerization peak appeared around 15th minute.

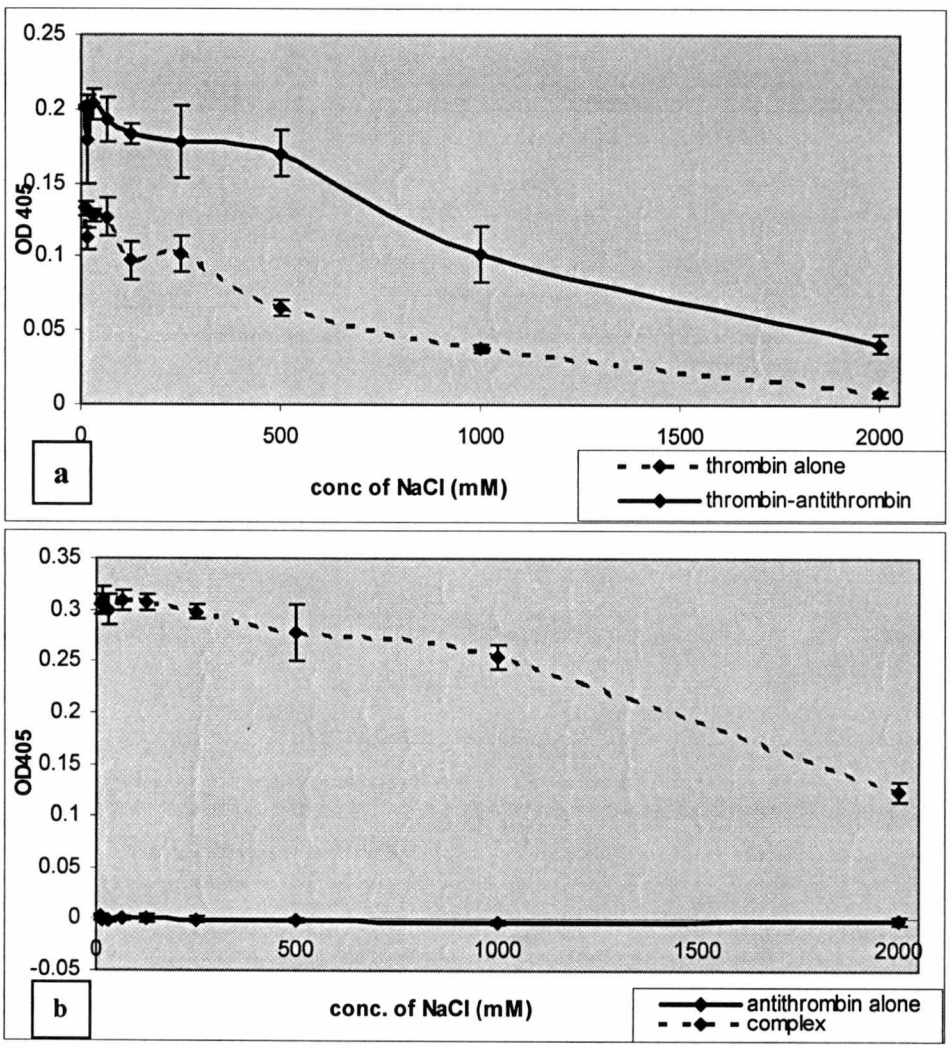


Figure 3.12: Solid phase assays analyzing NaCl effect on interaction between vitronectin and thrombin antithrombin pair, alone or in the complex. **(a)** NaCl effect on thrombin, or complex binding to VN, detected with anti-thrombin polyclonal antibody **(b)** NaCl effect on antithrombin or complex binding to vitronectin, detected with anti-antithrombin polyclonal antibody.

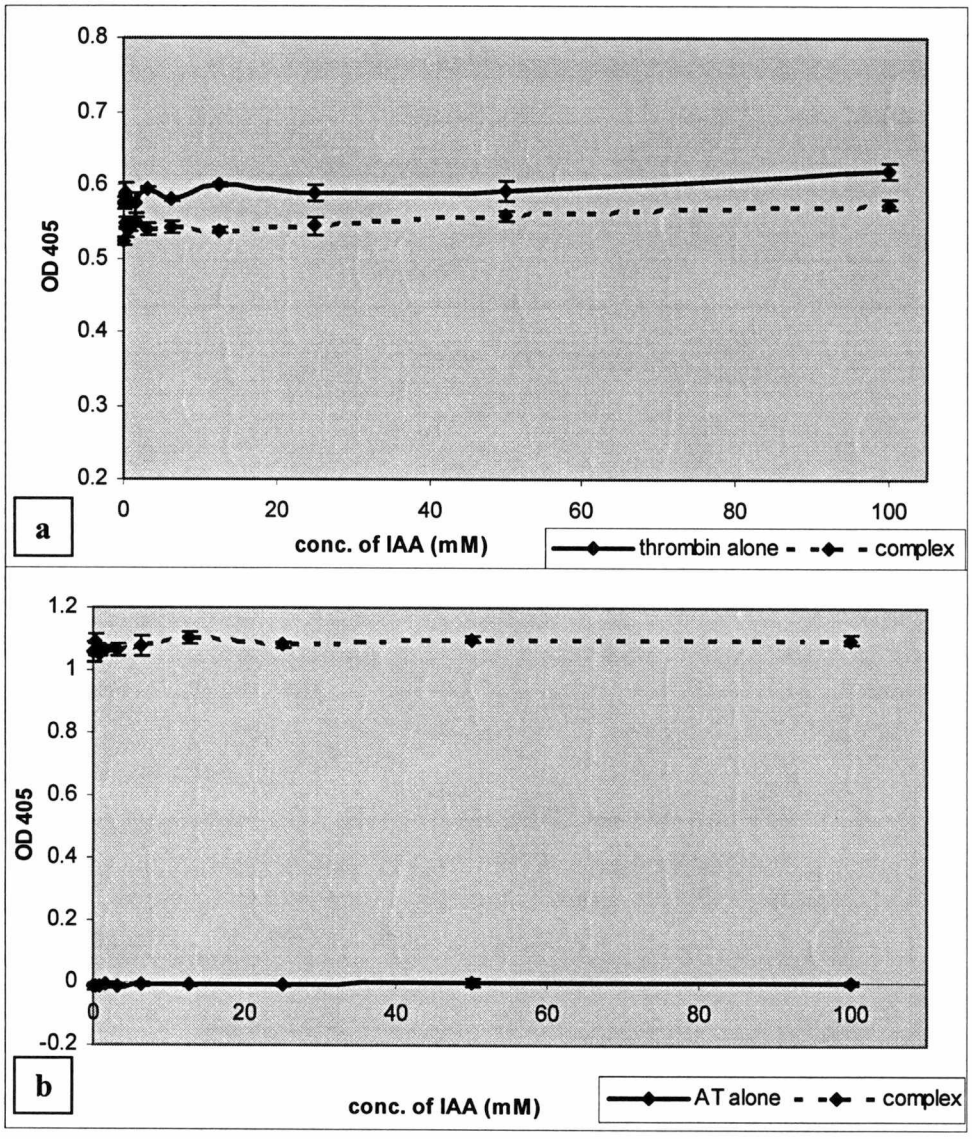


Figure 3.13: Solid phase assays analyzing IAA effect on interaction between vitronectin and thrombin antithrombin pair, alone or in the complex. **(a)** IAA effect on thrombin or complex binding to VN, detected with anti-thrombin polyclonal antibody **(b)** IAA effect on antithrombin or complex binding to vitronectin, detected with anti-antithrombin polyclonal antibody.

blocks sulfydryl groups of the protein. If disulfide bond formation is critical for interaction of T-AT with vitronectin, involvement of iodoacetamide would unable this complex formation. Since NaCl affected the complex formation and iodoacetamide had no effect at all, we can conclude that the binding of T-AT to vitronectin is probably not disulfide mediated but rather it is an ionic interaction.

