From the Department of Laboratory Medicine, Division of Clinical Chemistry and Blood Coagulation, Karolinska Hospital, Karolinska Institute S-171 76 Stockholm, Sweden

STRUCTURE - FUNCTION STUDIES OF PLASMINOGEN ACTIVATOR INHIBITOR-1

Guang-Chao Sui

Karolinska Institutet

Stockholm 1998

ABSTRACT

STRUCTURE-FUNCTION STUDIES OF PLASMINOGEN ACTIVATOR INHIBITOR-1

Thesis by Guang-Chao Sui, the Department of Laboratory Medicine, Division of Clinical Chemistry and Blood Coagulation, Karolinska Hospital, Karolinska Institute, S-171 76 Stockholm, Sweden

Plasminogen activator inhibitor-1 (PAI-1) is an important physiological inhibitor of tissue-type plasminogen activator (tPA) and urine-type plasminogen activator (uPA). High plasma levels of PAI-1 correlate to thrombotic disease, and low levels can be connected to a bleeding tendency. The native PAI-1 molecule is functionally active, but it spontaneously transforms into an inactive "latent" form with a half-life of about 2 h at physiological conditions. To investigate the relationship between structure and function of PAI-1, we used site-directed mutagenesis to produce more than forty PAI-1 mutants. Most of them carry a single amino acid change and are from three different regions in the PAI-1 molecule. These PAI-1 mutants, as well as wild type PAI-1 (wtPAI-1), have been expressed in an *E. coli* expression system and the mutant proteins were purified by heparin-Sepharose and anhydrotrypsin agarose chromatographies. Using this method, active PAI-1 proteins have been purified and subsequently characterized regarding PAI-1 inhibitory activity, interactions with tPA and vitronectin, and stability.

The transformation of active PAI-1 to its latent form is accompanied by the insertion of the reactive center loop (RCL) into the A β-sheet of the PAI-1 molecule. The study of the Phe^{113} - Asp^{138} stretch indicated that, even if this stretch is located at the opposite end of RCL on the PAI-1 molecule, it is still important for functional activity of PAI-1. This stretch seems to be involved in PAI-1 stability (Asp^{125}, Arg^{133}), substrate behavior when incubated with tPA (Asp^{125}, Phe^{126}, Arg^{133}) and heparin binding (Arg^{115}, Arg^{118}).

In the three-dimensional structure, the B β-sheet in PAI-1 is located beneath the A β-sheet, and the s2B and s3B strands are situated in the vicinity where RCL is believed to be inserted. Therefore, the role of the amino acids in the s2B and s3B strands for PAI-1 stability was extensively studied. Mutations of the residues in the central portions of both strands caused a significant decrease in stability with half-lives of about 10 ~ 25% as compared with that of wtPAI-1. However, mutations at both sides of the central portion in each of the two strands frequently resulted in an increased PAI-1 stability, by a factor up to 7-fold. This demonstrated that the residues on s2B and s3B are of major importance for PAI-1 stability, most likely by affecting the reactive center loop insertion rate into the A β-sheet of the molecule, either in a positive or a negative direction.

The increased stability of active PAI-1 at slightly acidic pH suggests that one or more histidine residue(s) may be involved in stabilizing the active form. Our study indicated that His^{229} indeed might be involved in this process, since substitutions of this residue significantly decreased the pH dependence of PAI-1 stability. Mutations at His^{143} gave results suggesting that this residue may also play a role regarding the pH dependence of PAI-1 stability. Our data also suggested that Tyr^{221} and Tyr^{228} may interact with the histidine(s) since the mutants of these two tyrosines were very stable at neutral pH but less stable at acidic pH.

Two patients with a bleeding tendency and low plasma PAI-1 concentrations were investigated for mutations in the PAI-1 structural gene. In both patients, a mutation in the PAI-1 gene (1334G → A) was observed. This mutation caused an exchange of the alanine residue at –9 of the PAI-1 propeptide to threonine. However, it was demonstrated subsequently that this constituted a common polymorphism, with an allele frequency of about 15%. Thus, it is not likely that the polymorphism is directly involved in the cause of the bleeding tendency.

Key words: PAI-1, site-directed mutagenesis, structure and function, stability.

ISBN 91-628-3255-7

Dedicated to my parents,

to my wife Meimei

and to my daughter Xinxin

TABLE OF CONTENTS

LIST OF PAPERS

I. Sui GC, Sun H, Zhang M and Hu MH. High level expression of recombinant plasminogen activator inhibitor-1 in *Escherichia coli* and generation of its mutants involving Asp^{125}, Glu^{128} and Glu^{130}. *Biochem.Mol.Biol.Int.* 1997; 42:621-629.

II. Sui GC and Wiman B. Functional effects of single amino acid substitutions in the region of Phe^{113} to Asp^{138} in the plasminogen activator inhibitor 1 molecule. *Biochem.J.* 1998; 331:409-415.

III. Sui GC and Wiman B. Stability of plasminogen activator inhibitor-1: role of $tyrosine^{221}$. *FEBS Lett.* 1998; 423:319-323.

IV. Sui GC and Wiman B. The B β–sheet in the PAI-1 molecule plays an important role for its stability. *Eur.J.Biochem.* (submitted).

V. Falk G, Sui GC, Schulman S and Wiman B. Detection of a new polymorphism in the propeptide region of the PAI-1 gene in two individuals with bleeding tendencies. *Fibrinolysis & Proteolysis* (submitted).

In this thesis, the above publications and manuscripts are referred to in the text by their roman numbers.

ABBREVIATIONS

PAI-1	plasminogen activator inhibitor-1
wtPAI-1	wild type PAI-1
tPA	tissue-type plasminogen activator
uPA	urine-type plasminogen activator
serpin	serine protease inhibitor
Mr.	relative molecular mass
E. coli	*Escherichia coli*
RCL	reactive center loop
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
bp	base pair
BSA	bovine serum albumin
$t_{½}$	half-life
PCR	polymerase chain reaction

INTRODUCTION

THE FIBRINOLYTIC SYSTEM

A schematic figure of the fibrinolytic system is shown in Fig. 1. In this system, plasminogen is activated by tissue-type plasminogen activator (tPA) or urine-type plasminogen activator (uPA) that cleave the peptide bond of plasminogen between Arg^{561}-Val^{562} (Wiman and Wallen 1975). The generated plasmin can degrade fibrin, e.g. in clots of the blood vessels, into soluble fibrin degradation products (FDP). The inhibitors for the fibrinolytic system are α_2-antiplasmin (Collen 1976, Moroi and Aoki 1976, Mullertz and Clemmensen 1976) and plasminogen activator inhibitor-1 (PAI-1) (Loskutoff *et al* 1983, Chmielewska *et al* 1983, Kruithof *et al* 1984).

Tissue-type plasminogen activator (tPA) and urine-type plasminogen activator (uPA)

tPA is synthesized in the endothelial cells of blood vessels. It is believed that tPA not only participates in the thrombolysis of human blood vessels, but also is involved in multiple physiological functions, such as tissue repair (Astrup 1968), macrophage function (Reich 1975), ovulation and embryo implantation (Strickland and Beers 1976). The tPA concentration in blood is about 70 pmol/L. As a serine protease, tPA is a glycoprotein and has a relative molecular mass (*Mr.*) of 68,000. The single-chain tPA (sctPA) can be converted to two-chain tPA (tctPA) by cleaving the peptide bond between Arg^{275}-Ile^{276}. The structure of tPA consists of a finger domain, an epidermal growth factor (EGF) domain, two kringle domains (K1, K2) and a catalytic domain. tPA can bind to the surface of fibrin via kringle 2 and this binding stimulates the activity of tPA (Rånby *et al* 1982, Kaneko *et al* 1992, Berg *et al* 1993).

uPA is also a glycoprotein with a *Mr.* of 54,000 and its concentration in plasma is about 150 pmol/L. The structure of uPA consists of a receptor binding region (named E domain), one kringle and a catalytic region. Unlike two-chain uPA, the single-chain uPA (scuPA, or prourokinase) has very low activity (Nielsen *et al* 1982, Wun *et al* 1982). However, it has been demonstrated that scuPA has higher specificity for blood clots, when compared with other plasminogen activators, and is resistant to the PAI-1 inhibition (Gurewich 1989). As a clinical thrombolytic agent, scuPA can be used together with tPA to get efficient thrombolysis. The cleavage of the peptide bond of Lys^{158}-Ile^{159} leads to the formation of a low *Mr.* form (33,000) of uPA, which remains active.

Plasminogen and plasmin

As the inactive precursor of plasmin, plasminogen is a glycoprotein with a *Mr.* of 92,000 and has a concentration of 1 ~ 2

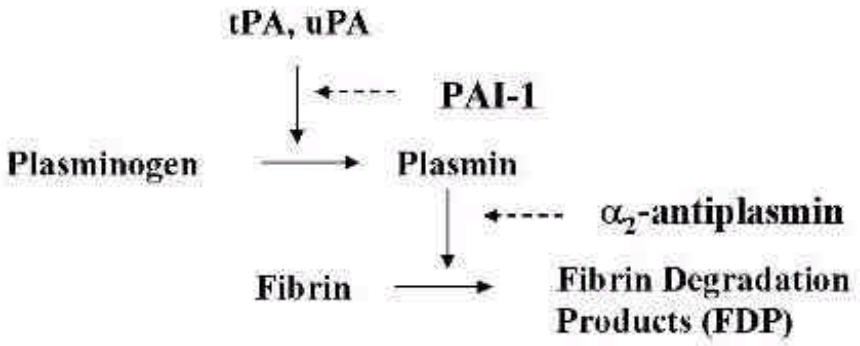


Fig. 1. The fibrinolytic system

μmol/L in plasma. Plasminogen contains five kringles and the serine protease catalytic triad (Ser^{741}, His^{603} and Asp^{646}) is located in the C-terminal domain. Plasminogen can exist in two forms: glu-plasminogen and lys-plasminogen. The glu-plasminogen is the natural form, which can be cleaved by plasmin at the Arg^{68}-Met^{69}, Lys^{77}-Lys^{78}, or Lys^{78}-Val^{79} to form lys-plasminogen (Wallen and Wiman 1972). It has been indicated that lys-plasminogen is more readily convertible to plasmin than glu-plasminogen. When compared with lys-plasminogen, glu-plasminogen has less affinity to the intact fibrin but it has higher affinity to the plasmin-digested fibrin.

The conversion from plasminogen to plasmin is conducted by tPA and uPA. The two plasminogen activators can cleave the peptide bond Arg^{561}-Val^{562} in plasminogen (Summaria *et al* 1967, Wiman and Wallen 1975) and subsequently generate active plasmin consisting of two polypeptides connected by a disulfide bridge. α_2-Antiplasmin is the physiological inhibitor of plasmin.

α_2-Antiplasmin

α_2-Antiplasmin is a glycoprotein with a single polypeptide of *Mr.* 65,000 (Collen 1976) and its concentration in plasma is about 1 μmol/L. α_2-Antiplasmin can form an irreversible complex with plasmin and therefore acts as a regulator in the fibrinolytic system. It belongs to the serpin family and the reactive center, P1-P1', consists of Arg^{364}-Met^{365} (Wiman and Collen 1979).

Plasminogen activator inhibitors

There are several kinds of plasminogen activator inhibitors in plasma, including PAI-1, PAI-2 (placental plasminogen activator inhibitor), PAI-3 (protein C inhibitor) and protease nexin (Kruithof 1988). Among them, PAI-1 is the most important inhibitor of the plasminogen activators. A separated section for the review of PAI-1 is given below.

SERPINS: STRUCTURE AND FUNCTION

Serpins

The family of **ser**ine **p**rotease **in**hibitors (**serpin**s) consists of more than 40 members that have about 35% homology regarding their primary structure. Even though these inhibitors were identified from different organisms: viruses, insects, plants and animals, most of the members in the family contain about 400 residues (with *Mr.* around 50,000) and they are believed to have evolved from a common ancestral protein. Not all of the members in the serpin family are functional protease inhibitors. Therefore, the proteins in this family can be divided into two groups (Table 1): inhibitory members (e.g. PAI-1, α_1-antitrypsin, antithrombin III, etc.) and non-inhibitory members (e.g. ovalbumin, angiotensinogen, maspin, etc.).

Each inhibitory serpin has its own specific target protease, even if they always to some extent exhibit inhibitory activity to some other serine proteases. Most of the serpins play important roles in the regulation of the proteolysis in many physiological processes, such as coagulation (e.g. antithrombin III) (Patston *et al* 1994a), fibrinolysis (e.g. PAI-1) and inflammation (e.g. α_1-antichymotrypsin) (Potempa *et al* 1994). Therefore, they maintain the hemostatic balance between coagulation and fibrinolysis in blood. The deficiencies of some serpins could cause clinical problems (for a review, see Gettins *et al* 1992).

Some serpins, such as PAI-1 (Sigurdardottir and Wiman 1990), α_2-antiplasmin, antithrombin III and C1-inhibitor (Stief *et al* 1988), are susceptible to oxidants, such as chloramine T. This is due to the oxidation of certain methionines in the serpin molecules.

The structural properties of serpins

The serpin family is one of the well-characterized protein families. Even though each protein in this family has its own

Table 1. The inhibitory and non-inhibitory members of the serpin family

Serpins	Target proteases	Function	References
Inhibitory members			
α_1-Antitrypsin	Neutrophil elastase	Proteolysis protection	Carrell and Owen 1985
Antithrombin III	Thrombin, factor Xa	Inhibition of coagulation	Rosenberg and Rosenberg 1984
PAI-1	tPA, uPA	Inhibition of fibrinolysis	Loskutoff *et al* 1983, Chmielewska *et al* 1983
α_2-Antiplasmin	Plasmin	Inhibition of fibrinolysis	Wiman and Collen 1977, Iwamoto and Abiko 1970
C1-inhibitor	C1-esterase	Control of complement cascade	Johnson *et al* 1971
α_1-Antichymotrypsin	Cathepsin G	Inflammation	Travis *et al* 1978
CrmA (cowpox)	Interleukin converting enzyme	Virulence factor	Nava *et al* 1998
SERP1(myxoma virus)	tPA, uPA, plasmin, thrombin, factor Xa	Anti-inflammation	Macen *et al* 1993
PNiXa (Xenopus oocytes and Embryos)	(Bovine) chemotrypsin	?	Kotyza *et al* 1998
Non-inhibitory members			
Ovalbumin	None	Storage protein	Stein *et al* 1990
Maspin	None	Prevention of tumorigenesis	Sager *et al* 1996
Caspin	Bind to type I collagen	Formation of bone, teeth and cartilage	Kozaki *et al* 1998
Angiotensinogen	None	Precursor of angiotensin II	Cohen *et al* 1996
Cortisol binding globulin	None	Carrier of cortisol	Pemberton *et al* 1988
Thyroxin-binding globulin	None	Carrier of thyroxin	Janssen *et al* 1995
HSP47	None	Chaperone in protein folding	Hosokawa *et al* 1993
Protein Z (wheat grain)	None	?	Rosenkrands *et al* 1994

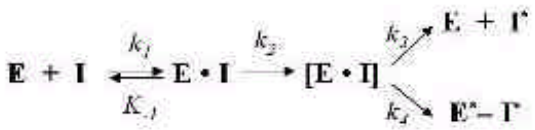
distinct properties and may not always be used as an accurate model for the serpin family, the individual studies still largely contribute to the increasing knowledge of serpin structure and function.

The tertiary structure model was generated from the crystallographic studies of some proteins in serpin family, such as α_1-antitrypsin (i.e. α_1-proteinase inhibitor) (Loebermann *et al* 1984), ovalbumin (Stein *et al* 1990, Stein *et al* 1991), latent form PAI-1 (Mottonen *et al* 1992), and antithrombin III (Mourey *et al* 1993). Besides the homologous primary sequences of serpins, the information obtained from X-ray crystal structure indicated that they also share similarity in three-dimensional structure: three dominant β-sheets (A, B, C), nine α-helixes (A to I) and one reactive center loop (RCL) (Huber and Carrell 1989, Baumann *et al* 1991). A stretch of about 27 residues (P16 to P10'), located 30~40 amino acids from the C-terminal, defines a RCL, a strained loop that is exposed outside the molecule and can mimic a substrate to bind to a cognate protease. This is the typical conformation for a native or active serpin and it is known as the S state (stressed state). The putative active center (P1-P1') is expected to exist on the RCL and is denoted as "bait" peptide bond. The RCL, which is believed to form an isolated helix (Stein *et al* 1990), has a tendency to be inserted into serpin molecule and form a new strand (s4A) in the A β-sheet. This hypothesis has been confirmed by the structural studies of ovalbumin (Stein *et al* 1990) and latent form PAI-1 (Mottonen *et al* 1992). The RCL insertion is also accompanied by other conformation change, even not as obvious as this insertion movement. It subsequently leads to the transition from the S state to the R state (relaxed state), which is a relatively thermal-stable conformation. Up to date, PAI-1 is the only native serpin observed to spontaneously transform from the active PAI-1 to its latent form. Some other serpins, such as antithrombin and α_1-antitrypsin, could be converted to a latent form with heating treatment under special conditions (Wardell *et al* 1993, Lomas *et al* 1995, Wardell *et al* 1997). Also, a natural mutant of antithrombin, Asn187Asp, was indicated to undergo very slow latency decay (Bruce *et al* 1994). The intermolecular RCL insertion has also been observed (Chang *et al* 1997) and the polymerizations of some serpins correlated with some diseases (Lomas *et al* 1992, Lomas *et al* 1993, Faber *et al* 1993, Aulak *et al* 1993, Bruce *et al* 1994, Eldering *et al* 1995).

The interaction of serpins and proteases

The mechanism of interaction between serpins and proteases has been extensively studied in the last few years. A large number of recombinant wild type and mutated serpins have been generated in the last decade (for a review, see Storck *et al* 1996). With the employment of various technologies, such as fluorescence resonance energy transfer measurement and NMR experiment, the mechanism of this interaction is gradually revealed. However, the crystal structure of a serpin/protease complex is not yet available.

The reaction of a protease and a serpin can be represented as in scheme 1. The first stage of the interaction between the protease (E) and the serpin (I) is an initial binding step that is normally fast and reversible. This leads to the formation of a non-covalent complex (E•I) and the serpin molecule is still intact at this stage. Evidence was provided by a thrombin mutant showing that the serine in the active



Scheme 1

site was not involved in the initial binding of thrombin and its inhibitors (Stone and Le Bonniec 1997). The second step consists of the further interaction between the two molecules. As a result, an acyl intermediate [E•I] between the non-covalent complex E•I and the final covalent complex, $E^*–I^*$, is formed. This was proven by clear kinetic evidence (Wiman and Collen 1978, O'Malley *et al* 1997) and also by the analysis of proteolytic digestion (Egelund *et al* 1998). This step is believed to be rate-limiting in the whole procedure (i.e. $k_2 << k_1$).

After the formation of [E•I], there are two pathways for the intermediate to go: (1) the inhibitor pathway, which leads to the cleavage of P1-P1' peptide bond of the serpin followed by the formation of a covalent complex, $E^*–I^*$; (2) the substrate pathway, which forms a P1-P1' cleaved inhibitor (I^*) and also releases the free enzyme (E).

Unlike other families of protease inhibitors, serpins undergo a major conformational change upon the binding of proteases (Gettins *et al* 1993). This conformational change also happens to the proteases in the inhibitor pathway. When the two proteins bind to each other, the covalent interaction involving the protease's catalytic serine and the serpin's bait peptide bond is essential for the serpin conformational change to bind to the enzyme tightly. This was proven by the phenomenon that anhydrotrypsin could not compete with trypsin for binding to some serpins (Olson *et al* 1995).

Studies of some protease/serpin complexes (Nilsson and Wiman 1982, Wilczynska *et al* 1995, Lawrence *et al* 1995) provided strong evidence that an acyl-intermediate was formed after the protease cleaved the P1-P1' peptide bond, although some researchers have speculated that a tetrahedral intermediate existed (Matheson *et al* 1991). Meanwhile, a rapid insertion of the RCL into the A β-sheet is conducted and consequently the covalently attached protease is dragged from the initial docking position on the serpin to the opposite side. The translocation of the protease and the insertion of the RCL into the A β-sheet have been proven by NMR experiments (Plotnick *et al* 1996) and the fluorescence resonance energy transfer measurements (Stratikos and Gettins 1997, Stratikos and Gettins 1998). Due to the attachment of the protease, the insertion of the RCL is perhaps not complete and results in about 60 Å separation of the P1 and P1' (Wilczynska *et al* 1997), in comparison with the 70 Å in the free cleaved serpins (Wilczynska *et al* 1997, Aertgeerts *et al* 1997). It is suggested that the reactive center of the protease can be distorted while dragged by the inserted RCL. As a result, the acyl-intermediate is prevented from deacylation and consequently the stable complex is generated.

The procession for the inhibitor pathway depends very much on the rate of the cleaved RCL insertion into the A β-sheet. For the non-inhibitory member of the serpin family, ovalbumin, both in the native form or the P1-P1' cleaved form, its RCL cannot be inserted (Wright *et al* 1990, Huntington *et al* 1997), or even just partially (P14-P10) inserted (for the native form) (Huntington *et al* 1995), into the A β-sheet, and consequently it does not have inhibitory activity to any protease.

The hypothesis for the substrate pathway is that, when the RCL insertion into the serpin molecule is delayed, or the loop insertion is slower than the deacylation (i.e. $k_4 < k_3$), the deacylation can occur before the RCL reaches its inserted position (Lawrence 1997). Blocking the insertion of RCL by the presence of an analogy peptide of P14-P7 residues could lead to the substrate behavior of PAI-1 in the reaction with tPA (Kvassman *et al* 1995a). In this case, it generates a cleaved inhibitor and releases the enzyme. As a result, the serpin molecule acts as a substrate of the protease. The X-ray crystal structure of the complex between PAI-1

and RCL-derived peptide was studied recently (Xue *et al* 1998). In the cleaved serpins, the RCL is fully inserted. With the regard for the PAI-1 molecule, this conformational change is larger than the active to latent transition (Aleshkov *et al* 1996) and the distance between P1 and P1' is the maximal (70 Å) (Wilczynska *et al* 1997, Aertgeerts *et al* 1997). It was also observed that the cleaved form PAI-1 was completely resistant to the digestion of non-target proteases, such as trypsin, proteinase K (Egelund *et al* 1997).

Due to the structural homology of the serpins, a serpin always has inhibitory activity to several proteases although its cognate protease is the most optimal for the inhibition. The P2 residue on RCL of serpins was proposed to be involved in deciding the specificity of serpins to the proteases (Rezaie 1996, Rezaie 1997). However, the specificity of a serpin was also proposed to be determined by the sequence and length of RCL, the "secondary binding site" for proteases and the intra molecular interactions (Djie *et al* 1997).

The reaction between the serpins and the proteases has also been extensively studied by protein mutagenesis, mainly focused on the RCL region. So far, all of the observed *inhibitory* members in serpin family have a threonine residue at P14 position. The substitution of the P14 threonine by an arginine led to the substrate behavior of PAI-1 (Lawrence *et al* 1994) or α_1-antitrypsin (Hood *et al* 1994) in the reaction with their cognate proteases. Ovalbumin is not an inhibitor since the RCL cannot be properly inserted into the molecule. It was proposed that the inability of the loop insertion in ovalbumin is due to the presence of an arginine residue at P14 position that cannot get into the A β-sheet (Wright *et al* 1990). Recent studies (Huntington *et al* 1997) demonstrated that the substitution of the P14 arginine in ovalbumin by a serine residue could only cause a partial insertion of the loop for the cleaved ovalbumin but it could not create any inhibitory ovalbumin variant. The explanation for this phenomenon is that the insertion of the cleaved RCL was not rapid enough to avoid deacylation. It was also reported that when the P14 (Arg), P12 (Val) and P11 (Val) of ovalbumin were substituted by Thr, Ala and Ala, respectively, the generated mutants did not gain any inhibitory activity (McCarthy and Worrall 1997).

The extensive insertion of the RCL is also believed to be of importance for the pathway of the inhibitor. The complete or partial substitutions of the P12-P2 part of α_1-antitrypsin by the corresponding region of ovalbumin indicated that the P6-P2 region was critical for the rapid inhibition to form stable serpin-protease complex (Chaillan-Huntington *et al* 1997). A recent report from the same group (Chaillan-Huntington and Patston 1998) indicated that the P5 residue is important in maintaining the RCL in an optimal conformation for attacking proteases and also for a rapid RCL insertion.

It was reported that the flexibility of RCL is believed to decided the activity (Carrell *et al* 1991) and specificity (Ehrlich *et al* 1990) of serpins, and it required at least five residues on each side of the P1-P1' bond (Hubbard *et al* 1994). However, the introduction of a disulfide bridge involving the P3' position to lock the RCL, generated an α_1-antitryspin variant with normal inhibitory activity (Hopkins *et al* 1997). This indicated that the initial binding of the inhibitor and the enzyme does not need the mobility of RCL, at least at the P1' side. It also proved that the RCL insertion happens after the cleavage of P1-P1' peptide, as previously suggested (Wilczynska *et al* 1995, Lawrence *et al* 1995).

PLASMINOGEN ACTIVATOR INHIBITOR-1

Biological functions of PAI-1

An inhibitor of plasminogen activators, named plasminogen activator inhibitor-1 (PAI-1), was firstly discovered in human

or bovine endothelial cells (Loskutoff *et al* 1983, Philips *et al* 1984), in human plasma (Chmielewska *et al* 1983, Kruithof *et al* 1984) and also indicated in rat hepatoma cells (Gelehrter *et al* 1983). PAI-1 is synthesized by several kinds of cells, such as the vascular endothelial cells and the liver cells. The synthesis and secretion of PAI-1 are stimulated by several agents. These agents include dexamethasone, endotoxin, lipopoly-saccharide, growth factor, thrombin, interleukin-1, tumor necrosis factor, insulin, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and lipoprotein (a) (Colucci *et al* 1985, Morrison and Ulevitch 1978, Hamsten and Wiman 1987b). The concentration of PAI-1 in normal human plasma is about 10~20 ng/mL (Chmielewska *et al* 1983) and PAI-1 exists in a non-covalent complex with vitronectin (Declerck *et al* 1988, Wiman *et al* 1988).

PAI-1 is the main physiological inhibitor of tPA and uPA. Increased plasma levels of PAI-1 are correlated with the development of thrombotic diseases, both myocardial infarction (Hamsten *et al* 1987a, Held *et al* 1997, Cortellaro *et al* 1993) and deep vein thrombosis (Paramo *et al* 1985, Eriksson *et al* 1989, Schulman and Wiman 1996). Decreased levels of PAI-1 are connected to the bleeding tendency (Stankiewicz *et al* 1991). PAI-1 is not only a regulator of the fibrinolytic system, it is also correlated to inflammation (Eitzman *et al* 1996), tumor invasion (Pappot *et al* 1995) and obesity (Vague *et al* 1986).

PAI-1 can bind to vitronectin and heparin (Ehrlich *et al* 1991). Heparin stimulates the inhibition of uPA by PAI-1 (Urano *et al* 1994). Several groups (Ehrlich *et al* 1991, Keijer *et al* 1991, Ehrlich *et al* 1992, Patston and Schapira 1994b, van Meijer *et al* 1997a) have demonstrated that heparin could enhance the inhibition of thrombin by PAI-1 by nearly 2 orders of magnitude, perhaps by acting as a template to assemble PAI-1 and thrombin, and consequently causing an acceleration of their interaction (Gebbink *et al* 1993). However, it was not applicable to the PAI-1 inhibition to some other proteases, such as Factor Xa or Factor XIIa (Keijer *et al* 1991). Also, similar effects were observed in the presence of heparan sulfate and dermatan sulfate (Gebbink *et al* 1993). PAI-1 can also bind to fibrin and, in this respect, active PAI-1 has a better affinity than the latent form (Wagner *et al* 1989, Braaten *et al* 1993). It was reported that PAI-1 also has affinity to low density lipoprotein receptor-related protein (Stefansson *et al* 1998).

Properties and purification of PAI-1

The synthesized PAI-1 protein in the endothelial cells and human hepatocytes has a signal peptide of 23 amino acids and the mature PAI-1 is a protein of 379 amino acids. There is no cystein in the PAI-1 molecule. PAI-1 is a glycoprotein and has an apparent *Mr.* of 50,000 when it is produced in plasma or endothelial cells. Recombinant PAI-1 expressed in *E. coli* has a *Mr.* of about 43,000 due to the lack of glycosylation in the bacteria. The peptide bond Arg^{346}-Met^{347} constitutes the "bait" region (Lindahl *et al* 1990). The reaction between PAI-1 and plasminogen activators is very fast, with a second-order rate constant of about 2×10^7 mol^{-1} s^{-1} L (Chmielewska *et al* 1988).

When PAI-1 is purified from human plasma, it co-purifies with its binding protein, vitronectin (Sigurdardottir and Wiman 1990). Since PAI-1 has high affinity to heparin, chromatography on heparin-Sepharose was used to purify PAI-1 (Lindahl and Wiman 1989, Ehrlich *et al* 1991), but a mixture of active, latent and substrate forms of PAI-1 was obtained. Several methods have been reported for the separation of the active PAI-1 from its latent form, including immobilized anhydrourokinase (Wun *et al* 1989), anhydrotrypsin (Fa *et al* 1995) and hydrophobic interaction (Kvassman and Shore 1995b) chromatographies. In our study, the anhydrotrypsin agarose chromatography was used and it was

observed that both active and substrate form PAI-1 could bind to the anhydrotrypsin column. This indicated that these two forms have a similar conformation in the binding region that interact with the proteases (Paper II).

Structure and function of PAI-1

1. The three forms of PAI-1

PAI-1 has some unique aspects regarding its conformational properties. It has been observed that PAI-1 has three inter-convertible conformations: active, latent and substrate forms (Declerck *et al* 1992, Urano *et al* 1992). When PAI-1 is synthesized in endothelial cells and released into blood, it is in a functionally active form (Lindahl and Wiman 1989), which is the native conformation and has the inhibitory activity towards its target proteases. In the *in vitro* experiments at physiological conditions, active PAI-1 spontaneously undergoes a conformational change and decay to a latent form with a half-life of about 2 h (Levin and Santell 1987, Lindahl *et al* 1989). Normally, in plasma, PAI-1 is completely bound to another protein, vitronectin (Wiman *et al* 1988, Declerck *et al* 1988) that stabilizes the active form of PAI-1 and prolongs the half-life to 4 h. An increase in stability of PAI-1 can also be achieved by lowering the environmental pH (Lindahl *et al* 1989).

An X-ray crystallographic study of PAI-1 indicated that it has the conformation which is typically observed for the serpin family (Mottonen *et al* 1992). The active conformation of PAI-1 is shown in Fig. 2. As can be seen, the RCL of active PAI-1 is exposed on the surface of the molecule. The conversion from the active form to latent form PAI-1 is accompanied by insertion of RCL (P16-P4) into the molecule forming a new strand in the A β-sheet (Aertgeerts *et al* 1994). The detailed mechanism for this transition is summarized in the “Serpins” section. This conformational change from active PAI-1 to the latent form results in a decreased content of α-helix from 26% to 22% and an increase in β-sheet from 23% to 34% (Sancho *et al* 1995).

When exposed to the high concentration of certain denaturants, such as guanidinium chloride, urea, followed by refolding in a slightly acidic dialysis buffer, latent form PAI-1 can be partially reactivated to the active form (Hekman and Loskutoff 1985, Lindahl *et al* 1989, Vaughan *et al* 1992). Vitronectin was also observed to partially restore the activity of latent PAI-1 by incubating the two proteins for days either at 25 °C, pH 5.5 (Wiman *et al* 1990) or at 37 °C, pH 7.5 (Reilly *et al* 1992). It was also reported that phospholipid might convert latent PAI-1 to the active form (Lambers *et al* 1987) and PAI-1 synthesized in bovine endothelial cells could be reactivated by heating it at 100 °C (Katagiri *et al* 1988).

Substrate form of PAI-1 was previously identified by three groups (Declerck *et al* 1992, Urano *et al* 1992, Munch *et al* 1993). Since then, some believe that the substrate form of PAI-1 is a unique conformation that is generated due to the *in vitro* treatment. A panel of monoclonal antibodies (Debrock and Declerck 1997a), or even its single-chain variable fragment (Debrock *et al* 1997b), exhibited an interesting property to switch active PAI-1 to its substrate form. Very recently, it was reported that the P13 residue of the RCL in PAI-1 also significantly contributed to the active to the substrate form transition (Gils *et al* 1998).

With the advances in the studies of the interaction between serpins and proteases, the presence of an invalid substrate form has become controversial. With the evidence for the presence of an acyl-intermediate and the insertion of cleaved RCL into the serpin molecule, researchers from other groups have suggested that the formation of the cleaved serpins is due to the delay or the blocking of RCL insertion (Lawrence 1997). Substitution of Thr^{333} at

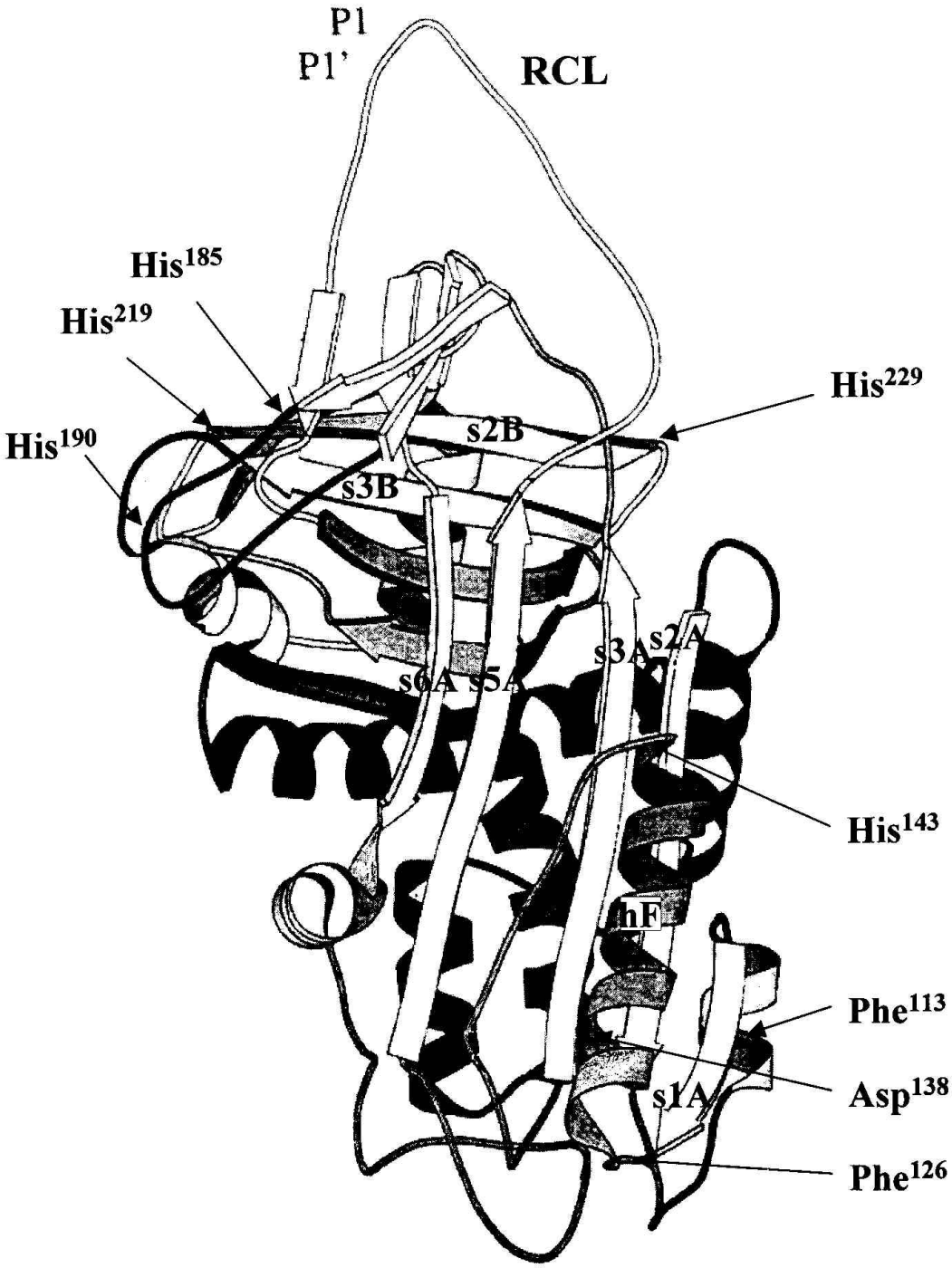


Fig. 2. Ribbon diagram of the model of the active form PAI-1
(adapted from Aertgeerts *et al* 1994, *J.Struct.Biol.,* 113:239-245.)