Competition between γ-thrombin and PAI-1 with Thrombin-Antithrombin complex for their binding to vitronectin: Competition between the α-thrombin-antithrombin complex and γ-thrombin for binding to vitronectin was analyzed in the solid-phase assay. When a fixed amount of γ-thrombin was assayed with varying amounts of the antithrombin/ α-thrombin complex, the competition was distinctive (Figure 3.14).

With the same approach, competition between PAI-1 and antithrombin-thrombin complex was studied on vitronectin coated ELISA plates. Binding sites for PAI-1 have been localized to the somatomedin B domain and to the connecting region (Deng, et. al., 1996b, Mimuro, et. al., 1993). Figure 3.15, the graphical representation of these results, indicates that the antithrombin-thrombin complex and PAI-1 do not compete for binding to vitronectin. These data demonstrate that binding sites of T-AT and PAI-1 on vitronectin are not the same.

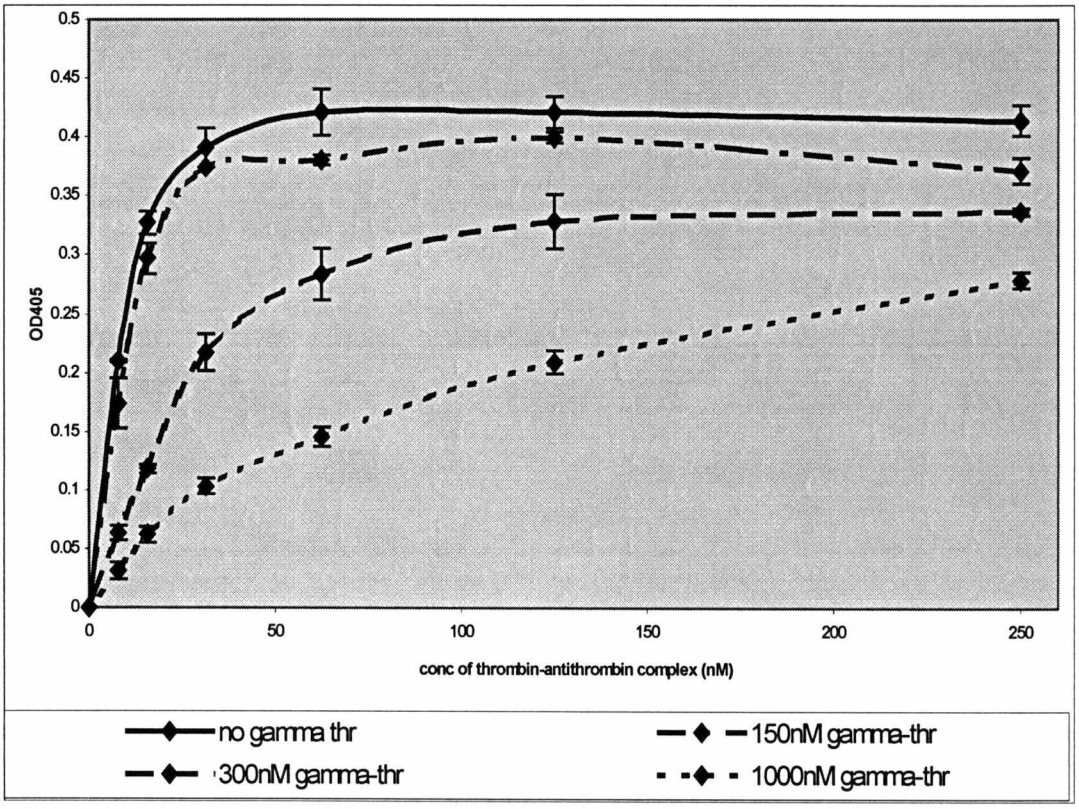


Figure 3.14: gamma thrombin-AT/alpha thrombin competition. Thrombin-antithrombin complex was diluted on the plate and assayed with fixed amounts of gamma thrombin up to 1000nM final concentration. For detection, goat anti-antithrombin antibody was used, and absorbances were measured at 405nm.

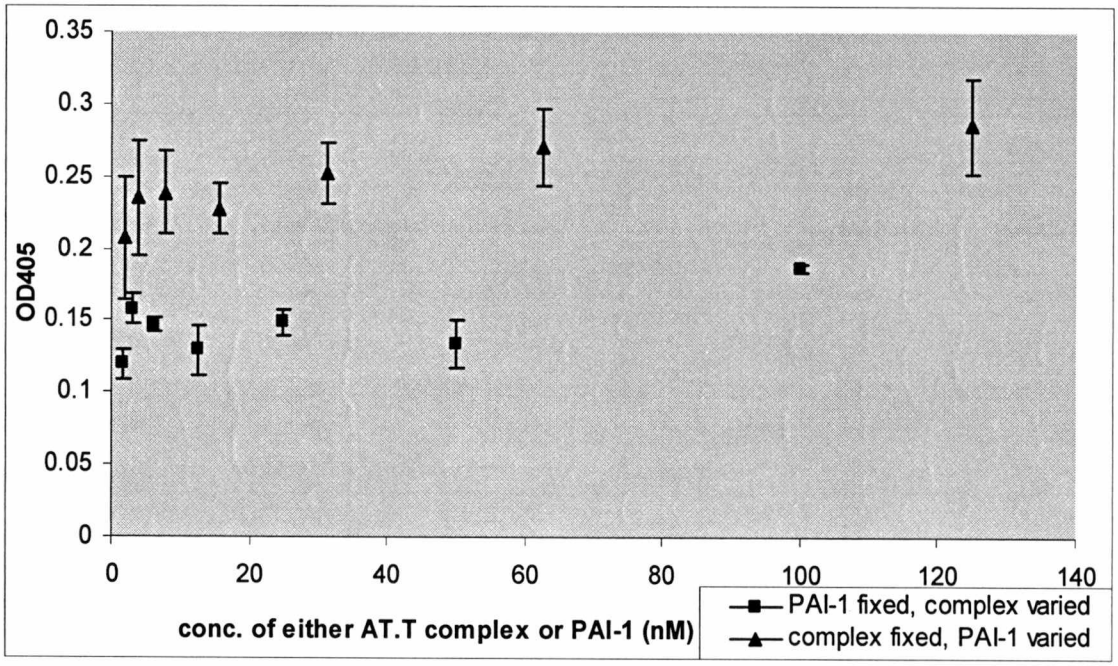


Figure 3.15: PAI-1 competition with antithrombin-thrombin complex. Either PAI-1 or thrombin- antithrombin complex amount fixed and the other varied on the plate and competition measured. For thrombin-antithrombin detection goat-anti-antithrombin antibody used. For PAI-1 detection mouse-anti PAI-1 antibody used. Absorbances were measured at 405 nm.

Evaluation of complex formation between T-AT and vitronectin by fluorescence studies: We first tested different methods for introducing extrinsic labels onto thrombin that can be used as fluorescent probes to follow binding interactions in solution. Texas Red and FITC were used for labeling thrombin. Activity assays showed that Texas Red labeled thrombin is not active compared to FITC labeled and unlabeled thrombin (see thrombin-antithrombin kinetics assay in experimental procedures). Also, Texas Red labeled thrombin showed decreased binding affinity towards vitronectin that was immobilized on ELISA plates. We studied the interaction between T-AT and vitronectin using FITC-labeled thrombin. Although we got increased intensity over time when antithrombin was added to FITC-labeled thrombin, addition of vitronectin to this complex did not result in a significant intensity change.

After the thrombin labeling experiments, we labeled vitronectin to evaluate suitable probes to use fluorescence to monitor the interaction with the thrombin-antithrombin pair. Labeled monomeric vitronectin was purified on HPLC (using Phenomenex Biosep-SEC-3000 column) which is depicted on figure 3.16. FITC was also used to label vitronectin. Monomeric FITC labeled vitronectin fractions were pooled according to the Western blot result. Binding of the antithrombin-thrombin complex and vitronectin was monitored using a fluorescence-based assay with FITC-labeled vitronectin. Figure 3.16 shows the chromatogram of separated peaks of labeled multimeric and monomeric vitronectin obtained on a Phenomenex Biosep-SEC-3000 column (300x 7.8mm).

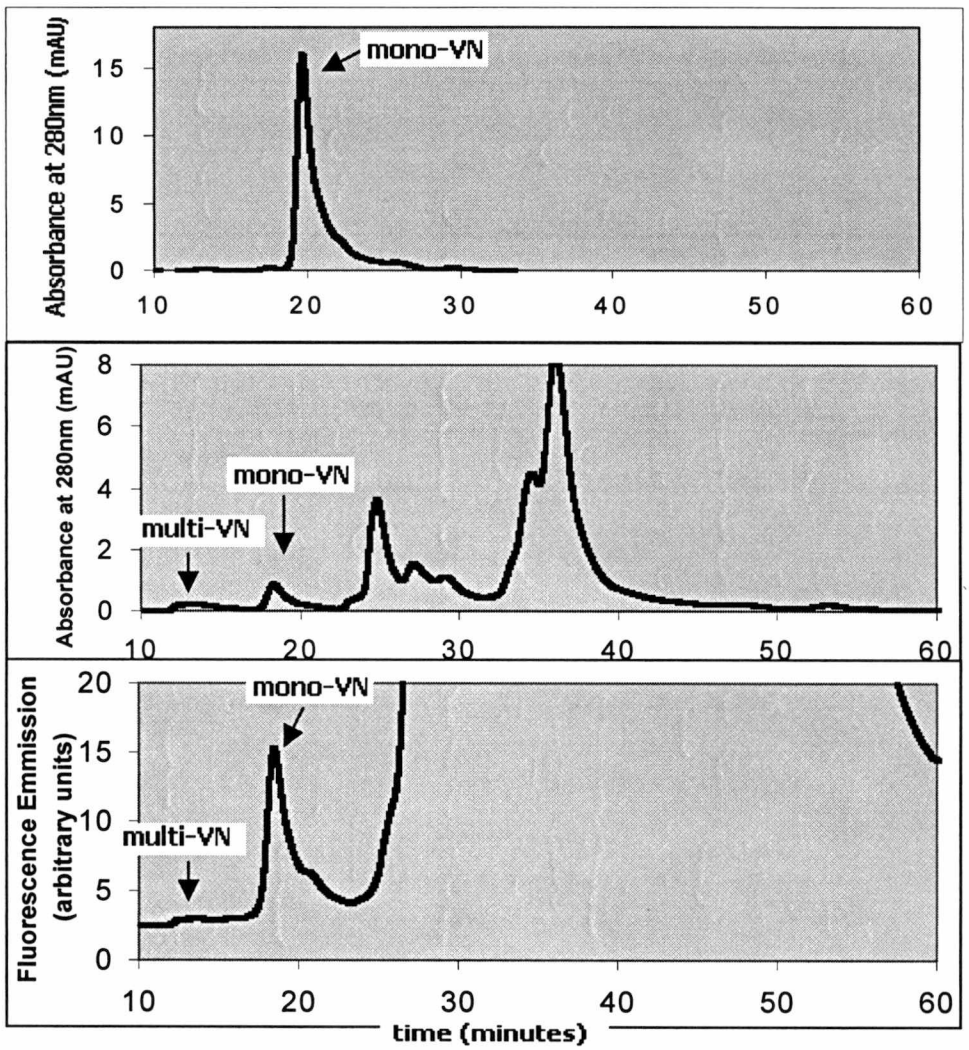


Figure 3.16: Separation of monomeric FITC labeled vitronectin on size-exclusion HPLC. The top graph is showing the 280nm spectrum of monomeric vitronectin that is not labeled. The middle graph is the 280nm spectrum of FITC labeled vitronectin. The bottom graph is the fluorescence emission spectrum of FITC labeled vitronectin (Ex = 494nm and Em = 518nm). Multimeric vitronectin was eluted between 12th and 15th minute and monomeric vitronectin was eluted between 18th and 21st minute. Labeled monomeric vitronectin between this range was run on a SDS-PAGE and western blotted using polyclonal antibody raised against vitronectin. Fractions containing FITC labeled monomeric vitronectin were pooled according to western result. Monomeric and multimeric vitronectin peaks were indicated as mono-VN and multi-VN, respectively.

Addition of the thrombin-antithrombin complex to FITC-vitronectin resulted in increase in the intensity of the fluorescence probe. Fluorescence intensity increased to 10% within the first minute at different concentrations of antithrombin-thrombin complex additions to FITC-vitronectin (15nM). This increase in intensity was saturable with respect to the concentration of antithrombin-thrombin complex. Figure 3.17a depicts the first minute percent increase of the fluorescence intensity at different complex concentrations. The intensity increase slowed down after first 5 minute, yet continued over a long period of time (Figure 3.17b).

Discussion

The objective of this study was to analyze the complexes formed between antithrombin-thrombin and vitronectin. In a first approach, enzyme-linked immunosorbant assays were performed to charactherize the binding of thrombin and antithrombin together and alone to vitronectin under different conditions. In the second part, analytical experiments were used to evaluate the binding kinetics and to clarify the stiochiometry of the species involved. This type of analysis is difficult to determine in a solid-phase experiment since one of the reactant substances should be immobilized on a surface, which may result in variable binding affinities. Therefore, we combined both solid and solution-phase experiments to answer some of the questions posed about antithrombin-thrombin interactions with vitronectin.