P14 in PAI-1 by a charged residue decreases the efficiency of RCL insertion and consequently leads to the cleavage of PAI-1 (Lawrence *et al* 1994). With the binding of a synthetic peptide (mimicking P14-P7 of RCL), active PAI-1 displayed substrate properties in the reaction with tPA (Kvassman *et al* 1995a). However, a PAI-1 variant with absolute substrate behavior could really have its cleaved RCL be inserted into PAI-1 molecule, shown by its X-ray structure (Aertgeerts *et al* 1995). This made the theories for the substrate form PAI-1 more ambiguous.

In our study, we found that the PAI-1 mutant Arg133Asp could exist both in an active form, but also in the substrate form. This supports the theory of the presence of the substrate form of PAI-1. Certainly, it does not exclude the possibility that the cleaved form PAI-1 can also be generated by the hindrance of RCL insertion.

2. PAI-1 stability

Both vitronectin and arginine can effectively bind to active PAI-1 and consequently stabilize its conformation. This provides an explanation for that high concentrations of arginine could extract PAI-1 from subendothelial matrix (Mimuro *et al* 1987) and also from anhydrotrypsin agarose column (Fa *et al* 1995).

PAI-1 also has a significantly increased stability at slightly acidic pH (Lindahl *et al* 1989). Its half-life at pH 5.5 is about 17 h at 37 °C (Lindahl *et al* 1989, Paper III). Since a decrease in pH is accompanied by protonation of side-chain groups of histidine residues in a protein, it is possible that histidine is involved in stabilizing the conformation of active PAI-1. It was reported (Kvassman *et al* 1995a) that the acidic stabilization was contributed by the protonation of a single group (histidine) with a p*K*a 7.6. This histidine was tentatively speculated to be His^{143} in helix F that is situated in the vicinity of the β-sheet A in the three-dimensional structure of PAI-1. In a recent study, we found that substitutions of His^{143} by Asp or Phe, but not by Lys or Thr, decreased the pH dependence of PAI-1 stability. Some positively charged amino acids located on the turn connecting strands s4C and s3C were analyzed by generating double-site changed PAI-1 mutants (Gils *et al* 1997). These PAI-1 variants, concerning Arg^{186}, Arg^{187}, His^{190} and Lys^{191}, exhibited much shorter half-lives. In our studies, we also found that substitutions of His^{185} and His^{190} decreased the PAI-1 stability while mutations at His^{229} diminished pH dependence of PAI-1 stability (Paper IV). Furthermore, the replacement of Tyr^{221} or Tyr^{228} by hydrophilic residues even reverted the pH dependence (Paper III, Paper IV).

To search for the residues in PAI-1 that are responsible for stabilizing the active conformation of PAI-1, Berkenpas *et al* (1995) randomly mutated PAI-1 cDNA and screened from the generated library for PAI-1 variants with prolonged half-lives. It was observed that a PAI-1 mutant with four residues mutated exhibited a half-life of 145 h. They concluded that the combination of several residues all over the PAI-1 molecule contributed to the stability. Very recently, an interesting phenomenon was observed by Declerck's group (Gils and Declerck 1998). They found that the presence of a non-ionic detergent, Triton X-100, had a dramatic effect in decreasing PAI-1 stability. The kinetic evaluation indicated that the transition caused by Triton X-100 consisted of two consecutive steps: active → substrate → latent, and only the first step was strongly dependent on the concentration of Triton X-100.

3. The interaction of PAI-1 with plasminogen activators

The RCL of active form PAI-1 is solvent exposed and thereby accessible to the catalytic groove of tPA and uPA. However, the mobility of the RCL is only required for the inhibitory function of PAI-1, but not for its initial recognition of the plasminogen activators (Lawrence *et al*

1994). The evidence of a "second-site" for the interaction between PAI-1 and tPA was very early observed (Chmielewska *et al* 1988).

Regarding tPA, it was reported that Arg^{298}, Arg^{299} and Arg^{304} of tPA are involved in the interaction with PAI-1 since substitutions of these three residues to negatively charged amino acids generated tPA mutants that were resistant to the inhibition of PAI-1 (Madison *et al* 1989, Madison *et al* 1990a). Subsequently a PAI-1 mutant, Glu350Arg, was observed to rapidly inhibit the tPA mutant Arg304Glu (Madison *et al* 1990b), indicating that Arg^{304} in tPA is forming a salt bridge with Glu^{350} in PAI-1 when the two proteins bind to each other. Therefore, based on these findings, a tetra-site changed tPA mutant was generated and its inhibition by PAI-1 was about 120,000 times slower than that of wild type tPA (Tachias and Madison 1997).

Most of the studies concerning the interaction between PAI-1 and tPA or uPA were focused on the reactive region of PAI-1. The requirement for the P1-P1' residues to PAI-1 inhibitory activity have been extensively studied. The presence of a basic amino acid at P1 position is essential for the PAI-1 inhibitory activity (Shubeita *et al* 1990, York *et al* 1991, Sherman *et al* 1992) and the double substitutions of P1, P1' residues generated PAI-1 variants with different specificity to either tPA or uPA (Sherman *et al* 1992). It was observed that the substitutions of the residues at P3 (Ser), P6 (Val), P10 (Ser) and P18 (Asn) also altered the specificity of PAI-1 to the two plasminogen activators (York *et al* 1991, Gils and Declerck 1997). It is clear that the initial attachment of tPA to PAI-1 involves the insertion of PAI-1's RCL into the catalytic groove of the target enzyme. However, an anthranilic acid derivative, AR-H029953XX, acts as an inhibitor for the tPA/PAI-1 complex formation even if this inhibitor was indicated to bind the opposite side of RCL on PAI-1 (Björquist *et al* 1998).

4. Interaction of PAI-1 with vitronectin and heparin

Human vitronectin is a glycoprotein with 459 amino acid residues and a *Mr.* 75,000 (Wiman *et al* 1988, Mimuro and Loskutoff 1989). PAI-1 and vitronectin are believed to be co-localized in the extracellular matrix (Preissner *et al* 1990, Seiffert *et al* 1990). Therefore, when PAI-1 was first demonstrated in human plasma by chromatography, it was observed to exist both in a free form and in a complex with its binding protein (vitronectin), which was named as high molecular weight plasminogen activator inhibitor (Wiman *et al* 1984).

Active PAI-1 has a high affinity for vitronectin, but the latent, cleaved, chloramine T-inactivated or protease-attacked PAI-1 exhibits very weak (over 100-fold less) binding affinity (Sigurdardottir and Wiman 1990, Aleshkov *et al* 1996, Lawrence *et al* 1997). Vitronectin can stabilize half-life of the active PAI-1, but the second-order association rate constant (k_1) for the inhibition of tPA or uPA by PAI-1 is not affected (Keijer *et al* 1991, van Meijer *et al* 1997b). It has been demonstrated that the vitronectin binding could lead to the conformational change in the reactive center of PAI-1 (Fa *et al* 1995).

Native vitronectin also accelerates the inhibition of human α-thrombin by PAI-1 (Naski *et al* 1993). A recent report indicated that this rate enhancement was about 100-fold (van Meijer *et al* 1997a). However, although urea-treated vitronectin can bind PAI-1, it did not exhibit this effect.

The studies of the PAI-1 binding sites on vitronectin have given conflicting results from different groups. Overall, it can be concluded that vitronectin has several regions involved in PAI-1 binding, probably with quite different affinities. The region between Asp^1 and Met^{51} (containing somatomedin B, SMB, domain) was suggested to be a binding site by several

investigators (Seiffert and Loskutoff 1991, Sigurdardottir and Wiman 1994, Seiffert *et al* 1994, Deng *et al* 1995, Deng *et al* 1996). However, by using synthetic polypeptides from certain regions in vitronectin, it was suggested that two binding sites of PAI-1 were situated in vitronectin among the residues from Gly^{115} to Glu^{121} (Mimuro *et al* 1993) and from Lys^{348} to Arg^{370} (Gechtman *et al* 1993, Gechtman *et al* 1997). The latter one is adjacent to the heparin binding site (Kost *et al* 1992). The arginine-rich region at vitronectin C-terminal of vitronectin was suggested to be responsible for stabilizing PAI-1 (Keijer *et al* 1990).

There could be two reasons for these conflicting results. Firstly, the synthesized polypeptides did not keep the natural conformation of vitronectin and, therefore, much higher concentration of PAI-1 (up to two million folds) was needed to observe an effect as compared to intact vitronectin or the SMB fragment (Deng *et al* 1995). Secondly, since urea-treated vitronectin binds to many different surfaces, the assays using urea-treated vitronectin could give different results as compared to that using native vitronectin.

The binding site(s) of vitronectin on PAI-1 molecule has also been studied by several groups. Vitronectin does not seem to approach PAI-1 from the same orientation as tPA. It was observed by fluorescent labelling that the bait peptide of PAI-1 was more solvent exposed after that PAI-1 bound to vitronectin (Gibson *et al* 1997). Chimeric PAI-1 variants with RCL substituted by the same region from other serpins did not interfere the vitronectin binding (Lawrence *et al* 1990). The presence of vitronectin binding site(s) on PAI-1 in the region from Gln^{55} to Lys^{145} was reported by several groups (van Meijer *et al* 1994, Lawrence *et al* 1994, Padmanabhan and Sane 1995). In our study, substitutions of the residues (Phe^{113}-Asp^{138}) overlapping this region of PAI-1 did not cause any dramatic change in the affinity to vitronectin-coated microtiter plates (Paper II).

Heparin also has high affinity to PAI-1, but, unlike vitronectin, all of the active, latent and cleaved forms of PAI-1 can bind to heparin (Lindahl and Wiman 1989, Aleshkov *et al* 1996). It was suggested that heparin provides a template for PAI-1 and thrombin, which subsequently enhances the inhibition of thrombin by PAI-1 (Gebbink *et al* 1993).

Kost *et al* (Kost *et al* 1992) reported that the regions on immobilized vitronectin for PAI-1 and heparin binding are overlapping each other. However, this result is in conflict with earlier reports from the same group, in which PAI-1 did not compete with heparin for the binding to a CNBr fragment of vitronectin (Preissner *et al* 1990). A recent publication (Gibson *et al* 1997) also criticized this result by showing evidence for separate binding sites of PAI-1 and heparin on vitronectin. With site-directed mutagenesis, the positively charged residues (Lys^{65}, Lys^{69}, Arg^{76}, Lys^{80} and Lys^{88}) were proposed to constitute major determinants for heparin binding in PAI-1 (Ehrlich *et al* 1992). Our study indicated that two arginine residues, Arg^{115} and Arg^{118}, were involved in PAI-1 binding to heparin (Paper II). Interestingly, a low molecular weight inhibitor of PAI-1, AR-H029953XX, was also supposed to bind to these two residues (Björquist *et al* 1998), but it obviously had different effect to PAI-1 inhibitory activity as compared to heparin.

The PAI-1 gene

The human PAI-1 gene is located on chromosome 7 (q21.3-q22) consisting of nearly 16 kilo base pairs (kbp). It contains 9 exons and 8 introns. Several groups have analyzed and sequenced the whole PAI-1 gene (Loskutoff *et al* 1987, Bosma *et al* 1988, Strandberg *et al* 1988, Riccio *et al* 1988, Bruzdzinski *et al* 1990). The gene may be transcribed into mRNAs with two sizes, 1.2 and 1.4 kbp, which differs in length in the 3'-untranslated region

(Klinger *et al* 1987, Bosma *et al* 1988). Since PAI-1 is an important regulatory factor in the fibrinolytic system, abnormal changes of PAI-1 gene might lead to clinical phenomena.

Several polymorphisms have been found in the PAI-1 gene, including a *Hind*III polymorphism (Bosma *et al* 1988) and a (CA)n dinucleotide repeat polymorphism (Dawson *et al* 1991). A common polymorphism in the promoter region of PAI-1 was also observed in 1993 (Dawson *et al* 1993). This polymorphism is due to a single-base-pair difference, four or five guanosines in a row (i.e. 4G/5G), which is localized at 675 bps upstream (-675) of the start codon of PAI-1 gene. The individuals with the 5G allele, either homozygous or heterozygous, had lower activity level of plasma PAI-1. It was speculated that the presence of the 5G allele sequence creates a binding site for a repressing nuclear protein that consequently reduced the transcription of the PAI-1 (Eriksson *et al* 1995).

In 1992, a child with complete deficiency of PAI-1 due to a frame-shift mutation in exon 4 was reported (Fay *et al* 1992). In a recent study, nineteen individuals who were heterozygous for this mutation and seven homozygous individuals with complete PAI-1 deficiency have been observed (Fay *et al* 1997). The homozygous individuals had abnormal bleeding tendencies occurring only after trauma or surgery, but the heterozygous individuals did not have any clinical signs of bleeding. Other types of PAI-1 deficiencies in plasma have also been reported in several families from different countries (Dieval *et al* 1991, Lee *et al* 1993, Takahashi *et al* 1996). The affected members in these families typically had a lifelong bleeding tendency with low PAI-1 antigen and PAI-1 activity. However, no further study was reported on the genetic mechanism for these observations.

Recently, four other polymorphisms have also been found (Henry *et al* 1997) among healthy men from France and Northern Ireland. These polymorphisms are located: at –844 (G/A substitution) with a strong positive linkage disequilibrium to the 4G/5G polymorphism; at +9,785 (G/A substitution); at +11,053 (T/G substitution); a 9-nucleotide insertion/ deletion between +11,320 and +11.345. Recently, we also found a G/A polymorphism at +1334 of PAI-1 gene in two patients with a mild bleeding tendency (Paper V). This mutation resulted in a substitution of the alanine residue at –9 of the PAI-1 propeptide to a threonine but it was not directly linked to the PAI-1 concentration in plasma.

THE PRESENT INVESTIGATIONS

AIMS OF THE STUDY

1. To increase the knowledge about the transition of active PAI-1 to the latent form by seeking the residues or regions involved in this process.
2. To analyze the interaction of PAI-1 and tPA, especially regarding the switch between substrate and inhibitory forms.
3. To study the vitronectin binding site on PAI-1.

MATERIALS AND METHODS

Site-directed mutagenesis of PAI-1 cDNA

To analyze the residues in any region of the PAI-1 molecule, a method, called two-step PCR, was used to introduce mutations in PAI-1 cDNA (Paper II). As shown in Fig. 3, the first PCR (PCR1) was performed with the mutation primer and another primer on the plasmid that should be located outside the first restriction enzyme (E1) site. The products were purified by low melting temperature gel to remove the residual primers. The second PCR (PCR2) was performed by using the purified PCR products from the first PCR as a new primer. The other primer was localized outside the second restriction enzyme (E2) site on PAI-1 cDNA (Fig. 3). The generated PCR products were cleaved by the two restriction enzymes (E1, E2) and transferred back to the corresponding region in the original PAI-1 cDNA by DNA ligation.

Two key points have been noticed in this two-step PCR mutagenesis. Firstly, to design a mutation primer, a complicating property of some thermal stable DNA polymerases (e.g. *Taq* DNA polymerase, DyNAZyme) must be considered. Without a requirement for a template, these polymerases can add an extra nucleotide, mostly an A, to the 3'-end of double stranded DNA (Clark 1988, Gurr and McPherson 1991). This extra nucleotide could cause an unwanted mutation in the final DNA products. To avoid this potential problem, the first nucleotide outside the 5'-end of a mutation primer should be a wobble position of a codon in the gene or preferably a T on the strand of the primer. Secondly, the residual primers in the first step PCR must be completely removed before the products can be used in the second PCR. Otherwise, these residual primers, which cooperate with mutation primer in the first PCR, can dominantly participate in the amplification of the second PCR and generate DNA products that do not contain the required mutation.

Another source of error is an incomplete digestion of the expression vector, prior to the DNA ligation with the second PCR products. This can be due to the use of two restriction enzymes with different optimal cleaving conditions. Mostly, a small portion of the plasmids is only cleaved by one of the enzymes and they can easily undergo cyclization at the DNA ligation step to create non-mutated plasmids. It could create about 20% of background in screening for PAI-1 mutants from transformed bacteria.