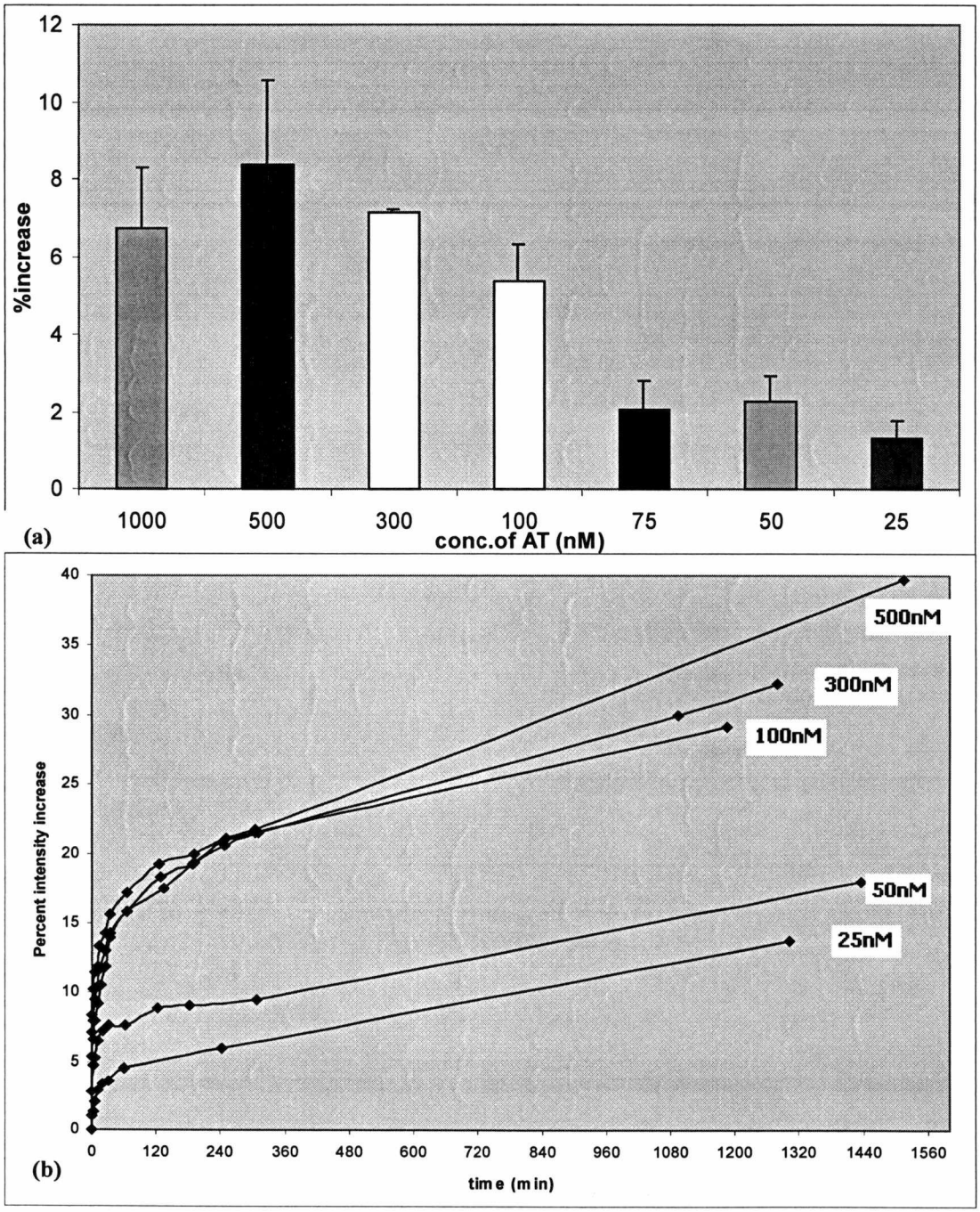


Figure 3.17: (a) Percent fluorescence intensity increase of FITC-vitronectin 1 minute after addition of thrombin-antithrombin complex. **(b)** Percent fluorescence intensity change of FITC-vitronectin when different concentrations of thrombin-antithrombin complex added over a long period of time.

The results of solid phase ELISA experiments showed that binding of the thrombin-antithrombin complex to vitronectin takes place through the thrombin part of this pair. Competition assays showed that in the presence of PAI-1, the binding of thrombin-antithrombin pair to vitronectin is not affected. Two PAI-1 binding sites have been located on vitronectin. One of them is on somatomedin-B domain and the second site is in the connecting region ((Deng, *et. al.*, 1996b, Mimuro, *et. al.*, 1993). Therefore, it can be concluded that, the thrombin-antithrombin binding site on vitronectin is somewhere other than somatomedin B domain and the connecting region. A possible site for thrombin-antithrombin interaction is the central hemopexin homology domain, which may mediate numerous protein-protein interactions.

The proteolytic derivative of α-thrombin, γ-thrombin was also used for competition studies. Compared to α-thrombin, γ-thrombin has reduced proteolytic activity and it does not have the clotting ability. Even though it forms complexes with antithrombin, it does so much more slowly compared to that of α-thrombin. Also, in contrast to α-thrombin, γ-thrombin has no heparin binding ability (Bezeaud, *et. al.,* 1985, Olson, *et. al.,* 1986). In a study by Tomasini and Mosher, γ-thrombin was shown to interact with vitronectin as efficient as thrombin-antithrombin complex at equal molar ratio, while α-thrombin interaction was not as effective (Tomasini, *et. al.,* 1989). As an extension of this work, the competition experiment carried on ELISA plates showed that the thrombin-antithrombin complex binding to vitronectin was inhibited as the amount of γ-thrombin was increased. γ-thrombin does not have proteolytic activity on vitronectin. Also, it binds to vitronectin as tightly as the

thrombin-antithrombin complex. Therefore, it can be used as a nice tool to study thrombin interaction with vitronectin without considering the proteolytic cleavage of the protein over time.

In the study of Tomasini and Mosher, it was suggested that the complex formation between thrombin-antithrombin and vitronectin take place through a disulfide bridge. However, our studies clearly show that it is not a disulfide mediated complex formation. In ELISA experiments in the presence of iodoacetamide, the complex formation between vitronectin and thrombin-antithrombin pair was not affected.

The time-course size-exclusion HPLC results showed that the binding of The thrombin-antithrombin pair to vitronectin leads to the multimerization of vitronectin over a long time period. The fluorescence experiments also support the multimerization of vitronectin where the fluorescence intensity of FITC-labeled vitronectin changed over a long period of time course similar to that observed in the HPLC experiments that demonstrate large complexes.

Heparin which binds to vitronectin, also interacts with thrombin and facilitates the complex formation with antithrombin. Therefore, in the presence of heparin, multimerization of vitronectin through its interaction with thrombin-antithrombin pair may increase the rate of clearance of inactive thrombin-antithrombin complexes from the circulation since glycosaminoglycans will trap these macromolecules and facilitate the interactions and clearance.

Concluding Remarks and Future Directions

Solution-phase analytical methods, such as ultracentrifugation will be a nice approach to determine the size and stiochiometry of T-AT-vitronectin complexes. These ultracentrifugation experiments appear feasible in pilot studies. HNB labeled antithrombin, FITC-thrombin and FITC-vitronectin, will be used to study complex formation on equilibrium experiments.

Monoclonal antibodies raised against vitronectin have been used on competitive ELISA experiments in order to determine the binding site for thrombin-antithrombin complex on vitronectin. Although inhibition of thrombin-antithrombin binding to vitronectin was observed with certain antibodies, we have not yet been able to locate binding sites on vitronectin because of an insufficient amount of material regarding antibody epitopes. Performing epitope mapping on vitronectin for these monoclonal antibodies will give clues about where thrombin-antithrombin pair binds to vitronectin.

In this thesis work, when the complex formation between thrombin-antithrombin pair and vitronectin was monitored using size-exclusion HLPC, this complex formation resulted in the incorporation of vitronectin into high-order species. Another line of work performed in this laboratory on interaction between PAI-1 and vitronectin gave the same type of vitronectin multimerization event (Minor and Peterson, manuscript submitted). Parallel to this study, it will be important to

examine the effects of thrombin-antithrombin-induced multimerization of vitronectin on its interactions with cell surface receptors and matrix components.

References

References:

Ahn, J. H., Lee, Y. P., Rhee, J. S. (1997). Investigation of refolding condition for *Pseudomonas fluorescens* lipase by response surface methodology. J. Biotechnol. *54*, 151-160.

Barnes, D. W., Silnutzer, J. See, C., Shaffer, M. (1983). Characterization of human spreading factor with monoclonal antibody. Proc. Natl. Acad. Sci. *80*, 1362-1366.

Becker, R. C., Bovill, E. G., Seghatchian, M. J., Samama, M. M. (1998). Pathobiology of thrombin in acute coronary syndromes. American Heart Journal *136*, 19-31.

Becker, R. C., Bovill, E. G., Seghatchian, M. J., Samama, M. M. (1998). Pathobiology of thrombin in acute coronary syndromes. American Heart Journal *136*.

Berliner, L. J., Sugawara, Y., Fenton, J. W. II. (1985). Human a-thrombin binding to non-polymerized fibrin-sepharose: evidence for an anionic binding region. Biochemistry *24*, 7005-7009.

Berliner, L. J., Birktoft, J. J., Miller, T. L., Musci, G., Scheffler, J. E., Shen, Y. Y>, Sugawara, Y. (1986). Thrombin: active-site topography. Ann. N. Y. Acad. Sci. *485*, 80-95.

Betemps, D., Mallet, F., Cheynet, V., Baron, T. (1999). Overexpression and purification of an immunologically reactive his BIV capsid fusion protein. Prot. Exp. Purif. *15*, 258-264.

Bezeaud, A., Denninger, M. H., Guillin, M. C. (1985). Interaction of human alpha-thrombin and gamma-thrombin with antithrombin III, protein C and thrombomodulin. Eur. J. Biochem. *153*, 491-496.

Bhakdi, S., Kaflein, R., Halstensen, T. S., Hugo, F., Preissner, K. T., Mollnes, T. E. (1988). Complement S-protein (vitronectin) is associated with cytolytic membrane-bound C5b-9 complexes. Clin. Exp. Immunol. *74*, 459-464.

Bjork, I., Danielsson, A., Fenton, J. W. II, Jornvall, H. (1981). the site in human anithrombin for functional proteolytic cleavage by human thrombin. FEBS Lett. *126*, 257-260.

Bjork, I., Lindahl, U. (1982). Mechanism of the anticoagulant action of heparin. Mol. Cell. Biochem. *48*, 161-182.

Blomback, B., Blomback, M., Hessel, B., Iwanaga, S. (1967). Structure of N-terminal fragments of fibrinogen and specificity of thrombin. Nature *215*, 1445-1448.

Bock, S. C., Wion, K. L., Vehar, G. A., Lawn, R. M. (1982). Cloning and expression of the cDNA for human antithrombin III. Nucleic Acids Res. *10*, 8113-8125.

Bode, W., Turk, D. and Karshikov, A. (1992). The refined 1.9-A^{o} x-ray crystal structure of D-Phe-Pro-Arg-chloromethyl ketone-inhibited human α-thrombin: structure analysis, overal structure, electrostatic properties, detailed active site geometry, and structure function relationships. Protein Sci. *1*, 426-471.

Cardamone, M., Puri, N. K., Brandon, M. R. (1995). Comparing the refolding and reoxidation of recombinant porcine growth hormone from a urea denatured state and from *E. coli* inclusion bodies. Biochem. *34*, 5773-5794.

Cardin, A. D., Weintraub, H. J. R. (1989). Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis *9*, 21-31.

Carrell, R. W., Stein, P. E., Fermi, G., Wardell, M. R. (1994). biological implications of a 3 A^{o} structure of dimeric antithrombin. Structure *2*, 257-270.

Carrell, R. W. (1999). How serpins are shaping up. Science *285*, 1861.

Chain, D., Kreizman, T., Shapira, H., Shaltiel, S. (1991). Plasmin cleavage of

vitronectin: Identification of the site and consequent attenuation in binding plasminogen activator inhibitor-1. FEBS Lett. *285*, 251-256.

Chandra, T., Stackhouse, R., Kidd, V. J>, Woo, S. L. (1983). Isolation and sequence charactherization of a cDNA clone of human antithrombin III. Proc. Natl. Acad. Sci. USA *80*, 1845-1848.

Chang, T.-L., Bauer, R. S., Berliner, L. J. (1980). Refolding of a three (noncovalently linked)-domain enzyme. human γ-thrombin. J. Biol. Chem. *255*, 5904-5906.

Chang, J. Y. (1986). The structures and proteolytic specificities of autolysed human thrombin. Biochem. J. *240*, 797-802.

Cherny, R. C., Honan, M. A., Thiagarajan, P (1993). Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion. J. Biol. Chem. *268*, 9725-9729.

Church, F. C., PRatt, C. W., Noyes, C. M., Kalayanamit, T., Sherrill, G. B., Tobin, R. B., Meade, J. B. (1989). Structural and functional properties of human α-thrombin, phosphopyridoxylated α-thrombin, and γ_T-thrombin. J. Biol. Chem. *264*, 18419-18425.

Cowley, D. J., Mackin, R. B. (1997). Expression, purification and characterization of recombinant human proinsulin. FEBS Lett. *402*, 124-130.

Dabrowski, S., Kur, J. (1999). Cloning, overexpression, and purification of the recombinant his-tagged SSB protein of E. coli and use in polymerase chain reaction amplification. Prot. Exp. Purif. *16*, 96-102.

Dahlback, B., Podack, E. R., Muller-Eberhard, H. J. (1985). Characterization of human S protein, an inhibitor of the membrane attack complex of complement. Demonstration of a free reactive thiol group. Biochem. *24*, 2368-2374.

Davie, E. W., Fujikawa, K., Kisiel, W. (1991). The coagulation cascade: Initiation, maintanence, and regulation. Biochemistry *30*, 10363-10370.

de Boer, H. C., Preissner, K. T., Bouma, B. N., de Groot, P. G. (1992). Binding of vitronectin-thrombin-anithrombin III complex to human endothelial cells is mediated by the heparin binding site of vitronectin. J. Biol. Chem. *267*, 2264-2268.

de Boer, H. C., de Groot, P. G., Bouma, B. N., Preissner, K. T. (1993). Ternary vitronectin-thrombin-antithrombin III complexes in human plasma. Detection and mode of association. J. Biol. Chem. *268*, 1279-1283.

Degen, S. J., McGillivray, R. T. A., Daive, E. W. (1983). Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin. Biochemistry *22*, 2087-2097.

Deng, G., Royle, G., Wang, S., Crain, K., Loskutoff, D. J. (1996(a)). Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin. J. Biol. Chem. *271*, 12716-12723.

Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., Loskutoff, D. J. (1996(b)). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J. Cell. Biol. *134*.

Eitzman, D. T., Ginsburg, D. (1997). Of mice and men- The function of plasminogen activator inhibitors (PAI1s) *in vivo.* Adv. Exp. Medicine and Biol. *425*, 131-141.

Esmon, C. T., Esmon, N. L., Kurusowa, S., Johnson, A. E. (1986). Interaction of thrombin with thrombomodulin. Ann. N. Y. Acad. Sci. *485*, 215-220.

Esmon, C. T., Lollar, P. T. (1996). Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII. J. Biol. Chem. *271*, 13882-13887.

Esmon, C. (2000). The protein C pathway. Critical Care Medicine *28*, 44-48.

Essex, D. W., Miller, A., Swiatkowska, M., Feinman, R. D. (1999). Protein disulfide isomerase catalyzes the formation of disulfide-linked complexes of vitronectin with thrombin-antithrombin. Biochemistry *38*, 10398-10405.

Faber, H. R., Groom, C. R., Baker, H. M., Morgan, T. M., Smith, A., and Baker E. N. (1995). 1.8 A° crystal structure of the C-terminal domain of rabbit serum haemopexin. Structure *3*, 551-59.

Felding-Habermann, B., Cheresh, D. A. (1993). Vitronectin and its receptors. Curr. Opin. Cell. Biol. *5*, 864-868.

Fenton, J. W. I., Fasco, M. J., Stackrow, A. B. (1977). Human thrombins. Production, evaluation, and properties of alpha-thrombin. J Biol Chem. *252*, 3587-3598.

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

Fenton, J. W. n., Ofosu, F. A., Moon, D. G., Maraganore, J. M. (1991). Thrombin structure and function: why thrombin is the primary target for antithrombotics. Blood

Coagul Fibrinolysis *1*, 69-75.

Fenton, J. W., Ofosu, F. A., Brezniak, D. V., Hassouna, H. I. (1998). Thrombin and antithrombotics. Seminars in Thrombosis and Hemostasis *24*, 87-91.

Frey, P. A., Whitt, S. A., Tobin, J. B. (1994). A low-barrier hydrogen bond in the catalytic triad of serine proteases. Science *264*, 1927-1930.

Fryklund, L., Sievertsson, H. (1978). Primary structure of somatomedin B- A growth hormone-dependent serum factor with protease inhibiting activity. FEBS Lett. *87*, 55-70.

Fulop, V., Jones, D. T. (1999). Beta propellers: structural rigidity and functional diversity. Curr. Opin. Struct. Biol. *9*, 715-721.