To overcome this problem in generating a large amount of PAI-1 mutants, a procedure that is schematically shown in Fig. 4, can be used. Firstly, a special plasmid was generated as shown in Fig. 4 (a): (1) to cleave the pBV220/PAI-1 with *Nco*I, which has a cleavage site in the middle of PAI-1 cDNA, (2) to fill the cohesive ends with nucleotides by using T_4 DNA polymerase, and (3) to ligate the blunted ends to get cycled plasmid. Secondly, the generated plasmid was used as the "original" plasmid to "catch" the cleaved PCR fragments. In other words, the cleaved PCR products replaced the "corresponding" region in this special plasmid. As shown in Fig. 4 (b), the elimination of the *Nco*I site caused a reading frame shift (4 bps insertion), and consequently this plasmid is invalid in the expression of PAI-1 protein. Since mutated PAI-1 cDNAs are derived from intact PAI-1 cDNA template via PCR, they should have the intact *Nco*I cleavage site and also

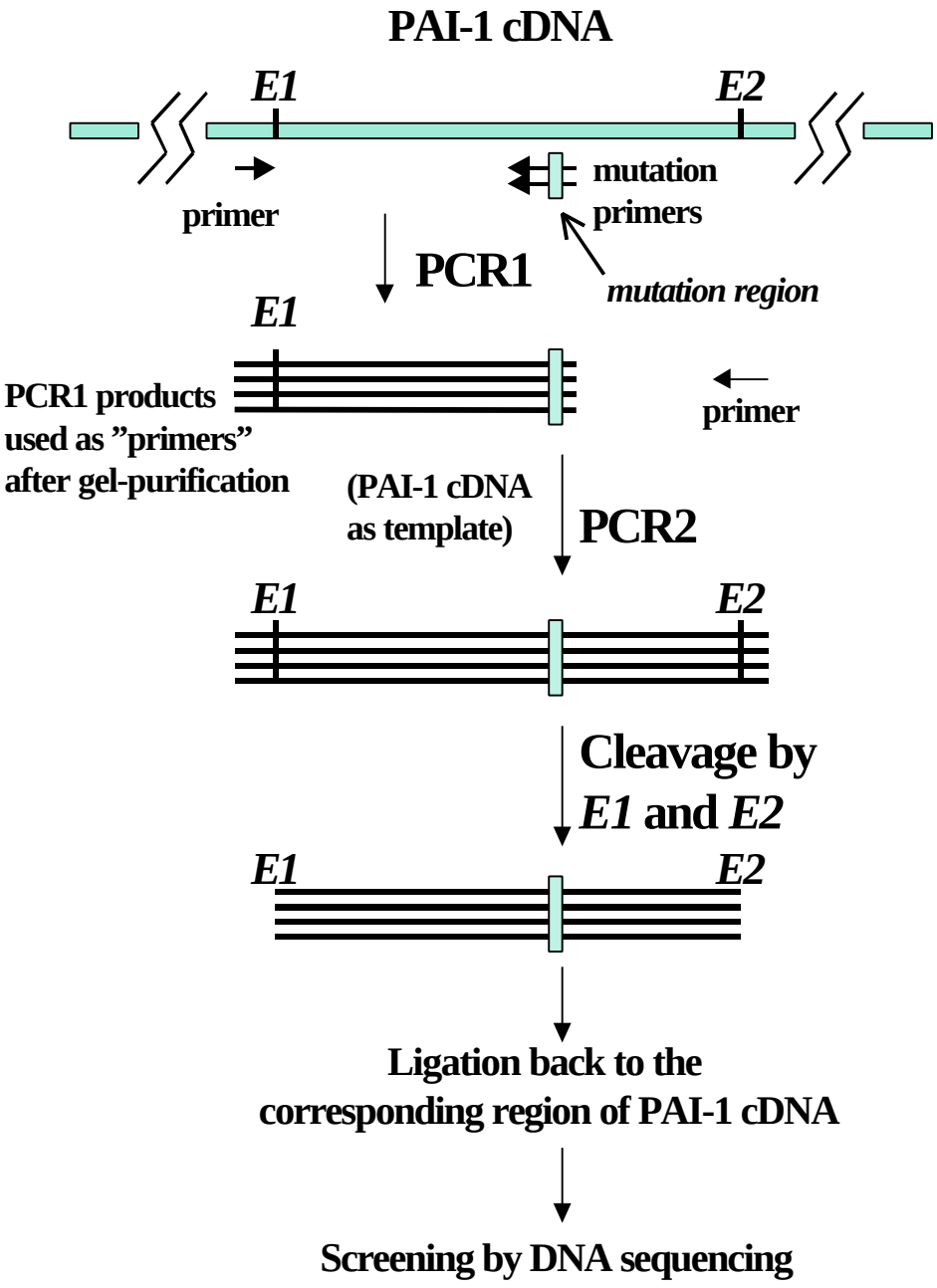


Fig. 3. Schematic presentation of the procedure to introduce mutations in PAI-1

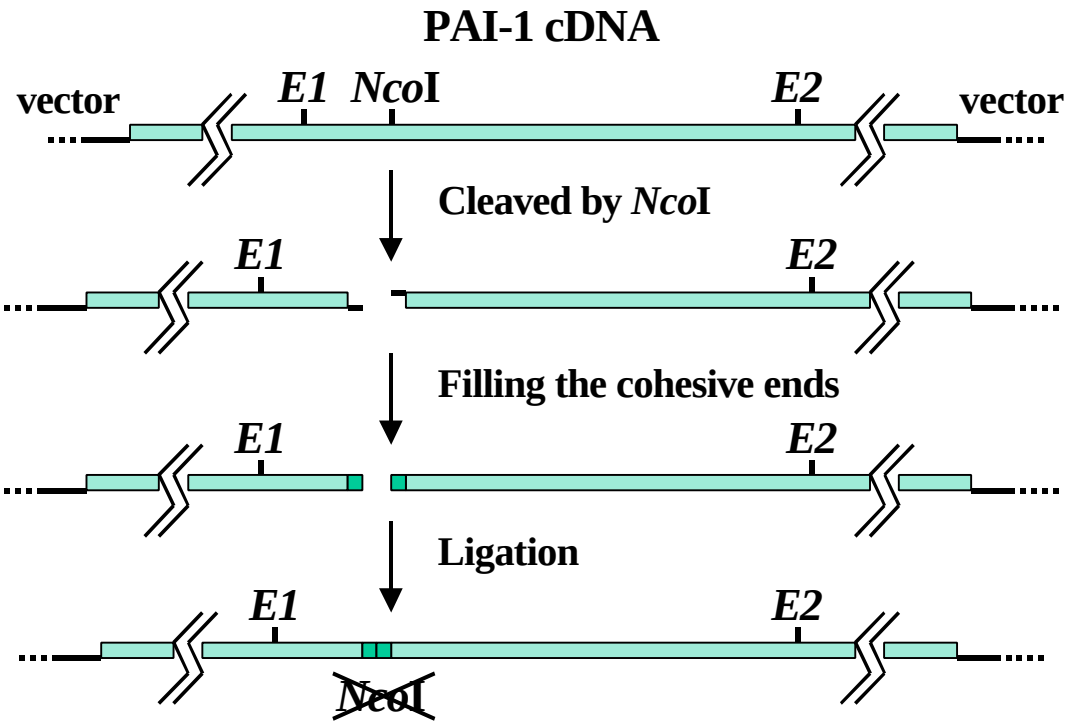


Fig. 4 (a) Schematic presentation of the procedure to generate pBV220/PAI-1 that does not contain *Nco*I cleavage site.

5'… ... CCATGG … … 3'
3'… … GGTACC … … 5'
6 bps
↓ *Nco*I
5'… ... C CATGG … … 3'
3'… … GGTAC C … … 5'
4dNTP ↓ T_4 DNA polymerase
5'… ... CCATG-CATGG … … 3'
3'… … GGTAC-GTACC … … 5'
10 bps

Fig. 4 (b) The change of DNA sequence after removing the *Nco*I cleavage site.

the ability to synthesize the PAI-1 variant proteins. Therefore, the "background" of this "original" plasmid can be easily discriminated by a pre-screening step in a large amount bacterial colonies either by *Nco*I digestion or by PAI-1 protein expression. It was found that the latter pre-screening method is more effective and practical than the former one. Finally, the resulting clones were sequenced in the PCR-replaced regions to make sure that the correct mutations were introduced, and to exclude the possibility of other undesired PCR-generated mutations.

Expression of PAI-1 variants in *E. coli*

The expression vector, pBV220, for producing PAI-1 proteins in *E. coli* contains two consecutive promoters, λP_R and λP_L derived from the λ phage. At a temperature of 30 °C, a repressor protein, *cIts*857, can bind to λP_L and consequently restrain the transcription of downstream DNA. However, this repressor protein is temperature-sensitive. At the temperature over 40 °C, it changes the conformation and loses the binding affinity to the λP_L (Buell *et al* 1985). Therefore, the initiation of λP_L is heat-inducible.

PAI-1 cDNA was cloned between *Eco*RI and *BamH*I restriction enzyme sites in the polylinker region of pBV220. This is the most suitable position for a cloned foreign gene to be expressed in *E. coli* since the distance between the SD (Shine-Dalgarno) sequence of the promoters and start codon ATG is optimal (7 bp). The expression of PAI-1 variants is schematically shown in Fig. 5. When the *E. coli* strain DH5α was used as a host cell, about 100 mg of recombinant PAI-1 could be produced from 1 liter of bacterial culture. In the *E. coli* strain XL1 Blue, a similar yield could be achieved but a larger portion (nearly 50%) of the synthesized PAI-1 stayed in a soluble form. The soluble form of wild type recombinant PAI-1 was functionally active while the insoluble PAI-1 did not have a natural structure or normal function. The PAI-1 in the inclusion bodies could be treated with 4 mol/L guanidinium chloride, followed by dialysis, to get partially reactivated PAI-1.

Purification of PAI-1 variants

The soluble form PAI-1 in the supernatant of the expressed and lysed bacteria was applied to a heparin-Sepharose CL-6B column (bed volume of about 10 mL), equilibrated with 0.05 mol/L of acetate buffer, 0.1M mol/L NaCl, 0.1 g/L Tween-80, pH 5.5. All of the operations were performed at 5 °C. After the unabsorbed proteins have been thoroughly washed out, the adsorbed material was eluted by a gradient of NaCl from 0.1 to 1.1 mol/L in 200 mL of the equilibration buffer. After dialysis against 0.15 mol/L sodium phosphate buffer, pH 6.6, containing 0.1 mol/L NaCl, the preliminarily purified PAI-1 protein was applied to an anhydrotrypsin agarose column (bed volume about 1 mL) equilibrated with the same phosphate buffer (Fa *et al* 1995). The adsorbed "active" PAI-1 was eluted with 0.3 mol/L arginine in the same phosphate buffer, pH 6.6. The fraction(s) with highest PAI-1 antigen concentrations was(were) dispensed in several tubes and kept at –70 °C until use.

PAI-1 antigen and activity

Since the PAI-1 variants expressed in *E. coli* seemed to have a different response to the monoclonal antibody in the Imulyse PAI-1 kit (for PAI-1 antigen) as compared to the PAI-1 from HT1080 cells, wild type PAI-1 (wtPAI-1) was used as a standard in all PAI-1 antigen measurements. The absorbance coefficient for wtPAI-1 at its absorbtion maximum (280 nm) was determined as 7.7 (for a 10 g/L solution) (Paper II). PAI-1 activity was determined using the Chromolize PAI-1 kit. In some cases, to measure activity of the samples from the PAI-1 stability assays in the presence of vitronectin, it was necessary to use the Spectrolyse/PL PAI-1 kit.

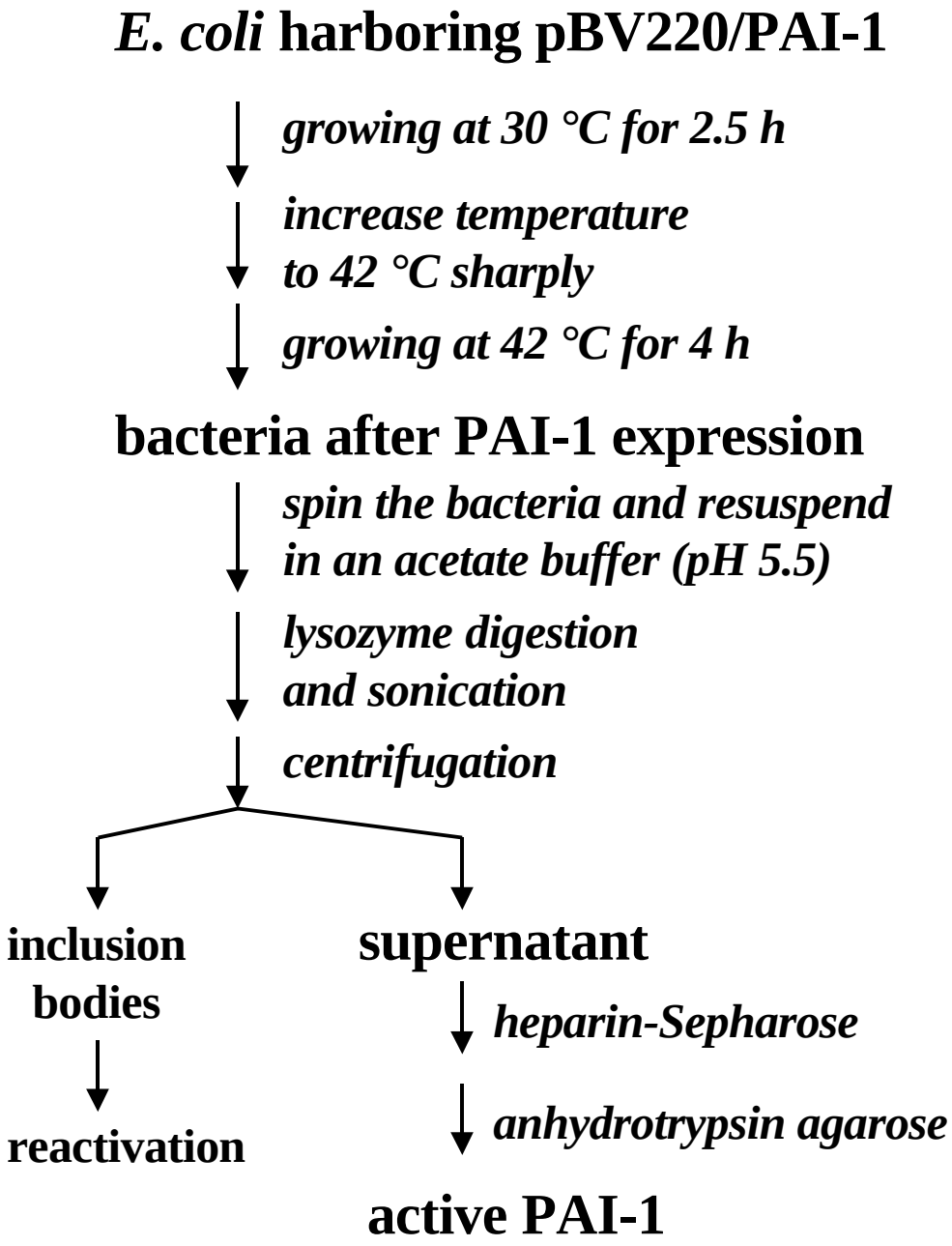


Fig. 5. The schematic procedure for the expression and purification of PAI-1

PAI-1 stability

When the stability of PAI-1 variants was measured at different pHs, the following buffer systems were used: 0.05 mol/L sodium acetate buffer, pH 5.5; 0.05 mol/L sodium phosphate buffer, pH 6.5 or pH 7.5, and 0.05 mol/L Tris-HCl buffer, pH 8.5. In all these buffers, 0.1 mol/L sodium chloride, 0.5 mg/mL bovine serum albumin and 0.1 g/L Tween-80 were also present. The PAI-1 variants were diluted in these buffers to final concentrations of about 250 U/mL in a final volume of 1 mL. When these PAI-1 variants were incubated at 37 °C, 100 μL samples were taken at various time intervals (0 to 24 h). For PAI-1 variants with shorter half-lives, the samples were just kept on ice and the activity was analyzed when all samples for one PAI-1 variant were taken. For the PAI-1 variants with longer half-lives, the samples were acidified, if necessary, by 1 mol/L sodium acetate buffer, pH 3.9, and stored at –70 °C until analysis.

Vitronectin-binding

Vitronectin was diluted to a final concentration of 30 μg/mL in 0.1 mol/L of $NaHCO_3$ buffer, pH 9.6, and used to coat microtiter plates overnight at room temperature. The plates were washed 4 times by 0.04 mol/L sodium phosphate, containing 0.1 mol/L NaCl, 5 mmol/L EDTA, 0.5 g/L Tween 80, pH 7.3, and 200 μL of the same phosphate buffer was added to each well. 10 μL of PAI-1 samples with different concentrations were also added to the plate to get final concentrations of PAI-1 from 0 to 600 U/mL. After incubation for 1 h at ambient temperature, the plates were washed and HRP-conjugated antibodies towards PAI-1 were added. After another one hour incubation, the plates were developed with o-phenylene diamine and H_2O_2. The absorbance at 492 nm was recorded and used as a measure of the PAI-1 binding to vitronectin.

RESULTS AND DISCUSSION

Modifications of the residues in the region of Phe^{113} to Asp^{138} in PAI-1

To introduce mutations at residues Asp^{125}, Glu^{128} and Glu^{130}, an *Apa*I site was previously introduced by a synonymous mutation at the nucleotide number 395 in PAI-1 cDNA. The synthesized primers containing the *Apa*I site and the degenerated codons concerning these three residues were used to perform PCR amplification. As a result, thirteen PAI-1 mutants were obtained (Paper I). They consisted of three single-site mutants, three double-site mutants and seven triple-site mutants. These PAI-1 variants could be expressed at a high level in *E. coli*. The preliminary characterization of these purified PAI-1 proteins demonstrated that these PAI-1 mutants exhibited similar activity as wtPAI-1 expressed in *E. coli*, with the exception that some of the triple-site mutants had completely lost their activity.

The continued study of this region was conducted by mutating the residues with either charged or large side groups on the stretch of Phe^{113} to Asp^{138} (Paper II). The site-directed mutageneses were performed by using the two-step PCR amplification. The generated single-site changed PAI-1 mutants and wtPAI-1 were expressed in *E. coli* system. With the XL1 Blue strain as host cells, PAI-1 proteins in soluble form were used as the source of purification. By using heparin-Sepharose and anhydro-trypsin agarose chromatographies, active PAI-1 variant proteins were purified and they were subsequently characterized by their inhibitory activity towards tPA, stability and the affinity to vitronectin.

Most of PAI-1 variants, except Asp125Lys, Phe126Ser and Arg133Asp, exhibited a high spontaneous inhibitory activity towards tPA and it did not change much upon the reactivation by 4 mol/L

guanidinium chloride followed by dialysis in a buffer with pH 5.5. Without the treatment of guanidinium chloride, PAI-1 mutants Asp125Lys, Phe126Ser and Arg133Asp had very low activity (0.1 ~ 8.4% the activity of wtPAI-1). The portions of these three mutants that were absorbed on anhydrotrypsin agarose could to a large extent be cleaved by tPA. The two mutants Asp125Lys and Arg133Asp gained considerably in inhibitory activity by the guanidinium chloride reactivation. While wtPAI-1 exhibited a half-life of 2 h, the reactivated PAI-1 mutants Asp125Lys and Arg133Asp had significantly decreased half-lives, 22 and 31 min, respectively. In the presence of vitronectin, their half-lives increased to 2.3 h, which was comparable to the half-life of wtPAI-1 (3.0 h). Both in the absence and presence of vitronectin, PAI-1 Glu130Lys showed increased stability with half-lives of 4.3 and 6.8 h, respectively. The stability of the other PAI-1 mutants did not differ much when compared to wtPAI-1.

When PAI-1 variants were purified from heparin-Sepharose column, most of the PAI-1 variants were eluted at the NaCl concentration of 0.7 ~ 0.8 mol/L. However, the two arginine PAI-1 variants, Arg115Asp and Arg118Asp, were eluted earlier than wtPAI-1, at the NaCl concentrations of 0.63 and 0.58 mol/L, respectively. This indicated that the Arg^{115} and Arg^{118} are involved in the heparin binding.

Two groups (van Meijer *et al* 1994, Lawrence *et al* 1994) have indicated that a binding site of vitronectin was located in the stretch from Met^{110} to Gln^{123}. However, in our study, none of the PAI-1 mutants with the substitutions of residues between Phe^{113} to Asp^{138} showed any significantly vitronectin binding difference as compared to wtPAI-1. PAI-1 Phe126Ser could not be tested due to its low activity. There are two possible explanations for our conflicting results. Firstly, the residues that we mutated in this region of PAI-1 are different from those published in the article by Lawrence *et al* (Lawrence *et al* 1994). Secondly, we used vitronectin coated microtiter plate to test the affinity of PAI-1 variants to vitronectin. It was claimed that the coating vitronectin to plastic plate could somehow hide the high affinity PAI-1 binding site on vitronectin (Deng *et al* 1995). Therefore, those PAI-1 mutants should preferably be analyzed by another kind of vitronectin binding assay.

Modifications of the residues in s2B and s3B strands of B b-**sheet in PAI-1**

The B β-sheet of PAI-1 is located beneath the A β-sheet and the strands s2B, s3B are in the vicinity of what is believed to be the region where RCL insertion starts. Therefore, an investigation on the role of the B β-sheet for PAI-1 stability was conducted by introducing single-site mutations on the s2B and s3B strands.

The role of Tyr^{221} on s2B strand to PAI-1 stability was first studied by substituting it with five different amino acids, His, Phe, Ser, Lys and Asp (Paper III). Active PAI-1 proteins were obtained using the same expression and purification procedures as described above. Characterization of these PAI-1 mutants and wtPAI-1 included stability in the absence and presence of vitronectin and at different pHs (5.5, 6.5, 7.5 and 8.5) or at different temperatures (37, 22 and 0 °C). It also included complex formation with tPA and binding affinity to vitronectin-coated microtiter plates.

In the absence of vitronectin at 37 °C and pH 7.5, the PAI-1 mutants Tyr221His and Tyr221Ser had prolonged half-lives of 14.8 and 4.1 h, respectively. Vitronectin had significantly effect in stabilizing these two PAI-1 mutants, especially to the serine variant, and dramatically prolonged their half-lives to 23.8 and 53.7 h, respectively.

In the absence of vitronectin, at 37 °C and at different pHs, wtPAI-1 showed a similar

stability pattern to the native PAI-1, with a longer half-life at acidic pH (e.g. 16.8 h at pH 5.5) and a shorter half-life at basic pH (e.g. 1.0 h at pH 8.5). However, the PAI-1 mutants Tyr221His and Tyr221Ser showed highest stability at neutral pH, with half-lives of 23.3 and 12.0 h, respectively, at pH 6.5, but their half-lives were decreased to just 2.6 and 2.5 h, respectively, at pH 5.5. Interestingly, in the presence of vitronectin, the PAI-1 mutants Tyr221His and Tyr221Ser considerably increased their stability at pH 5.5, which was even much higher than for wtPAI-1 (62.1 and 91.2 h, respectively, verse 25.9 h of wtPAI-1). The PAI-1 mutant Tyr221Phe exhibited similar half-lives as wtPAI-1 at different pHs. It seemed that vitronectin hardly had any effect on its stability, especially at neutral or basic pHs.

These results indicated that, in the absence and the presence of vitronectin, the mechanisms for the active to latent form transition of the PAI-1 mutants Tyr221His and Tyr221Ser are quite different. As speculated above, a histidine residue is probably involved in the transition from the active PAI-1 to its latent form. At neutral pH, in the absence of vitronectin, a non-protonated histidine (mentioned as His^x below) most likely has hydrophobic properties and therefore it might interact with the hydrophobic side chain of Tyr^{221}. However, when pH is decreased to 5.5, His^x will be protonated and become positively charged. As a result, it will lose the interaction with Tyr^{221}. This somehow could lead to a conformational change that is not favored by the RCL insertion and consequently causes a prolongation of the half-life of wtPAI-1.

When Tyr^{221} was mutated to a histidine or to a serine, the two generated PAI-1 mutants, Tyr221His and Tyr221Ser, had increased half-lives at pH 6.5 and 7.5, while PAI-1 Tyr221Phe exhibited similar stability as wtPAI-1. Therefore, the presence of hydrophilic residues at the position of residue number 221 in PAI-1 is favored to keep PAI-1 stable at neutral pH. However, if Tyr^{221} was substituted by an aspartic acid or a lysine, which are also hydrophilic residues, the generated PAI-1 mutants became insoluble, perhaps resulting from a large conformational change. Surprisingly, at acidic pH (pH 5.5), these two PAI-1 mutants, Tyr221His and Tyr221Ser, dramatically decreased their half-lives (Fig. 6). The explanation for this phenomenon can also come from the His^x that interacts with Tyr^{221} in wtPAI-1. As speculated above, the protonated form of His^x (at pH 5.5) could lose its connection with Tyr^{221} and consequently stabilize active form PAI-1. When Tyr^{221} was replaced by histidine or serine, this connection could still be kept at pH 5.5, since His^{221} or Ser^{221} in these two mutants might still form hydrogen bonds with the protonated His^x and keep the interaction, resulting in facilitating the formation of latent PAI-1.

Interestingly, the substitution of another tyrosine residue, Tyr^{220}, by serine, which generated the PAI-1 mutant Tyr220Ser, exhibited quite similar stability property to wtPAI-1 (Paper IV). It is reasonable to assume that the side chain of Tyr^{220} must point out in a different direction and therefore could not have the same function as Tyr^{221}.

In the presence of vitronectin, the effect caused by the interaction of Tyr^{221} and the speculated His^x could be interfered by the vitronectin binding. It has been suggested that the binding of vitronectin to PAI-1 can somehow prevent the insertion of RCL into PAI-1 molecule. Since the binding of PAI-1 and vitronectin tends to involve multiple sites, the portion of vitronectin that stabilizes active form PAI-1 could be just one or few residues, while the other binding sites are used to attach the two proteins together. From the phenomenon that we observed in PAI-1 Tyr^{221} mutants, it can be assumed that, in the case of

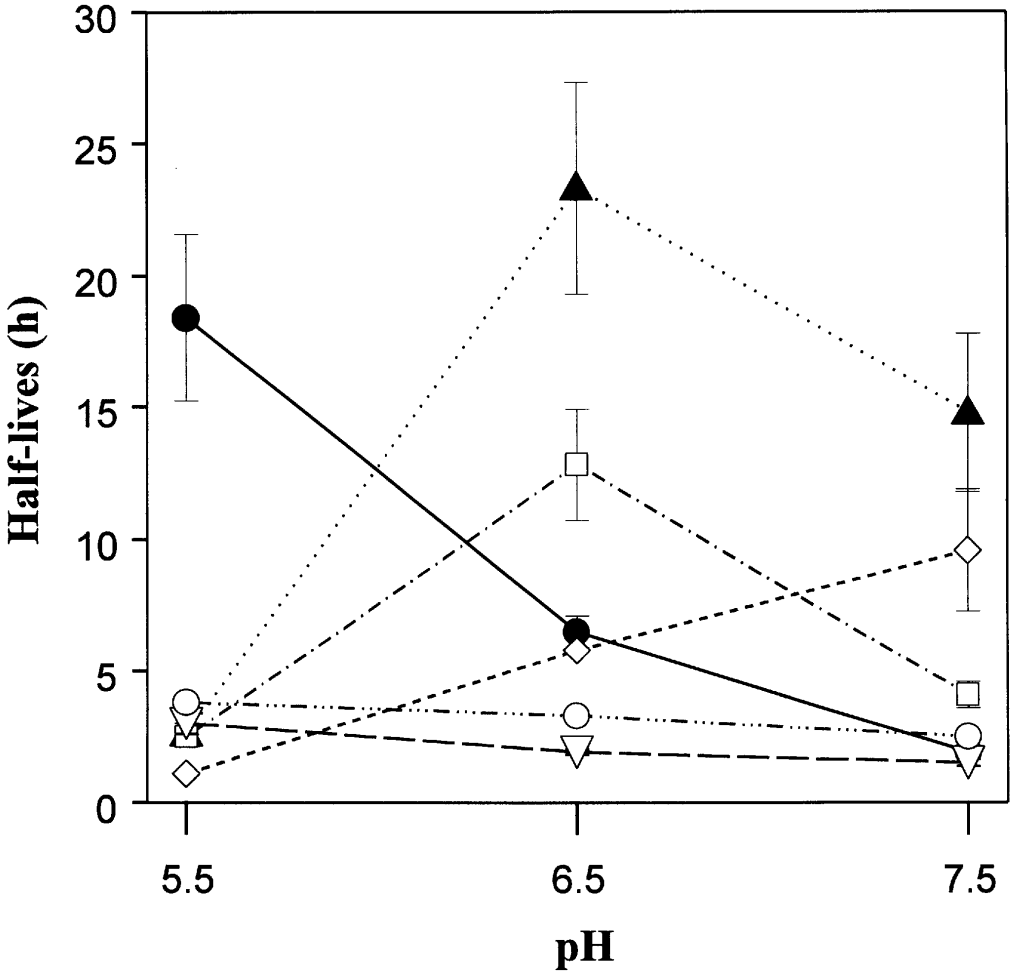


Fig. 6. PAI-1 stability at 37 °C, different pH and in the absence of vitronectin. The PAI-1 variants are indicated by: wtPAI-1 (●), Tyr221His (▲), Tyr221Phe (□), Tyr228Ser (◇), His229Asp (▽) and His229Phe (○).

wtPAI-1 bound to vitronectin, the Tyr^{221} could use its hydroxyl group to bind a group in vitronectin. In other words, this group in vitronectin takes the place of that speculated His^{x} in PAI-1 to interact with Tyr^{221} and this binding can somehow block the insertion of RCL into the molecule.

In the case of PAI-1 mutants Tyr221His and Tyr221Ser, the His^{221} or Ser^{221} might interact with vitronectin in a unique way that leads to a larger conformational change and consequently results in a dramatic increase of the stability for these two mutants at all pHs. This interaction might be due to the formation of a hydrogen bond, since histidine and serine are both better donors of hydrogen ions than tyrosine. However, this difference in the interaction with vitronectin is not large enough to be significantly observed in the vitronectin binding assay. This is perhaps also because that the residue at number 221 position in PAI-1 is just an "effecting" residue but not a "binding" site. Since Tyr^{221} can either interact with His^{x} in PAI-1 in the absence of vitronectin, or bind to a group in vitronectin to stabilize the active PAI-1 form, the side group of Tyr^{221} should point to the outside of PAI-1 molecule. Also, the His^{x} that is speculated to interact with Tyr^{221} ought to be present on the surface of PAI-1.

In the case of the PAI-1 mutant Tyr221Phe, the Phe^{221} loses this interaction, since Phe does not have a hydroxyl group, and consequently cannot form a hydrogen bond with a corresponding site in vitronectin, which may be the case in wtPAI-1 and in the other two Tyr^{221} mutants. As a result, vitronectin does not have any effect on the stability of PAI-1 mutant Tyr221Phe.

The three Tyr^{221} PAI-1 mutants formed SDS-stable complex with tPA and also a small portion of cleaved material was formed. They exhibited dramatically increased stability at lower temperature. At 0 °C they were virtually stable. The PAI-1 mutants Tyr221Lys and Tyr221Asp were insoluble and could not be further analyzed.

To study the function of the B β-sheet in PAI-1 for its stability, other residues between His^{219} and Tyr^{241}, except Gly^{230} and Pro^{240}, were also substituted by residues with opposite properties, i.e. positively charged to negatively charged, hydrophobic to hydrophilic, or vice versa (Paper IV). These residues cover the s2B and s3B strands of the B β-sheet. Also, to analyze the function of two other histidine residues in this vicinity, His^{185} and His^{190} were studied after substituted by aspartic acid, respectively. The mutagenesis was performed using the two-step PCR method (Fig. 3). The active PAI-1 mutants were obtained by the same purification procedure described above. Their stability was analyzed at three different pHs (5.5, 6.5 and 7.5) at 37 °C.

At pH 7.5, substitutions in the central portions of both strands s2B and s3B in PAI-1 (Ile^{223} to Leu^{226} on s2B and Met^{235} to Ile^{237} on s3B) caused a significant decreased stability. The half-lives were typically below 25% of that for wtPAI-1. On the other hand, the mutations on both sides of the central portions of the two strands (Tyr^{221}, Asp^{222}, Tyr^{228} and Thr^{232}) frequently resulted in an increased PAI-1 stability. The half-lives were increased up to 12.7 h. At pH 6.5 or 5.5, most of the PAI-1 mutants had prolonged half-lives following the similar stability pattern of wtPAI-1 (Fig. 6). However, the PAI-1 mutant Tyr228Ser had a completely opposite stability pattern when compared to wtPAI-1. Its half-lives at pH 7.5, 6.5 and 5.5 were 9.5, 5.8 and 1.1 h, respectively (Fig. 6). A similar property was also observed for the two Tyr^{221} mutants (to His and Ser) as mentioned above. Interestingly, these two tyrosine residues are both situated on the s2B strand but on the opposite ends. Therefore, they must act in

a quite similar manner to influence PAI-1 stability.

Another interesting phenomenon was observed regarding the stability of the two His^{229} mutants, His229Asp and His229Phe. They had comparable half-lives as for wtPAI-1 at pH 7.5, but their pH dependence of the stability was much less than that of wtPAI-1. Thus, when pH was decreased from 7.7 to 5.5, the half-lives of these two mutants were just increased by less than 2-fold. This indicated that protonation of His^{229} stabilizes the conformation of active PAI-1. The other two histidine mutants, PAI-1 His185Asp and PAI-1 His190Asp, exhibited largely decreased half-lives of 0.6 and 0.1 h, respectively, at pH 7.5, but their stability was significantly improved at pH 6.5 and 5.5.

SDS-PAGE analysis showed that all of the PAI-1 mutants and wtPAI-1 could form SDS-stable complexes with tPA and there was a small amount of substrate form present for most of the PAI-1 variants. However, some of the leucine mutants had interesting properties. PAI-1 Leu224Ser and PAI-1 Leu233Ser did not exhibit any cleaved form upon the reaction with tPA. Meanwhile, large portions of the PAI-1 mutants Leu224Ser, Leu226Ser and Leu233Ser did not form SDS-stable complexes. They exhibited largely “latent” behavior on SDS-PAGE analyses, even though they had the ability to bind to anhydrotrypsin agarose and were analyzed just after eluted from the column. An explanation for this phenomenon might be that these PAI-1 variants rapidly form reversible complexes with tPA, but that the reaction rate to form the covalent bond was somehow decreased. Also, the mutant Pro227Gly displayed a normal specific activity, but it mainly acted as a substrate when incubated with tPA. It is possible that the interaction with tPA and peptide bond cleavage in this case occurs normally, but the quality of the formed complex may be affected in a way that it dissociates as a result of the treatment with SDS.

Since the strands s2B and s3B are located at a very important position where the RCL is believed to start its insertion, it is reasonable to speculate that the residues on these two strands could effect the transition of the active PAI-1 to its latent form. The properties of the PAI-1 mutants concerning the residues on these two strands proved this speculation.

The RCL of PAI-1 contains a relatively hydrophilic cluster of residues from P17 to P8. However, hydrophobic residues are dominating on the s2B and s3B strands, and the groove of PAI-1 for RCL to be inserted was also indicated to be in a hydrophobic milieu (Shore *et al* 1995). Therefore, the insertion of the hydrophilic RCL should not be favored by the hydrophobic interior of PAI-1. Since the procedure of RCL insertion, which leads to the latency decay of active PAI-1, is a spontaneous transition, another force that drags or finally anchors the inserted RCL in the molecule can be assumed to exist. This could come from the stress state of the active PAI-1 conformation or the binding of RCL to another residue. Nevertheless, the substitutions of the hydrophobic residues on the s2B and s3B strands by hydrophilic residues could damage the hydrophobic core of PAI-1 and subsequently facilitate the RCL insertion. This can explain our observation that the substitutions of the residues in the central part of these two strands mostly generate PAI-1 variants with shorter half-lives.