Gechtman, Z., Belleli, A., Lechpammer, S., Shaltiel, S. (1997). The cluster of basic amino acids in vitronectin contributes to its binding of plasminogen activator inhibitor-1: evidence from thrombin, elastase and plasmin cleaved vitronectins and anti-peptide antibodies. Biochem. J. *325*, 339-349.

Gibson, A., Baburaj, K., Day, D.E., Verhamme, I., Shore, J. D., and Peterson, C. B. (1997). The use of fluorescent probes to characterize conformational changes in the

interaction between vitronectin and plasminogen activator inhibitor-1. J. Biol. Chem *272*, 5112-5121.

Gohlke, U., Gomis-Ruth, F. X., Crabbe, T., Murphy, G., Docherty, A. J. P., Bode, W. (1996). The C-terminal (haemopexin-like) domain structure of human gelatinase-A (MMP2): structural implications for its function. FEBS Letters *378*, 126-130.

Gomis-Ruth, F. X., Gohlke, M., Betz, M., Knauper, V., Murphy, G., Lopez-Otin, C., and Bode, W. (1996). The helping hand of collagenase-3 (MMP-13): 2.7A crystal structure of its C-terminal haemopexin-like domain. J. Mol. Biol. *264*, 556-66.

Griffith, M. J. (1982). Measurement of the heparin-enhanced antithrombin III/ thrombin reaction rate in the presence of synthetic substrate. Thromb. Res. *25*, 245-253.

Gustafson, M. E., Junger, K. D., Wun, T., Foy, B. A., Diaz-collier, J. A., Welsch, D. J., Obukowicz, M. G., Bishop, B. F., Bild, G. S., Leimgruber, R. M., Palmier, M. O., Matthews, B. K., Joy, W. D., Frazier, R. B., Galluppi, G. R., Grabner, R. W. (1994). Renaturation and purification of human tissue factor pathway inhibitor expressed in recombinant E. coli. Prot. Exp. Purif. *5*, 233-241.

Hayman, E. G., Pierschbacher, M. D., Suzuki, S., Ruoslahti, E. (1985). Vitronectin: a

major cell attachment-promoting protein in fetal bovine serum. Exp. Cell. Res. *160*, 245-258.

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

Hedstrom, L. S., L., Rutter, W. J. (1992). Converting trypsin to chymotrypsin: the role of surface loops. Science *255*, 1249-1253.

Hlavac, F., Rouer, E. (1997). Expression of the protein-tyrosine kinase p56lck by the pTRX vector yeilds a highly soluble protein recovered by mild sonication. Prot. Exp. Purif. *11*, 227-232.

Hofsteenge, J., Braun, P. J., Stone, S. R. (1988). Enzymatic properties of proteolytic derivatives of human alpha-thrombin. Biochemistry *27*, 2144-2151.

Hogasen, K., Mollnes, T. E., Brandtzaeg, P. (1994). Low levels of vitronectin and clustrin in acute meningococcal disease are closely associated with formation of the terminal complement complex and the vitronectin-thrombin-antithrombin cpomplex. Infection and Immunity *62*, 4874-4880.

Hogg, P. J., Jackson, C. M. (1990(a)). Heparin promotes the binding of thrombin to fibrin polymer: quantitative charactherization of a thrombin-fibrin polymer-heparin ternary complex. J. Biol. Chem. *265*, 241-247.

Holmes, R. (1967). Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells *in vitro*. J. Cell Biol. *32*, 297-308.

Hsieh, K.-h. (1997). Thrombin interaction with fibrin polymerization sites. Thrombosis Research *86*, 301-316.

Huber, R., Carrell, R. W. (1989). Implications of the three-dimensional structure of alpha 1-antityripsin for structure and function of serpins. Biochem. *28*, 8951-8966.

Hunt, L. T., Dayhoff, M. O. (1980). A suprising new protein superfamily containing ovalalbumin, anithrombin III, and alpha$_1$-proteinase inhibitor. Biochem. Biophys. Res. Commun. *95*, 864-871.

Hunt, L. T., Barker, W. C., Chen, H. R. (1987). A domain structure common to hemopexin, vitronectin, interstitial collagenase and a collagenase homolog. Protein Seq. Data Anal. *1*, 3841-59.

Ill, C. R., Rouslahti, E. (1985). Association of thrombin-anithrombin III complex with

vitronectin in serum. J. Biol. Chem. *260*, 15610-15615.

Ishikawa-Sakurai, M., Hayashi, M. (1993). Two collagen binding domains of vitronectin. Cell Struct. Funct. *18*, 253-259.

Jenne, D., Ferdinand, H., Bhakdi, S. (1985). Interaction of complement S-protein with thrombin-snithrombin complexes: A role for the S-protein in haemostasis. Thromb. Res. *38*, 401-407.

Jenne, D., Stanley, K. K. (1987). Nucleotide sequence and organization of the human S-protein gene: repeating peptide motifs in the 'pexin' family and a model for their evolution. Biochemistry *26*, 6735-42.

Jenne, D., Hille, A., Stanely, K. K., Huttner, W. B. (1989). Sulfation of two tyrosine residues in human complement S-protein (vitronectin). Eur. J. Biochem. *185*, 391-395.

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

Kaminski, M., McDonagh, J. (1983). Studies of the mechanism of thrombin

interaction with fibrin. Biochem. J. *242*, 10530-10535.

Kost, C., Stuber, W., Ehrich, H. J., Pannekoek, H., and Preissner, K. T. (1992). Mapping of binding sites for heparin, plasminogen activator inhibitor-1, and plasminogen to vitronectin's heparin-binding region reveals a novel vitronectin-dependent feedback mechanism for the control of plasmin formation. J. Biol. Chem *267*, 12098-105.

Kost, C., Benner, K., Stockmann, A., Linder, D., Preissner, K. T. (1996). Limited plasmin proteolysis of vitronectin. Characterization of the adhesion protein as morpho-regulatory and angiostatin binding factor. Eur. J. Biochem. *236*, 682-688.

Kurucz, I., Titus, J. A., Jost, C. A., Segal, D. M. (1995). Correct disulfide pairing and efficient refolding of detergent-solubilized single-chain Fv proteins from bacterial inclusion bodies. Mol. Immunol. *12*, 1443-1452.

Lane, D. A., Erdjument, H., Thompson, E., Panico, M., di Marzo, V., Morris, H. R>, Leone, G. de Stefano, V., Thrin, S. L. (1989). A novel amino acid substitution in the reactive site of a congenital variant antithrombin. Antithrombin pescara, Arg 393 to Pro, caused by a CGT to CCT mutation. J. Biol. Chem. *264*, 10200-10204.

Lewis, S. D., Lorand, L., Fenton, J. W. II, Shafer, J. A. (1987). Catalytic competence

of human α– and γ-thrombin in the activation of firinogen and factor XIII. Biochemistry *26*, 7597-7603.

Li, N., Lui, Y., Li, Q., Gu, H., Chen, Z. (1999). The refolding, purification, and activity analysis of a rice bowman-birk inhibitor expressed in E. coli. Prot. Exp. Purif. *15*, 99-104.

Liang, O. D., Rosenblatt, S., Chhatwal, G. S., Preissner, K. T. (1997). Identification of novel heparin-binding domains of vitronectin. FEBS Lett. *407*, 169-172.

Longas, M. O., Finlay, T. H. (1980). The covalent nature of the human antithrombin-thrombin bond. Biochem. J. *189*, 481-489.

Maeda, Y., Yamada, H., Ueda, T., Imoto, T. (1996). Effect of additives on the renaturation of reduced lysozyme in the presence of 4M urea. Protein Eng. *9*, 461-465.