Substitutions at both sides of the central portion in s2B and s3B strands frequently resulted in an increased PAI-1 stability. It can be assumed that those residues situated outside the central portions of the two strands could be of importance in keeping the positions of s2B and s3B by interacting with some other residues in PAI-1. The mutations of these “anchoring” residues

might lead to the movement of the two strands and consequently interfere the insertion of RCL, which results in increasing the half-lives of these PAI-1 mutants.

Modifications of His^{143} on the hF helix in PAI-1

The hF helix of PAI-1 is located just beyond the A β-sheet, and His^{143} on the hF helix is situated in the vicinity where the inserted RCL forms the s4A strand. This histidine residue was believed to determine the pH dependence of PAI-1 stability by some investigators (Kvassman *et al* 1995a). To study the role of His^{143} to PAI-1 functions, we substituted this histidine by Lys, Asp, Phe and Thr, respectively. The generated single-site changed PAI-1 mutants were purified and analyzed for their stability and reactions with tPA. When His^{143} was substituted by Asp or Phe, the stability of the generated mutants showed much less pH dependence when compared to wtPAI-1 (Table 2). These two His^{143} mutants had very similar properties to the two His^{229} mutants (Paper IV) regarding the pH dependence of PAI-1 stability. However, after purification on the anhydrotrypsin agarose column, the two mutants His143Asp and His143Phe were to a large extent cleaved by tPA (Fig. 7). The other two PAI-1 mutants, His143Lys and His143Thr, showed similar properties to wtPAI-1. Our results indicated that His^{143}, at least partly, may be involved in the pH dependence of PAI-1 stability. In addition, a substrate behavior of PAI-1 was observed when a negatively charged or hydrophobic residue was present at this position.

We have studied several regions of PAI-1 and some of the generated mutants had deviating properties regarding the interaction with tPA, stability (in the absence or presence of vitronectin, pH dependence), binding to heparin, etc. In conclusion, these results indicated that residues outside the RCL in PAI-1 might also be of importance for PAI-1 function. To further investigate the mechanisms of the behavior of these PAI-1 mutants, combinations of selected mutations leading to deviating properties need to be produced and analyzed.

Detection of a new polymorphism in the propeptide region of PAI-1

Two patients with zero PAI-1 activity and a mild bleeding tendency were investigated for mutations in the PAI-1 structural gene (Paper V). The PAI-1 genes from these two patients and also a healthy individual were analyzed by DNA sequencing. As a result, in both patients a mutation in the PAI-1 gene (1334G → A) was observed. This causes a substitution of the alanine residue at –9 in the PAI-1 propeptide to threonine. Subsequently, a PCR method, utilizing allele specific primers, was developed and used to genotype 152 healthy individuals for this mutation in the propeptide. It was found that 16% of the healthy individuals in this study were heterozygous for the mutation/polymorphism, and that 1.9% were homozygous. The distribution follows Hardy-Weinberg equilibration. Twenty-three of the healthy individuals, 5 of them were heterozygous for the 1334A allele, were also analyzed for the plasma PAI-1 concentrations. Three of the healthy individuals, 2 of whom were heterozygous for the 1334A allele, had zero PAI-1 activity in plasma. However, the remaining three individuals with the 1334A allele had a normal or even elevated plasma PAI-1 level. Therefore, the 1334A allele does not seem to be directly involved in the low PAI-1 concentration or the bleeding tendency in the patients. The presence of the 1334A allele may be in linkage dysequilibrium to another mutation in the PAI-1 gene of importance for the PAI-1 concentration in plasma. This must be continued in forthcoming studies.

Table 2. The half-lives (h) of the PAI-1 variants at pH 5.5, 6.5 and 7.5.
The data were obtained from at least three separate experiments and expressed as mean ± standard deviation (SD).

PAI-1	pH 5.5	pH 6.5	pH 7.5
wtPAI-1	18.4 " 3.76	7.4 " 0.32	2.1 " 0.10
His143Lys	9.3 " 1.38	5.6 " 0.48	1.8 " 0.34
His143Asp	2.5 " 0.26	2.2 " 0.85	1.1 " 0.31
His143Phe	2.2 " 0.42	2.2 " 0.79	1.4 " 0.12
His143Thr	11.6 " 1.93	6.6 " 0.29	2.3 " 0.48

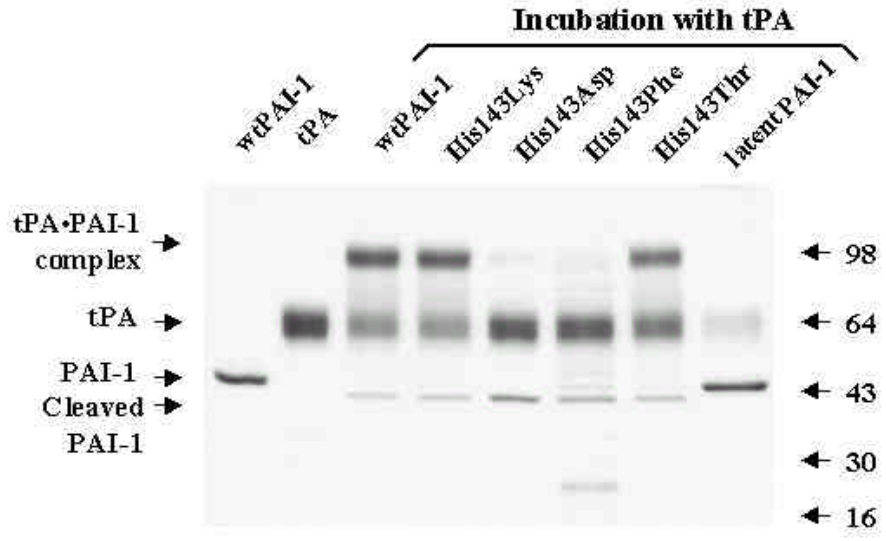


Fig. 7. Analysis of tPA, wtPAI-1 and mixtures of tPA (in about 50% excess) and the PAI-1 variants by SDS-PAGE.

ACKNOWLEDGEMENTS

I arrived at the Arlanda Airport in Stockholm on October 4th, 1995, with my wife, Meimei. We did not know what was waiting for us and what kind of people we would meet here. Now we know.

Since I am really not good in expressing my feeling orally, it gives me a relief to have this chance to give my gratitude to everyone who has helped me.

I want to thank Professor Björn Wiman, my tutor, for his elegant instruction on my research and his kindness to my family. I have very deep impression for his broad knowledge in scientific studies and his enthusiasm in the research. I admire his attitude to life. I am glad that I have really learned a lot from him on many aspects during the three-year study.

Dr. Gunnar Falk, my “office-mate”, has continuously been giving me help, especially when I started my work here. As a friend, his good humor sense makes my work more interesting. I really gained a lot in the discussion with him. Meanwhile, I learned to say “good morning” anytime during the day. Also thanks to him for reading my thesis and giving me valuable suggestions.

Dr. Margareta Sten-Linder, a typical intellectual Swedish woman, always talks with me with great patience and kindness. I got a lot of encourage from her.

Dr. Arvid Nordenhem is a person with great humor to life. As a friend, he would like to give me help whenever I encounter difficulties.

Dr. Anna Yu, who was working with me closely, helped me very much in reading my manuscripts and this thesis, and gave me valuable advice on them. She also gave a lot of help to my family.

Ms Birgitta Samppala always helps me with multiple things. I cannot remember how many times I went to her office asking for help. She always modifies my manuscripts before they are submitted. With her magical hands, my manuscripts all become pretty brides before they were sent to the journals.

Ms Helen Svensson, a nice Swedish girl, gave me excellent technical support. I am impressed very much with her kindness and pure mind.

Miss Helena Mångs, a clever and pretty Swedish girl, was latterly involved in our research with her graduation project from Linköping University. I am glad that we have had very good cooperation and also got some promising achievements in the study.

Ms Iréne Sjöström helped me in the protein purification when I started my work. She has also been very kind in helping me to solve many other problems.

I am very glad to work in this very friendly atmosphere that is also contributed by these nice ladies: Åsa Almqvist, Caroline Netré, Marie Haegerstrand-Björkman, Anette Dahlin, Marie Ståhlberg, Tuija Lindblom, Tova Dahlström, Eva-Maria Idehaag. It seems that I am always lucky to meet kind people and also beautiful ladies. Unfortunately, I cannot always write these words in my personal work experience or the acknowledgements of my articles, but here should be an exception.

Dr. Shu He and Dr. Hong-Lie Cao gave me help on many aspects all the time. I cannot imagine how I could have handled some problems without their support. As friends, they have really made my life in Stockholm easier.

Professor Per Wallén gave me deep impression by his elegant scholar attitude. I am very grateful for the valuable advice that he gave me in my research.

Professor Margareta Blombäck is also a person that I have great respect for. Her

devotion to the scientific work always gives me encourages in my research. I also want thank her for the kindness to my family.

Dr. Kamaran Fatah, Dr. Maria Bokarewa gave me much help and we had very good discussions, not only on scientific research but also on life.

Ms Inga Karlsson from the department of Microbiology gave me much help when I started my work here. Whenever I say “thanks” to her, her answer is always “I am glad to help you!” Very nice lady!

Also thanks to Ms Hanna Stina Hansson, Dr. Klas Jönsson, Mr. Thomas Eberhardt from the department of Microbiology for the help and nice talks.

I want to thank Professor Paul Declerck from the University of Leuven, Belgium, for providing the human PAI-1 cDNA and also for some nice three-dimensional pictures of PAI-1.

I also want to say thanks to my former tutor, Professor Mei-Hao Hu, in the department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University. I spent five years in her lab. For the rest of my life, I will benefit the knowledge and techniques that I learned during that time.

Dr. Zhen Chai, my alumna in Peking University, who was studying in Stockholm University when I came here. As a good friend, she guided and helped us about the life in Stockholm at the beginning. That was very helpful.

I want to give thanks to my parents, even if my father perhaps can only watch me from the heaven. They told me how to be an honest person and how to get alone well with other people. I always feel regretful since I could not stay with my mother for long time during the years when I was studying in Peking or Stockholm. Also thanks to my sisters for taking care of my mother when I was not home.

My wife, Meimei Wan, also contributes very much to my life and my work in multiple ways. Nearly half of the time during our ten years marriage did she live alone without me. I always concentrated on my work and study too much without being able to take care of her. She is very comprehensible on this aspect and always firmly supports me. I also want to thank my daughter, Xinxin. She is also one of my achievements after I came to Sweden. She brought vigorous atmosphere to my family and made my life more meaningful. With my own child, I realized the feeling and the responsibility for being a parent. I am glad to have such a happy family. It is the most important part in my life.

Our research program was conducted with the financial supports from the Swedish Medical Research Council (project no 05193), *Konung Gustaf V och Drottning Victorias Stiftelse*, Funds from Karolinska Institute and the Swedish Institute. All these financial supports are gratefully acknowledged.

REFERENCES

Aertgeerts K, De Bondt HL, De Ranter C and Declerck PJ. (1994) A model of the reactive form of plasminogen activator inhibitor-1. *J.Struct.Biol.,* 113:239-245.

Aertgeerts K, De Bondt HL, De Ranter CJ and Declerck PJ. (1995) Mechanisms contributing to the conformational and functional flexibility of plasminogen activator inhibitor-1. *Nature Structural Biology,* 2:891-897.

Aertgeerts K, De Ranter CJ, Booth NA and Declerck PJ. (1997) Rational design of complex formation between plasminogen activator inhibitor-1 and its target proteinases. *J.Struct.Biol.,* 118:236-242.

Aleshkov SB, Fa M, Karolin J, Strandberg L, Johansson LB, Wilczynska M and Ny T. (1996) Biochemical and biophysical studies of reactive center cleaved plasminogen activator inhibitor type 1. The distance between P3 and P1' determined by donor-donor fluorescence energy transfer. *J.Biol.Chem.,* 271:21231-21238.

Astrup T. (1968) Blood coagulation and fibrinolysis in tissue culture and tissue repair. In: Houck JC, Forscher BK, eds. Chemical biology of inflammation. Pergamon, New York, pp. 242-257.

Aulak KS, Eldering E, Hack CE, Lubbers YP, Harrison RA, Mast A, Cicardi M and Davis AE. (1993) A hinge region mutation in C1-inhibitor (Ala436→Thr) results in nonsubstrate-like behavior and in polymerization of the molecule. *J.Biol. Chem.,* 268:18088-18094.

Baumann U, Huber R, Bode W, Grosse D, Lesjak M and Laurell CB. (1991) Crystal structure of cleaved human α_1-antichymotrypsin at 2.7 Å resolution and its comparison with other serpins. *J.Mol.Biol.,* 218:595-606.

Berg DT, Burck PJ, Berg DH and Grinnell BW. (1993) Kringle glycosylation in a modified human tissue plasminogen activator improves functional properties. *Blood,* 81:1312-1322.

Berkenpas MB, Lawrence DA and Ginsburg D. (1995) Molecular evolution of plasminogen activator inhibitor-1 functional stability. *EMBO J.,* 14:2969-2977.

Björquist P, Ehnebom J, Inghardt T, Hansson L, Lindberg M, Linschoten M, Strömqvist M and Deinum J. (1998) Identification of the binding site for a low-molecular-weight inhibitor of plasminogen activator inhibitor type 1 by site-directed mutagenesis. *Biochemistry,* 37:1227-1234.

Bosma PJ, van den Berg EA, Kooistra T, Siemieniak DR and Slightom JL. (1988) Human plasminogen activator inhibitor-1 gene. Promoter and structural gene nucleotide sequences. *J.Biol.Chem.,* 263: 9129-9141.

Braaten JV, Handt S, Jerome WG, Kirkpatrick J, Lewis JC and Hantgan RR. (1993) Regulation of fibrinolysis by platelet-released plasminogen activator inhibitor 1: light scattering and ultrastructural examination of lysis of a model platelet-fibrin thrombus. *Blood,* 81: 1290-1299.

Bruce D, Perry DJ, Borg JY, Carrell RW and Wardell MR. (1994) Thromboembolic disease due to thermolabile conformational changes of antithrombin Rouen-VI (187 Asn→Asp). *J.Clin.Invest.,* 94:2265-2274.

Bruzdzinski CJ, Riordan-Johnson M, Nordby EC, Suter SM and Gelehrter TD. (1990) Isolation and characterization of the rat plasminogen activator inhibitor-1 gene. *J.Biol.Chem.,* 265:2078-2085.

Buell G, Schulz MF, Selzer G, Chollet A, Movva NR, Semon D, Escanez S and Kawashima E. (1985) Optimizing the expression in *E. coli* of a synthetic gene encoding somatomedin-C (IGF-I). *Nucleic Acids Res.,* 13:1923-1938.

Carrell RW, Evans DL and Stein PE. (1991) Mobile reactive centre of serpins and the control of thrombosis [published erratum appears in Nature 1993 Aug 19;364(6439): 737]. *Nature,* 353:576-578.

Carrell RW and Owen MC. (1985) Plakalbumin, α_1-antitrypsin, antithrombin and the mechanism of inflammatory thrombosis. *Nature,* 317:730-732.

Chaillan-Huntington CE, Gettins PGW, Huntington JA and Patston PA. (1997) The P6-P2 region of serpins is critical for proteinase inhibition and complex stability. *Biochemistry,* 36:9562-9570.

Chaillan-Huntington CE and Patston PA. (1998) Influence of the P5 residue on α_1-

proteinase inhibitor mechanism. *J.Biol.Chem.,* 273:4569-4573.

Chang WS, Whisstock J, Hopkins PC, Lesk AM, Carrell RW and Wardell MR. (1997) Importance of the release of strand 1C to the polymerization mechanism of inhibitory serpins. *Protein Science,* 6:89-98.

Chmielewska J, Rånby M and Wiman B. (1983) Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. *Thromb.Res.,* 31:427-436.

Chmielewska J, Rånby M and Wiman B. (1988) Kinetics of the inhibition of plasminogen activators by the plasminogen-activator inhibitor. Evidence for 'second-site' interactions. *Biochem.J.,* 251:327-332.

Clark JM. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic.Acids.Res.,* 16:9677-9686.

Cohen P, Badouaille G, Gimenez-Roqueplo AP, Mani JC, Guyene TT, Jeunemaitre X, Menard J, Corvol P, Pau B and Simon D. (1996) Selective recognition of M235T angiotensinogen variants and their determination in human plasma by monoclonal antibody-based immuno-analysis. *J.Clin.Endocrinol.Metab.,* 81: 3505-3512.

Collen D. (1976) Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. *Eur.J.Biochem.,* 69:209-216.

Colucci M, Paramo JA and Collen D. (1985) Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. *J.Clin.Invest.,* 75:818-824.

Cortellaro M, Cofrancesco E, Boschetti C, Mussoni L, Donati MB, Cardillo M, Catalano M, Gabrielli L, Lombardi B, Specchia G and *et al.* (1993) Increased fibrin turnover and high PAI-1 activity as predictors of ischemic events in atherosclerotic patients. A case-control study. The PLAT Group. *Arterioscler. Thromb.,* 13:1412-1417.

Dawson S, Hamsten A, Wiman B, Henney A and Humphries S. (1991) Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arterioscler.Thromb.,* 11:183-190.

Dawson SJ, Wiman B, Hamsten A, Green F, Humphries SX and Henney AM. (1993) The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. *J.Biol.Chem.,* 268:10739-10745.

Debrock S and Declerck PJ. (1997a) Neutralization of plasminogen activator inhibitor-1 inhibitory properties: identification of two different mechanisms. *Biochim.Biophys.Acta,* 1337:257-266.

Debrock S, Sironi L and Declerck PJ. (1997b) Cloning of a single-chain variable fragment (scFv) switching active plasminogen activator inhibitor-1 to substrate. *Gene,* 189:83-88.

Declerck PJ, De Mol M, Alessi MC, Baudner S, Paques EP, Preissner KT, Müller-Berghaus G and Collen D. (1988) Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). *J.Biol.Chem.,* 263:15454-15461.

Declerck PJ, De Mol M, Vaughan DE and Collen D. (1992) Identification of a conformationally distinct form of plasminogen activator inhibitor-1, acting as a noninhibitory substrate for tissue-type plasminogen activator. *J.Biol.Chem.,* 267: 11693-11696.

Deng G, Royle G, Seiffert D and Loskutoff DJ. (1995) The PAI-1/vitronectin interaction: two cats in a bag? [Review]. *Thromb.Haemost.,* 74:66-70.

Deng G, Royle G, Wang S, Crain K and Loskutoff DJ. (1996) 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.

Dieval J, Nguyen G, Gross S, Delobel J and Kruithof EK. (1991) A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. *Blood,* 77:528-532.

Djie MZ, Stone SR and Le Bonniec BF. (1997) Intrinsic specificity of the reactive

site loop of α_1-antitrypsin, α_1-antichymotrypsin, antithrombin III, and protease nexin I. *J.Biol.Chem.,* 272:16268-16273.

Egelund R, Rodenburg KW, Andreasen PA, Rasmussen MS, Guldberg RE and Petersen TE. (1998) An ester bond linking a fragment of a serine proteinase to its serpin inhibitor. *Biochemistry,* 37:6375-6379.

Egelund R, Schousboe SL, Sottrup-Jensen L, Rodenburg KW and Andreasen PA. (1997) Type-1 plasminogen-activator inhibitor -- conformational differences between latent, active, reactive-centre-cleaved and plasminogen-activator-complexed forms, as probed by proteolytic susceptibility. *Eur.J.Biochem.,* 248:775-785.

Ehrlich HJ, Gebbink RK, Keijer J, Linders M, Preissner KT and Pannekoek H. (1990) Alteration of serpin specificity by a protein cofactor. Vitronectin endows plasminogen activator inhibitor 1 with thrombin inhibitory properties. *J.Biol.Chem.,* 265:13029-13035.

Ehrlich HJ, Gebbink RK, Keijer J and Pannekoek H. (1992) Elucidation of structural requirements on plasminogen activator inhibitor 1 for binding to heparin. *J.Biol.Chem.,* 267:11606-11611.

Ehrlich HJ, Keijer J, Preissner KT, Gebbink RK and Pannekoek H. (1991) Functional interaction of plasminogen activator inhibitor type 1 (PAI-1) and heparin. *Biochemistry,* 30:1021-1028.

Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D and Simon RH. (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J.Clin.Invest.,* 97:232-237.

Eldering E, Verpy E, Roem D, Meo T and Tosi M. (1995) COOH-terminal substitutions in the serpin C1 inhibitor that cause loop overinsertion and subsequent multimerization. *J.Biol.Chem.,* 270:2579-2587.

Eriksson BI, Eriksson E, Gyzander E, Teger-Nilsson AC and Risberg B. (1989) Thrombosis after hip replacement. Relationship to the fibrinolytic system. *Acta Orthop.Scand.,* 60:159-163.

Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P and Hamsten A. (1995) Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc.Natl.Acad.Sci. USA,* 92:1851-1855.

Fa M, Karolin J, Aleshkov S, Strandberg L, Johansson LB and Ny T. (1995) Time-resolved polarized fluorescence spectroscopy studies of plasminogen activator inhibitor type 1: conformational changes of the reactive center upon interactions with target proteases, vitronectin and heparin. *Biochemistry,* 34:13833-13840.

Faber JP, Poller W, Olek K, Baumann U, Carlson J, Lindmark B and Eriksson S. (1993) The molecular basis of α_1-antichymotrypsin deficiency in a heterozygote with liver and lung disease. *J.Hepatol.,* 18:313-321.

Fay WP, Parker AC, Condrey LR and Shapiro AD. (1997) Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood,* 90:204-208.

Fay WP, Shapiro AD, Shih JL, Schleef RR and Ginsburg D. (1992) Brief report: complete deficiency of plasminogen-activator inhibitor type 1 due to a frame-shift mutation. *N.Engl.J.Med.,* 327:1729-1733.

Gebbink RK, Reynolds CH, Tollefsen DM, Mertens K and Pannekoek H. (1993) Specific glycosaminoglycans support the inhibition of thrombin by plasminogen activator inhibitor 1. *Biochemistry,* 32:1675-1680.

Gechtman Z, Belleli A, Lechpammer S and 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.

Gechtman Z, Sharma R, Kreizman T, Fridkin M and Shaltiel S. (1993) Synthetic peptides derived from the sequence around the plasmin cleavage site in vitronectin. Use

in mapping the PAI-1 binding site. *FEBS Lett.,* 315:293-297.

Gelehrter TD, Barouski-Miller PA, Coleman PL and Cwikel BJ. (1983) Hormonal regulation of plasminogen activator in rat hepatoma cells. *Mol.Cell Biochem.,* 53-54:11-21.

Gettins P, Patston PA and Schapira M. (1992) Structure and mechanism of action of serpins. [Review] [60 refs]. *Hematol.Oncol. Clin.North Am.,* 6:1393-1408.

Gettins P, Patston PA and Schapira M. (1993) The role of conformational change in serpin structure and function. [Review] [55 refs]. *Bioessays,* 15:461-467.

Gibson A, Baburaj K, Day DE, Verhamme I, Shore JD and Peterson CB. (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.

Gils A and Declerck PJ. (1997) Proteinase specificity and functional diversity in point mutants of plasminogen activator inhibitor 1. *J.Biol.Chem.,* 272: 12662-12666.

Gils A and Declerck PJ. (1998) Modulation of plasminogen activator inhibitor 1 by Triton X-100 -- identification of two consecutive conformational transitions [In Process Citation]. *Thromb. Haemost.,* 80:286-291.

Gils A, Lu J, Aertgeerts K, Knockaert I and Declerck PJ. (1997) Identification of positively charged residues contributing to the stability of plasminogen activator inhibitor 1. *FEBS Lett.,* 415:192-195.

Gils A, Vleugels N, Tobback K, Knockaert I and Declerck PJ. (1998) Characterization of plasminogen activator inhibitor 1 mutants containing the P13 to P10 region of ovalbumin or antithrombin III: evidence that the P13 residue contributes significantly to the active to substrate transition. *Biochim.Biophys.Acta,* 1387:291-297.

Gurewich V. (1989) Importance of fibrin specificity in therapeutic thrombolysis and the rationale of using sequential and synergistic combinations of tissue plasminogen activator and pro-urokinase. [Review] [40 refs]. *Semin.Thromb.Hemost.,* 15:123-128.

Gurr SJ and McPherson MJ. (1991) PCR-directed cDNA libraries. In: McPherson MJ, Quirke P, Taylor GR, eds. PCR, a practical approach. Oxford: Oxford University Press, pp. 147-170.

Hamsten A, de Faire U, Walldius G, Dahlen G, Szamosi AX, Landou C, Blombäck M and Wiman B. (1987a) Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. *Lancet,* 2:3-9.

Hamsten A and Wiman B. (1987b) Fibrinolysis and atherosclerotic cardio-vascular disease. In: Francis RB, Jr., ed. Atherosclerotic, cardiovascular disease, hemostasis and endothelial function. New York: Marcel Dekker Inc., pp. 107-129.

Hekman CM and Loskutoff DJ. (1985) Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J.Biol.Chem.,* 260:11581-11587.

Held C, Hjemdahl P, Rehnqvist N, Wallen NH, Björkander I, Eriksson SV, Forslund L and Wiman B. (1997) Fibrinolytic variables and cardiovascular prognosis in patients with stable angina pectoris treated with verapamil or metoprolol. Results from the Angina Prognosis study in Stockholm. *Circulation,* 95:2380-2386.

Henry M, Chomiki N, Scarabin PY, Alessi MC, Peiretti F, Arveiler D, Ferrieres J, Evans A, Amouyel P, Poirier O, Cambien F and Juhan-Vague I. (1997) Five frequent polymorphisms of the PAI-1 gene: lack of association between genotypes, PAI activity, and triglyceride levels in a healthy population. *Arterioscler.Thromb.Vasc.Biol.,* 17:851-858.

Hood DB, Huntington JA and Gettins PG. (1994) α_1-Proteinase inhibitor variant T345R. Influence of P14 residue on substrate and inhibitory pathways. *Biochemistry,* 33:8538-8547.

Hopkins PC, Chang WS, Wardell MR and Stone SR. (1997) Inhibitory mechanism of serpins. Mobility of the C-terminal region of the reactive-site loop. *J.Biol.Chem.,* 272:3905-3909.

Hosokawa N, Takechi H, Yokota S, Hirayoshi K and Nagata K. (1993) Structure of the gene encoding the mouse 47-kDa heat-shock protein (HSP47). *Gene,* 126:187-193.

Hubbard SJ, Eisenmenger F and Thornton JM. (1994) Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Sci.,* 3:757-768.

Huber R and Carrell RW. (1989) Implications of the three-dimensional structure of α_1-antitrypsin for structure and function of serpins. [Review]. *Biochemistry,* 28:8951-8966.

Huntington JA, Fan B, Karlsson KE, Deinum J, Lawrence DA and Gettins PG. (1997) Serpin conformational change in ovalbumin. Enhanced reactive center loop insertion through hinge region mutations. *Biochemistry,* 36:5432-5440.

Huntington JA, Patston PA and Gettins PG. (1995) S-ovalbumin, an ovalbumin conformer with properties analogous to those of loop-inserted serpins. *Protein Science,* 4:613-621.

Iwamoto M and Abiko Y. (1970) Plasminogen-plasmin system. VI. Preparation of α_2-macroglobulin anti-plasmin from human plasma. *Biochim. Biophys.Acta,* 214:402-410.

Janssen OE, Chen B, Buttner C, Refetoff S and Scriba PC. (1995) Molecular and structural characterization of the heat-resistant thyroxine-binding globulin-Chicago. *J.Biol.Chem.,* 270:28234-28238.

Johnson AM, Alper CA, Rosen FS and Craig JM. (1971) C1 inhibitor: evidence for decreased hepatic synthesis in hereditary angioneurotic edema. *Science,* 173:553-554.

Kaneko M, Sakata Y, Matsuda M and Mimuro J. (1992) Interactions between the finger and kringle-2 domains of tissue- type plasminogen activator and plasminogen activator inhibitor-1. *J.Biochem.,* 111:244-248.

Katagiri K, Okada K, Hattori H and Yano M. (1988) Bovine endothelial cell plasminogen activator inhibitor. Purification and heat activation. *Eur.J.Biochem.,* 176:81-87.

Keijer J, Ehrlich HJ, Linders M, Preissner KT and Pannekoek H. (1991) Vitronectin governs the interaction between plasminogen activator inhibitor 1 and tissue-type plasminogen activator. *J.Biol.Chem.,* 266:10700-10707.

Keijer J, Linders M, Ehrlich H, Klein Gebbink R and Pannekoek H. (1990) Stability of plasminogen activator inhibitor type 1 (PAI-1) activity by arginine: possible implications for the interaction of PAI-1 with vitronectin. *Fibrinolysis,* 4:153-159.

Keijer J, Linders M, Wegman JJ, Ehrlich HJ, Mertens K and Pannekoek H. (1991) On the target specificity of plasminogen activator inhibitor 1: the role of heparin, vitronectin, and the reactive site. *Blood,* 78:1254-1261.

Klinger KW, Winqvist R, Riccio A, Andreasen PA, Sartorio R, Nielsen LS, Stuart N, Stanislovitis P, Watkins P, Douglas R and *et al*. (1987) Plasminogen activator inhibitor type 1 gene is located at region q21.3-q22 of chromosome 7 and genetically linked with cystic fibrosis. *Proc.Natl.Acad.Sci.USA,* 84:8548-8552.

Kost C, Stuber W, Ehrlich HJ, Pannekoek H and Preissner KT. (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: 12098-12105.

Kotyza J, Varghese AH, Korza G and Sunderman FWJ. (1998) Interactions of serine proteinases with pNiXa, a serpin of Xenopus oocytes and embryos. *Biochim. Biophys.Acta,* 1382:266-276.

Kozaki K, Miyaishi O, Koiwai O, Yasui Y, Kashiwai A, Nishikawa Y, Shimizu S and Saga S. (1998) Isolation, purification, and characterization of a collagen-associated serpin, caspin, produced by murine colon adenocarcinoma cells. *J.Biol.Chem.,* 273: 15125-15130.

Kruithof EK. (1988) Plasminogen activator inhibitors--a review. [Review] [86 refs]. *Enzyme,* 40:113-121.

Kruithof EK, Tran-Thang C, Ransijn A and Bachmann F. (1984) Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. *Blood,* 64:907-913.

Kvassman JO, Lawrence DA and Shore JD. (1995a) The acid stabilization of plasminogen activator inhibitor-1 depends on protonation of a single group that affects loop insertion into β-sheet A. *J.Biol.Chem.,* 270:27942-27947.

Kvassman JO and Shore JD. (1995b) Purification of human plasminogen activator inhibitor (PAI-1) from *Escherichia coli* and separation of its active and latent forms by hydrophobic interaction chromatography. *Fibrinolysis,* 9:215-221.

Lambers JW, Cammenga M, Konig BW, Mertens K, Pannekoek H and van Mourik JA. (1987) Activation of human endothelial cell-type plasminogen activator inhibitor (PAI-1) by negatively charged phospho-lipids. *J.Biol.Chem.,* 262:17492-17496.

Lawrence DA. (1997) The serpin-proteinase complex revealed [news]. *Nature Structural Biology,* 4:339-341.

Lawrence DA, Berkenpas MB, Palaniappan S and Ginsburg D. (1994) Localization of vitronectin binding domain in plasminogen activator inhibitor-1. *J.Biol. Chem.,* 269:15223-15228.

Lawrence DA, Ginsburg D, Day DE, Berkenpas MB, Verhamme IM, Kvassman JO and Shore JD. (1995) Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. *J.Biol.Chem.,* 270: 25309-25312.

Lawrence DA, Olson ST, Palaniappan S and Ginsburg D. (1994) Serpin reactive center loop mobility is required for inhibitor function but not for enzyme recognition. *J.Biol.Chem.,* 269:27657-27662.

Lawrence DA, Palaniappan S, Stefansson S, Olson ST, Francis-Chmura AM, Shore JD and Ginsburg D. (1997) Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J.Biol. Chem.,* 272:7676-7680.

Lawrence DA, Strandberg L, Ericson J and Ny T. (1990) Structure-function studies of the SERPIN plasminogen activator inhibitor type 1. Analysis of chimeric strained loop mutants. *J.Biol.Chem.,* 265: 20293-20301.

Lee MH, Vosburgh E, Anderson K and McDonagh J. (1993) Deficiency of plasma plasminogen activator inhibitor 1 results in hyperfibrinolytic bleeding. *Blood,* 81:2357-2362.

Levin EG and Santell L. (1987) Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. *Blood,* 70:1090-1098.

Lindahl T and Wiman B. (1989) Purification of high and low molecular weight plasminogen activator inhibitor 1 from fibrosarcoma cell-line HT 1080 conditioned medium. *Biochim.Biophys.Acta,* 994:253-257.

Lindahl TL, Ohlsson PI and Wiman B. (1990) The mechanism of the reaction between human plasminogen-activator inhibitor 1 and tissue plasminogen activator. *Biochem.J.,* 265:109-113.

Lindahl TL, Sigurdardottir O and Wiman B. (1989) Stability of plasminogen activator inhibitor 1 (PAI-1). *Thromb.Haemost.,* 62: 748-751.

Loebermann H, Tokuoka R, Deisenhofer J and Huber R. (1984) Human α_1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J.Mol.Biol.,* 177:531-557.

Lomas DA, Elliott PR, Chang WS, Wardell MR and Carrell RW. (1995) Preparation and characterization of latent α_1-antitrypsin. *J.Biol.Chem.,* 270:5282-5288.

Lomas DA, Evans DL, Finch JT and Carrell RW. (1992) The mechanism of Z α_1-antitrypsin accumulation in the liver [see comments]. *Nature,* 357:605-607.

Lomas DA, Finch JT, Seyama K, Nukiwa T and Carrell RW. (1993) α_1-Antitrypsin Siiyama (Ser53→Phe). Further evidence for intracellular loop-sheet polymerization. *J. Biol.Chem.,* 268:15333-15335.

Loskutoff DJ, Linders M, Keijer J, Veerman H, van Heerikhuizen H and Pannekoek H. (1987) Structure of the human plasminogen activator inhibitor 1 gene: nonrandom distribution of introns. *Biochemistry,* 26:3763-3768.

Loskutoff DJ, van MJ, Erickson LA and Lawrence D. (1983) Detection of an unusually stable fibrinolytic inhibitor roduced by bovine endothelial cells. *Proc. Natl.Acad.Sci.U.S.A.,* 80:2956-2960.

Macen JL, Upton C, Nation N and McFadden G. (1993) SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology,* 195: 348-363.

Madison EL, Goldsmith EJ, Gerard RD, Gething MJ and Sambrook JF. (1989) Serpin-resistant mutants of human tissue-type plasminogen activator. *Nature,* 339: 721-724.

Madison EL, Goldsmith EJ, Gerard RD, Gething MJ, Sambrook JF and Bassel-Duby RS. (1990a) Amino acid residues that affect interaction of tissue-type plasminogen activator with plasminogen activator inhibitor 1. *Proc.Natl.Acad.Sci.USA,* 87: 3530-3533.

Madison EL, Goldsmith EJ, Gething MJ, Sambrook JF and Gerard RD. (1990b) Restoration of serine protease-inhibitor interaction by protein engineering. *J.Biol. Chem.,* 265:21423-21426.