Mast, A. E., Enghild, J. J., Pizzo, S. V., Salvasen, G. (1991). Analysis of the plasma elimination kinetics and conformational stabilities of native, proteinase-complexed, and reactive site cleaved serpins: comparison of alpha 1-proteinase, alpa 1-antichymotrypsin, antithrombin III, alpha 2-antiplasmin, angiotensionogen, and ovalbumin. biochemistry *30*, 1723-1730.

McGuire, E. A., Peacock, M. E., Inhorn, R. C., Siegel, N. R., Tollefsen, D., M. (1988). Phosphorylation of vitronectin by a protein kinase in human plasma. Identification of a unique phosphorylation site in the heparin-binding domain. J. Biol. Chem. *263*, 1942-1945.

Millis, L., Morris, C. A., Sheehan, M. C., Charlesworth, J. A., Pussell, B. A. (1993). Vitronectin-mediated inhibition of complement. Clin. Exp. Immunol. *92*, 114-119.

Mimuro, J., Loskutoff, D. J. (1989). Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. Evidence that the protein is vitronectin. J. Biol. Chem. *264*, 936-939.

Mimuro, J., Muramatsu, S., Kurano, Y., Uchida, Y., Ikadai, H., Watanabe, S., Sakata, Y. (1993). Identification of the plasminogen activator inhibitor-1 binding heptapeptide in vitronectin. Biochem. *32*, 2314-2320.

Minor, K. H., Peterson, C. B. (2001 (unpublished)). PAI-1 Promotes the Self-Association of Vitronectin into Complexes Exhibiting Altered Adhesive Properties. .

Moroder, L., Besse, D., Musiol, H. J., Rudolph-Bohner, S., Siedler, F. (1996).

Oxidative folding of cysteine-rich peptides vs regloselective cysteine pairing strategies. Biopolymers *40*, 207-234.

Morris, C. A., Underwood, P. A., Bean, P. A., Sheehan, M., Charlesworth, J. A. (1994). Relative topology of biologically active domains of human vitronectin. Evidence from monoclonal antibody epitope and denaturation studies. J. Biol. Chem. *269*, 23845-23852.

Mosesson, M. W. (1998). Fibrinogen structure and fibrin clot assembly. Semin Thromb Hemost. *24*, 169-174.

Olson, T. A., Sonder, S. A., Wilner, G. D., Fenton, J. W. (1986). Heparin binding in proximity to the catalytic site of human alpha-thrombin. Ann. N.Y. Acad. Sci. *485*, 96-103.

Olson, S. T., Bjork, I. (1991). Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects. J. Biol. Chem. *266*, 6353-6364.

Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., Choay, J. (1992). Role of the antithrombin binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the sntithrombin conformational change

contribution to heparin rate enhancement. J. Biol. Chem. *267*, 12528-12538.

Paduel, A., Cohen, N. C., Gertler, A., Elizur, A. (1999). Preparation and characterization of recombinant dolphin fish (*Coryphaena hippurus*) growth hormone. Prot. Exp. Purif. *16*, 417-423.

Peterson, C. B., Blackburn, M. N. (1987). Antithrombin conformation and the catalytic role of heparin. J. Biol. Chem. *262*, 7552-58.

Peterson, C. B. (1998). Binding sites on native and multimeric vitronectin exhibit similar affinity for heparin. The influence of self-association and multivalence on ligand binding. Trends in Cardiovascular Med. *8*, 124-131.

Preissner, K. T., Wassmuth, R., Muller-Berghaus, G. (1985(a)). Physicochemical characterization of human S-protein and its function in the blood coagulation system. Biochem. J. *231*, 349-355.

Preissner, K. T., Zwicker, L., Muller-Beghaus, G. (1985(b)). Formation, characterization and detection of a ternary complex between S-protein, thrombin and antithrombin III. Biochem. J. *243*, 349-351.

Preissner, K. T., Muller-Berghaus, G. (1986). S protein modulates the heparin-

catalyzed inhibition of thrombin by antithrombin III. Eur. J. Biochem. *156*, 645-650.

Preissner, K. T., Zwicker, L., Muller-Berghaus, G. (1987(a)). Formation, characterization and detection of a ternery complex between S protein, thrombin and antithrombin III in serum. Biochem. J. *243*, 105-111.

Preissner, K. T., Muller-Berghaus, G. (1987(b)). Neutralization and binding of heparin by S-protein/vitronectin in the inhibition of Factor Xa by antithrombin. Involvement of inducible heparin-binding domain of S-protein/vitronectin. J. Biol. Chem. *262*, 12247-12253.

Preissner, K. T., Anders, E., Grulich-Henn, J., and Muller-Berghaus, G. (1988). Attachment of cultured human endothelial cells is promoted by specific association with S protein (vitronectin) as well as with the ternary S protein-thrombin-antithrombin III complex. Blood *71*, 1581-1589.

Preissner, K. T., Grulich-Henn, J., Ehrlich, H. J., Declerck, P., Justus, C., Collen, D., Pannekoek, H., Muller-Berghaus, G. (1990(a)). Structural requirements for the extracellular interaction of plasminogen activator inhibitor-1 with endothelial cell matrix-associated vitronectin. J. Biol. Chem. *265*, 18490-18498.

Preissner, K. T. (1990(b)). Specific binding of plasminogen to vitronectin. Evidence

for a modulatory role of vitronectin on fibrin(ogen)-induced plasmin formation by tissue plasminogen activator. Biochem. Biophys. Res. Commun. *168*, 966-971.

Preissner, K. T. (1991(a)). Structure and biological role of vitronectin. Annu. Rev. Cell Biol. *7*, 275-310.

Preissner, K. T. (1991(b)). Structure of vitronectin and its biological role in haemostasis. Thrombosis and Haemostasis *66*, 123-132.

Rajan, S. S., Lackland, H., Stein, S., Denhardt, D. T> (1998). Presence of an N-terminal polyhistidine tag facilitates stable expression of an otherwise unstable N-terminal domain of mouse tissue inhibitor of metalloproteinase-1 in E. coli. Prot. Exp. Purif. *5*, 233-241.

Rosenberg, R. D., Damus, P. S. (1973). The purification and mechanism of action of human antithrombin-heparin cofactor. J. Biol. Chem. *248*, 6495-6505.

Rudolph, R., Lilie, H. (1996). *In vitro* folding of inclusion body proteins. FASEB J. *10*, 49-56.

Ruoslahti, E., and Pierschbacher, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238*, 491-97.

Rydel, T. J., Yin, M., Padmanabhan, K. P., Blankenship, D. T., Cardin, A. D., Correa, P. E., Fenton, J. W. II., Tulinsky, A. (1994). Crystallographic structure of human γ-thrombin. J. Biol. Chem. *269*, 22000-22006.

Sane, D. C., Moser, T. L., Greenberg, C. S. (1991). Limited proteolysis of vitronectin by plasmin destroys heparin. Thromb. Haemost. *66*, 310-314.

Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., Hol, W. G. J. (1994). The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. Nature Struc. Biol. *1*, 48-54.

Seddon, A. P., Hulmes, J. D., Decker, M. M., Kovesdi, I., Fairhurst, J. L., Backer, J., Vermazen, M. D., Bohlen, P. (1994). Refolding and characterization of human recombinant heparin-binding neurite-promoting factor. Prot. Exp. Purif. *5*, 14-21.

Seiffert, D., Keeton, M., Eguchi, Y., Saudey, M., Loskutoff, D. J. (1991(a)). Detection of vitronectin mRNA in tissues and cells of the mouses. Proc. Natl. Acad. Sci. *88*, 9402-9406.

Seiffert, D., Ciambrone, G., Wagner, N. V., Binder, B. R., Loskutoff, D. J. (19[illegible].

The somatomedin B domain of vitronectin. Structural requirements for the binding and stabilization of active type1 plasminogen activator inhibitor. J. Biol. Chem. *269*, 2659-2666.