Matheson NR, van Halbeek H and Travis J. (1991) Evidence for a tetrahedral intermediate complex during serpin-proteinase interactions. *J.Biol.Chem.,* 266: 13489-13491.

McCarthy BJ and Worrall DM. (1997) Analysis of serpin inhibitory function by mutagenesis of ovalbumin and generation of chimeric ovalbumin/PAI-2 fusion proteins. *J.Mol.Biol.,* 267:561-569.

Mimuro J and Loskutoff DJ. (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 and Sakata Y. (1993) Identification of the plasminogen activator inhibitor-1 binding heptapeptide in vitronectin. *Biochemistry,* 32:2314-2320.

Mimuro J, Schleef RR and Loskutoff DJ. (1987) Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type 1 plasminogen activator inhibitor. *Blood,* 70:721-728.

Moroi M and Aoki N. (1976) Isolation and characterization of α_2-plasmin inhibitor from human plasma. A novel proteinase inhibitor which inhibits activator-induced clot lysis. *J.Biol.Chem.,* 251:5956-5965.

Morrison DC and Ulevitch RJ. (1978) The effects of bacterial endotoxins on host mediation systems. A review. *Am.J.Pathol.,* 93:526-617.

Mottonen J, Strand A, Symersky J, Sweet RM, Danley DE, Geoghegan KF, Gerard RD and Goldsmith EJ. (1992) Structural basis of latency in plasminogen activator inhibitor-1. *Nature,* 355:270-273.

Mourey L, Samama JP, Delarue M, Petitou M, Choay J and Moras D. (1993) Crystal structure of cleaved bovine antithrombin III at 3.2 Å resolution. *J.Mol. Biol.,* 232:223-241.

Mullertz S and Clemmensen I. (1976) The primary inhibitor of plasmin in human plasma. *Biochem.J.,* 159:545-553.

Munch M, Heegaard CW and Andreasen PA. (1993) Interconversions between active, inert and substrate forms of denatured/ refolded type-1 plasminogen activator inhibitor. *Biochim.Biophys.Acta,* 1202:29-37.

Naski MC, Lawrence DA, Mosher DF, Podor TJ and Ginsburg D. (1993) Kinetics of inactivation of α-thrombin by plasminogen activator inhibitor-1. Comparison of the effects of native and urea-treated forms of vitronectin. *J.Biol. Chem.,* 268:12367-12372.

Nava VE, Rosen A, Veliuona MA, Clem RJ, Levine B and Hardwick JM. (1998) Sindbis virus induces apoptosis through a caspase-dependent, CrmA-sensitive pathway. *J.Virol.,* 72:452-459.

Nielsen LS, Hansen JG, Skriver L, Wilson EL, Kaltoft K, Zeuthen J and Dano K. (1982) Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. *Biochemistry,* 21:6410-6415.

Nilsson T and Wiman B. (1982) On the structure of the stable complex between plasmin and α_2-antiplasmin. *FEBS Lett.,* 142:111-114.

O'Malley KM, Nair SA, Rubin H and Cooperman BS. (1997) The kinetic mechanism of serpin-proteinase complex formation. An intermediate between the

michaelis complex and the inhibited complex. *J.Biol.Chem.,* 272:5354-5359.

Olson ST, Bock PE, Kvassman J, Shore JD, Lawrence DA, Ginsburg D and Björk I. (1995) Role of the catalytic serine in the interactions of serine proteinases with protein inhibitors of the serpin family. Contribution of a covalent interaction to the binding energy of serpin-proteinase complexes. *J.Biol.Chem.,* 270:30007-30017.

Padmanabhan J and Sane DC. (1995) Localization of a vitronectin binding region of plasminogen activator inhibitor-1. *Thromb.Haemost.,* 73:829-834.

Pappot H, Gardsvoll H, Romer J, Pedersen AN, Grondahl-Hansen J, Pyke C and Brunner N. (1995) Plasminogen activator inhibitor type 1 in cancer: therapeutic and prognostic implications. [Review]. *Biol.Chem.H-S.,* 376:259-267.

Paramo JA, Alfaro MJ and Rocha E. (1985) Postoperative changes in the plasmatic levels of tissue-type plasminogen activator and its fast-acting inhibitor--relationship to deep vein thrombosis and influence of prophylaxis. *Thromb.Haemost.,* 54:713-716.

Patston PA, Gettins PG and Schapira M. (1994a) Serpins are suicide substrates: implications for the regulation of proteolytic pathways. [Review] [82 refs]. *Semin. Thromb.Hemost.,* 20:410-416.

Patston PA and Schapira M. (1994b) Low-affinity heparin stimulates the inactivation of plasminogen activator inhibitor-1 by thrombin. *Blood,* 84:1164-1172.

Pemberton PA, Stein PE, Pepys MB, Potter JM and Carrell RW. (1988) Hormone binding globulins undergo serpin conformational change in inflammation. *Nature,* 336:257-258.

Philips M, Juul AG and Thorsen S. (1984) Human endothelial cells produce a plasminogen activator inhibitor and a tissue-type plasminogen activator-inhibitor complex. *Biochim.Biophys.Acta,* 802:99-110.

Plotnick MI, Mayne L, Schechter NM and Rubin H. (1996) Distortion of the active site of chymotrypsin complexed with a serpin. *Biochemistry,* 35:7586-7590.

Potempa J, Korzus E and Travis J. (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. [Review] [74 refs]. *J.Biol.Chem.,* 269:15957-15960.

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

Rånby M, Norrman B and Wallen P. (1982) A sensitive assay for tissue plasminogen activator. *Thromb.Res.,* 27: 743-749.

Reich E. (1975) Plasminogen activation by neoplastic cell and macrophages. In: Reith E, Rifkin DB, Shaw E, eds. Proteases and biological control. New York: Cold Spring Harbor Symposium, pp. 491-500.

Reilly TM, Forsythe MS, Dwivedi AM, Walton HL, Duke JL, Wade DL, Knabb RM and Mousa SA. (1992) Vitronectin effects on recombinant plasminogen activator inhibitor-1: structural and functional analysis. *Fibrinolysis,* 6:167-171.

Rezaie AR. (1996) Role of residue 99 at the S2 subsite of factor Xa and activated protein C in enzyme specificity. *J.Biol. Chem.,* 271:23807-23814.

Rezaie AR. (1997) Role of Leu99 of thrombin in determining the P2 specificity of serpins. *Biochemistry,* 36:7437-7446.

Riccio A, Lund LR, Sartorio R, Lania A, Andreasen PA, Dano K and Blasi F. (1988) The regulatory region of the human plasminogen activator inhibitor type-1 (PAI-1) gene. *Nucleic Acids Res.,* 16:2805-2824.

Rosenberg RD and Rosenberg JS. (1984) Natural anticoagulant mechanisms. *J.Clin. Invest.,* 74:1-6.

Rosenkrands I, Hejgaard J, Rasmussen SK and Björn SE. (1994) Serpins from wheat grain. *FEBS Lett.,* 343:75-80.

Sager R, Sheng S, Pemberton P and Hendrix MJ. (1996) Maspin: a tumor suppressing serpin. [Review] [14 refs]. *Curr.Topics Microbio.Immunol.,* 213:51-64.

Sancho E, Declerck PJ, Price NC, Kelly SM and Booth NA. (1995) Conformational studies on plasminogen activator inhibitor

(PAI-1) in active, latent, substrate, and cleaved forms. *Biochemistry,* 34:1064-1069.

Schulman S and Wiman B. (1996) The significance of hypofibrinolysis for the risk of recurrence of venous thromboembolism. Duration of Anticoagulation (DURAC) Trial Study Group. *Thromb.Haemost.,* 75:607-611.

Seiffert D, Ciambrone G, Wagner NV, Binder BR and Loskutoff DJ. (1994) The somatomedin B domain of vitronectin. Structural requirements for the binding and stabilization of active type 1 plasminogen activator inhibitor. *J.Biol.Chem.,* 269:2659-2666.

Seiffert D and Loskutoff DJ. (1991) Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. *J.Biol.Chem.,* 266: 2824-2830.

Seiffert D, Wagner NN and Loskutoff DJ. (1990) Serum-derived vitronectin influences the pericellular distribution of type 1 plasminogen activator inhibitor. *J.Cell Biol.,* 111:1283-1291.

Sherman PM, Lawrence DA, Yang AY, Vandenberg ET, Paielli D, Olson ST, Shore JD and Ginsburg D. (1992) Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. *J.Biol.Chem.,* 267:7588-7595.

Shore JD, Day DE, Francis-Chmura AM, Verhamme I, Kvassman J, Lawrence DA and Ginsburg D. (1995) A fluorescent probe study of plasminogen activator inhibitor-1. Evidence for reactive center loop insertion and its role in the inhibitory mechanism. *J.Biol.Chem.,* 270:5395-5398.

Shubeita HE, Cottey TL, Franke AE and Gerard RD. (1990) Mutational and immunochemical analysis of plasminogen activator inhibitor 1. *J.Biol.Chem.,* 265:18379-18385.

Sigurdardottir O and Wiman B. (1990) Complex formation between plasminogen activator inhibitor 1 and vitronectin in purified systems and in plasma. *Biochim. Biophys.Acta,* 1035:56-61.

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

Stankiewicz AJ, Crowley JP and Steiner M. (1991) Increased levels of tissue plasminogen activator with a low plasminogen activator inhibitor-1 in a patient with postoperative bleeding. [Review]. *Am.J.Hematol.,* 38:226-229.

Stefansson S, Muhammad S, Cheng XF, Battey FD, Strickland DK and Lawrence DA. (1998) Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J.Biol.Chem.,* 273:6358-6366.

Stein PE, Leslie AG, Finch JT and Carrell RW. (1991) Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J.Mol.Biol.,* 221:941-959.

Stein PE, Leslie AG, Finch JT, Turnell WG, McLaughlin PJ and Carrell RW. (1990) Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature,* 347:99-102.

Stief TW, Aab A and Heimburger N. (1988) Oxidative inactivation of purified human α_2-antiplasmin, antithrombin III, and C1-inhibitor. *Thromb.Res.,* 49:581-589.

Stone SR and Le Bonniec BF. (1997) Inhibitory mechanism of serpins. Identification of steps involving the active-site serine residue of the protease. *J.Mol.Biol.,* 265:344-362.

Storck J, Abdelrazek H and Zimmermann ER. (1996) Effect of polyvinyl chloride plastic on the growth and physiology of human umbilical vein endothelial cells. *Biomaterials,* 17:1791-1794.

Strandberg L, Lawrence D and Ny T. (1988) The organization of the human-plasminogen-activator-inhibitor-1 gene. Implications on the evolution of the serine-protease inhibitor family. *Eur.J.Biochem.,* 176:609-616.

Stratikos E and Gettins PG. (1997) Major proteinase movement upon stable serpin-proteinase complex formation. *Proc.Natl. Acad.Sci.USA,* 94:453-458.

Stratikos E and Gettins PG. (1998) Mapping the serpin-proteinase complex using single cysteine variants of α_1-proteinase inhibitor Pittsburgh. *J.Biol. Chem.,* 273:15582-15589.

Strickland S and Beers WH. (1976) Studies on the role of plasminogen activator in ovulation. *In vitro* response of granulosa cells to gonadotropins, cyclic nucleotides,

and prostaglandins. *J.Biol.Chem.*, 251:5694-5702.

Summaria L, Hsieh B and Robbins KC. (1967) The specific mechanism of activation of human plasminogen to plasmin. *J.Biol. Chem.*, 242:4279-4283.

Tachias K and Madison EL. (1997) Variants of tissue-type plasminogen activator that display extraordinary resistance to inhibition by the serpin plasminogen activator inhibitor type 1. *J. Biol.Chem.*, 272:14580-14585.

Takahashi Y, Tanaka T, Minowa H, Ookubo Y, Sugimoto M, Nakajima MX, Miyauchi Y and Yoshioka A. (1996) Hereditary partial deficiency of plasminogen activator inhibitor-1 associated with a lifelong bleeding tendency. *Int.J.Hematol.*, 64:61-68.

Travis J, Garner D and Bowen J. (1978) Human α_1-antichymotrypsin: purification and properties. *Biochemistry*, 17:5647-5651.

Urano T, Serizawa K, Takada Y, Ny T and Takada A. (1994) Heparin and heparan sulfate enhancement of the inhibitory activity of plasminogen activator inhibitor type 1 toward urokinase type plasminogen activator. *Biochim.Biophys.Acta*, 1201:217-222.

Urano T, Strandberg L, Johansson LB and Ny T. (1992) A substrate-like form of plasminogen-activator-inhibitor-type 1. Conversions between different forms by sodium dodecyl sulphate. *Eur.J.Biochem.*, 209:985-992.

Vague P, Juhan-Vague I, Aillaud MF, Badier C, Viard R, Alessi MC and Collen D. (1986) Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects. *Metabolism*, 35:250-253.

van Meijer M, Gebbink RK, Preissner KT and Pannekoek H. (1994) Determination of the vitronectin binding site on plasminogen activator inhibitor 1 (PAI-1). *FEBS Lett.*, 352:342-346.

van Meijer M, Smilde A, Tans G, Nesheim ME, Pannekoek H and Horrevoets AJ. (1997a) The suicide substrate reaction between plasminogen activator inhibitor 1 and thrombin is regulated by the cofactors vitronectin and heparin. *Blood*, 90:1874-1882.

van Meijer M, Stoop A, Smilde A, Preissner KT, van Zonneveld AJ and Pannekoek H. (1997b) The composition of complexes between plasminogen activator inhibitor 1, vitronectin and either thrombin or tissue-type plasminogen activator. *Thromb.Haemost.*, 77:516-521.

Vaughan DE, Declerck PJ, Van Houtte E, De Mol M and Collen D. (1992) Reactivated recombinant plasminogen activator inhibitor-1 (rPAI-1) effectively prevents thrombolysis *in vivo*. *Thromb.Haemost.*, 68: 60-63.

Wagner OF, de Vries C, Hohmann C, Veerman H and Pannekoek H. (1989) Interaction between plasminogen activator inhibitor type 1 (PAI- 1) bound to fibrin and either tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Binding of t-PA/PAI-1 complexes to fibrin mediated by both the finger and the kringle-2 domain of t-PA. *J.Clin.Invest.*, 84:647-655.

Wallen P and Wiman B. (1972) Characterization of human plasminogen. II. Separation and partial characterization of different molecular forms of human plasminogen. *Biochim.Biophys.Acta*, 257: 122-134.

Wardell MR, Abrahams JP, Bruce D, Skinner R and Leslie AG. (1993) Crystallization and preliminary X-ray diffraction analysis of two conformations of intact human antithrombin. *J.Mol.Biol.*, 234: 1253-1258.

Wardell MR, Chang WSW, Bruce D, Skinner R, Lesk AM and Carrell RW. (1997) Preparative induction and characterization of L-antithrombin: A structural homologue of latent plasminogen activator inhibitor-1. *Biochemistry*, 36: 13133-13142.

Wilczynska M, Fa M, Karolin J, Ohlsson PI, Johansson LB and Ny T. (1997) Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins [letter]. *Nature Structural Biology*, 4:354-357.

Wilczynska M, Fa M, Ohlsson PI and Ny T. (1995) The inhibition mechanism of serpins. Evidence that the mobile reactive

enter loop is cleaved in the native protease-inhibitor complex. *J.Biol.Chem.,* 270: 29652-29655.

Wiman B, Almquist A, Sigurdardottir O and Lindahl T. (1988) Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma. *FEBS Lett.*, 242:125-128.

Wiman B, Chmielewska J and Rånby M. (1984) Inactivation of tissue plasminogen activator in plasma. Demonstration of a complex with a new rapid inhibitor. *J.Biol.Chem.,* 259:3644-3647.

Wiman B and Collen D. (1977) Purification and characterization of human antiplasmin, the fast-acting plasmin inhibitor in plasma. *Eur.J.Biochem.,* 78:19-26.

Wiman B and Collen D. (1978) On the kinetics of the reaction between human antiplasmin and plasmin. *Eur.J.Biochem.*, 84:573-578.

Wiman B and Collen D. (1979) On the mechanism of the reaction between human α_2-antiplasmin and plasmin. *J.Biol.Chem.*, 254:9291-9297.

Wiman B, Lindahl TL and Sigurdardottir O. (1990) Plasminogen activator inhibitor (PAI-1): interaction with vitronectin and plasminogen activators. In: Takada A, Samama MM, Collen D, eds. Protease Inhibitors. Amsterdam: Excerpta Medica, pp. 37-45.

Wiman B and Wallen P. (1975) Amino acid sequence around the arginyl-valyl bond in plasminogen which is cleaved during the second step of the activation process. *Thromb.Res.*, 7:239-243.

Wright HT, Qian HX and Huber R. (1990) Crystal structure of plakalbumin, a proteolytically nicked form of ovalbumin. Its relationship to the structure of cleaved α_1-proteinase inhibitor. *J.Mol.Biol.,* 213: 513-528.

Wun TC, Ossowski L and Reich E. (1982) A proenzyme form of human urokinase. *J.Biol.Chem.,* 257:7262-7268.

Wun TC, Palmier MO, Siegel NR and Smith CE. (1989) Affinity purification of active plasminogen activator inhibitor-1 (PAI-1) using immobilized anhydro-urokinase. Demonstration of the binding, stabilization, and activation of PAI-1 by vitronectin. *J.Biol.Chem.,* 264:7862-7868.

Xue Y, Björquist P, Inghardt T, Linschoten M, Musil D, Sjölin L and Deinum J. (1998) Interfering with the inhibitory mechanism of serpins: crystal structure of a complex formed between cleaved plasminogen activator inhibitor type 1 and a reactive-centre loop peptide. *Structure.,* 6:627-636.

York JD, Li P and Gardell SJ. (1991) Combinatorial mutagenesis of the reactive site region in plasminogen activator inhibitor I. *J.Biol.Chem.,* 266:8495-8500.