Sheiffert, D., and Loskutoff, D. J. (1991(b)). Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. J. Biol. Chem. *266*, 2824-30.

Sheiffert, D., Loskutoff, D. J. (1991(c)). Kinetic analysis of the interaction between type 1 plasminogen activator inhibitor and vitronectin and evidence that the bovine inhibitor binds to a thrombin-derived amino-terminal fragment of bovine vitronectin. Biochim. Biophys. Acta *1078*, 23-30.

Shifman, M. A., Pizzo, S. V. (1982). The *in vivo* metabolism of antithrombin III complexes in blood. J. Biol. Chem. *257*, 3243-3248.

Sigurdardottir, O., Wiman, B. (1994). Identification of a PAI-1 bindnig site in vitronectin. Biochim. Biophys. Acta. *1208*, 104-110.

Simmons, T., Newhouse, Y. M., Arnold, K. S., Innerarity, T. L., Weisgraber, K. H. (1997). Human low density lipoprotein receptor fragment. Successful refolding of a functionally active ligand-binding domain produced in *E. coli*. J. Biol. Chem. *272*,

25531-25536.

Skorstengaard, K., Halkier, T., Hojrup, P., Mosher, D. (1990). Sequence location of a putative transglutaminase cross-linking site in human vitronectin. FEBS Lett. *262*, 269-274.

Stanley, K. K. (1986). Homology with hemopexin suggests a possible scavenging function for S-protein/vitronectin. FEBS Lett. *199*, 249-253.

Stefansson, S., Lawrence, D. A. (1996). The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. Nature *383*, 441-443.

Stephens, A. W., Siddiqui, A., Hirs, C. H. (1988). Site-directed mutagenesis of the reactive center (serine 394) of antithrombin III. J. Biol. Chem. *263*, 15849-15852.

Stubbs, M. T., Bode, W. (1993). A player of many parts: the spotlight falls on thrombin's structure. Thromb. Res. *69*, 1-58.

Stubbs, M. T., Bode, W. (1995). The clot thickens: clues provided by thrombin structure. Trends Biochem. Sci. *20*, 23-28.

Suzuki, S., Pierschbacher, M. D., Hayman, E. G., Nguyen, K., Ohgren, Y., and

Ruoslahti, E. (1984). Domain structure of vitronectin. Alignment of active sites. J. Biol. Chem ***259***, 15307-14.

Suzuki, S., Oldberg, A., Hayman, E. G., Pierschbacher, M. D., and Ruoslahti, E. (1985). Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. EMBO J. *4*, 2519-24.

Tomasini, B. R., Mosher, D. F. (1988). Conformational states of vitronectin: Preferential expression of an antigenic epitope when vitronectin is covalently and noncovalently complexed with thrombin-antithrombin III or treated with urea. Blood *72*, 903-912.

Tomasini, B. R., Owen, M. C., Fenton, J. W. II., Mosher, D. F. (1989). Conformational lability of vitronectin: induction of an antigenic change by α-thrombin-serpin complexes and by proteolytically modified thrombin. Biochemistry *28*, 7617-7623.

Travis, J., Salvesen, G. S. (1983). Human plasma proteinase inhibitors. Annu. Rev. Biochem. *52*, 655-709.

Vali, Z., Scheraga, H. A. (1988). Localization of the binding site on fibrin for secondary binding site of thrombin. Biochemistry *27*, 1956-1963.

van Meijer, M., Stoop, A., Smilde, A., Preissner, K. T., van Zonneveld, A.J., Pannekoek, H. (1997). The composition of complexes between plasminogen activator inhibitor 1, vitronectin and either thrombin or tissue-type plasminogen activator. Thrombosis and Haemostatis *77*, 516-521.

Waltz, D. A., Natkin, L. R., Fujita, R. M., Wei, Y., chapman, H. A. (1997). Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. J. Clin. Invest. *100*, 58-67.

Wei, A., Rubin, H., Cooperman, B. S., Christianson, D. W. (1994). Crystal structure of an uncleaved serpin reveals the conformation of an inhibitory reactive loop. Nature Struct. Biol. *1*, 251-258.

Wetlaufer, D. B., Branca, P. A., Chen, G. X. (1987). The oxidative folding of proteins by disulfide plus thiol does not correlate with redox potential. Protein Eng. *2*, 141-146.

Wetlaufer, D. B., Xie, Y. (1995). Control of aggregation in protein refolding: a variety of surfactants promote renaturation of carbonic anhydrase II. Protein Sci. *4*, 1535-1543.

Wun, T.-C., Palmier, M. O., Siegel, N. R., Smith, C. E. (1989). Affinity purification of active plasminogen activator inhibitor-1 (PAI-1) using immobilized anhydrourokinase. Demonstration of the binding, stabilization, and activation o PAI-1 by vitronectin. J. Biol. Chem. *264*, 7862-7868.

Xie, Y., Lashuel, H. A., Miroy, G. J., Dikler, S., Kelly, J. W. (1998). Recombinant human retinol-binding protein refolding, native disulfide formation, and chaacterization. Protein Exp. Purif. *14*, 31-37.

Xu, D., Baburaj, K., Peterson, C. B., Xu, Y. (2001). Model for the three-dimensional structure of vitronectin: predictions for the multi-domain protein from threading and docking. Proteins *44*, 312-320.

Yamaguchi, Y., Okabe, K., Liang, J., Ohshiro, H., Ishihara, K., Uchino, S., Zhang, J. L., Hidaka, H., Yamada, S., Ogawa, M. (2000). Thrombin and factor Xa enhance neutrophil chemoattractant production after ischemia/reperfusion in the rat liver. J. Surg. Res. *92*, 96-102.

Zabin, I. (1982). β-galactosidase and α-complementation: a model of protein-protein interaction. Mol. Cell. Biol. *49*, 87-96.

Zheng, X., Saunders, T. L., Camper, S. A., Samuelson, L. C., and Gingsburg, D.

(1995). Vitronectin is not essential for normal mammalian development and fertility. Proc. Acad. Sci. USA *92*, 12426-30.

Zhuang, P., Blackburn, M. N., and Peterson, C. B. (1996(a)). Characterization of the denaturation and renaturation of human plasma vitronectin. I. Biophysical characterization of protein unfolding and multimerization. J. Biol. Chem. *271*, 14323-332.

Zhuang, P., Li, H., Williams, J. G., Wagner, N. V., Seiffert, D., and Peterson, C. B. (1996(b)). Characterization of the denaturation and renaturation of human plasma vitronectin. II. Investigation into the mechanism of the formation of multimers. J. Biol. Chem. *271*, 14333-343.

Zhuang, P., Chen, A. I., and Peterson C. B. (1997). Native and multimeric vitronectin exhibit similar affinity for heparin. Differences in heparin binding properties induced upon denaturation are due to self-association into a multivalent form. J. Biol. Chem. *272*, 6858-6867.

Vita

Secil was born on March 11th 1975. She completed her education until the end of high school in her hometown, Konya, which is the city of whirling dervishes. She moved to Ankara, the capital city of Turkey, for her undergraduate education. She entered to the Biological Sciences Department of the Middle East Technical University, and graduated on summer of 1997 with specialties on Molecular Biology and Biotechnology.

On August 1997, she moved to Washington, DC. She worked as a research associate in the Pharmacology Department of the George Washington University for a year. Secil got an acceptance from the Biochemistry and Cellular and Molecular Biology department of the University of Tennessee, and started as a graduate student there on 1998-fall semester. She worked in Dr. Cynthia Peterson's laboratory and carried out research about a blood protein called vitronectin. She got her master's degree at the end of 2001-fall semester.

Right now, Secil is working in a biotechnology company in Seattle as a Research Associate in the cardiovascular biology department.