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ANTIPLASMIN THE MAIN PLASMIN INHIBITOR IN BLOOD PLASMA

Studies on Structure-Function Relationships

Haiyao Wang

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ABSTRACT

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Antiplasmin is an important regulator of the fibrinolytic system. It inactivates plasmin very rapidly. The reaction between plasmin and antiplasmin occurs in several steps: first a lysine-binding site in plasmin interacts with a complementary site in antiplasmin. Then, an interaction occurs between the substrate-binding pocket in the plasmin active site and the scissile peptide bond in the RCL of antiplasmin. Subsequently, peptide bond cleavage occurs and a stable acyl-enzyme complex is formed. It has been accepted that the COOH-terminal lysine residue in antiplasmin is responsible for its interaction with the plasmin lysine-binding sites. In order to identify these structures, we constructed single-site mutants of charged amino acids in the COOH-terminal portion of antiplasmin. We found that modification of the COOH-terminal residue, Lys452, did not change the activity or the kinetic properties significantly, suggesting that Lys452 is not involved in the lysine-binding site mediated interaction between plasmin and antiplasmin. On the other hand, modification of Lys436 to Glu decreased the reaction rate significantly, suggesting this residue to have a key function in this interaction. Results from computerised molecular modelling indeed supported our experimental data. The interaction between immobilized plasminogen or an elastase degradation product from plasminogen, constituting "kringles" 1-3 and different purified variants of antiplasmin was then studied by surface plasmon resonance. Again, the data demonstrated that Lys452 is not involved in the lysine-binding site mediated interaction. On the other hand, solid evidence was produced, proving that Lys436 is indeed very important for this interaction. Some evidence was found that Glu443 may also be involved in this interaction. Molecular modelling experiments suggest that the negatively charged Glu443 is within the expected range from the positively charged Lys436 to form a complementary site to a lysine-binding site. Serine protease inhibitors may under certain conditions undergo conformational changes resulting in the insertion of RCL into the Aβ-sheet during formation of "latent" molecules or polymers. Antiplasmin is stable at neutral pH, but at acidic pH or at elevated temperatures it rapidly becomes inactivated. At decreased pH, antiplasmin activity declined following first-order kinetics. Analysis by PAGE under non-denaturing conditions demonstrated that only minor amounts of polymerized material had formed. However, on incubation at elevated temperatures a rapid formation of polymerized material was observed. Antiplasmin inactivated by treatment at pH ~5 could spontaneously regain activity if incubated at neutral pH. Furthermore, by treatment of such material with guanidinium chloride followed by dialysis, considerable activity was regained, in contrast to antiplasmin that had been inactivated by polymerization. To better understand these processes, site-directed mutagenesis was employed to produce some interesting variants of antiplasmin, which were purified and characterized. Five of the 11 mutants were found to have a deviating stability at decreased pH. One mutant was less stable as compared to wt-antiplasmin, but the other 4 were more stable. His341Thr was 7-fold more stable at pH 4.9, as compared to wt-antiplasmin. The wt-antiplasmin had a much more pronounced tendency to polymerize at decreased pH, as compared to "native" antiplasmin. However, many of the mutants were rather transformed to "latent" molecules, as judged both from PAGE-analysis at non-denaturing condition and reactivation experiments.

Key words: antiplasmin, lysine-binding-site, inactivation.

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I. Wang H, Yu A, Wiman B and Pap S. (2003) Identification of amino acids in antiplasmin involved in its non-covalent "lysine-binding site" dependent interaction with plasmin, *Eur. J. Biochem*., 270: 2023-2029

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ABBREVIATIONS

AP	antiplasmin
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
RCL	reactive centre loop
serpin	serine protease inhitor
SDS	sodium dodecyl sulphate
tPA	tissue-type plasminogen activator
uPA	urokinase
6-AHA	6-aminohexanoic acid
wt-AP	wild type antiplasmin

INTRODUCTION

The fibrinolytic system is capable of dissolving blood clots and removing excessive fibrin from the blood vessels. This system is also involved in tissue repair, ovulation, macrophage function and malignant transformation. The main components of the fibrinolytic system are serine proteases, such as plasmin (Wallen and Wiman, 1970, 1972; Wiman and Wallen, 1973, 1975a 1975b, 1975c), tissue-type plasminogen activator (t-PA) (Rijken and Collen, 1981; Pennica et al, 1983; Bykowskan et al, 1981; Pannekoek et al, 1988) or urokinase-type plasminogen activator (u-PA) (Wun et al, 1982; Nielsen et al, 1982). Serine protease inhibitors (Serpins) like antiplasmin (Collen, 1976; Moroi and Aoki, 1976; Mullertz and Clemmensen, 1976) and plasminogen activator inhibitor type-1 (PAI-1) (Loskutoff et al, 1983; Chmielewska et al, 1983; Kruithof et al, 1984) are important regulators of this system. The proteolytic enzyme plasmin is formed by activation of plasminogen by t-PA or u-PA. In the blood vessels, this occurs preferably at the fibrin surface, since both plasminogen and t-PA have a high affinity for fibrin. This is one important way to keep the fibrinolytic system localized to the fibrin surface. When fibrin has become degraded into soluble degradation products, free plasmin will rapidly become inactivated by antiplasmin (Wiman and Collen, 1977). This is another regulating step, also keeping the fibrinolytic process localized. In addition the plasminogen acitivator inhibitors (in plasma mainly PAI-1) regulate the activity of the activators.

Increased fibrinolytic activity, e.g. due to antiplasmin deficiency, is associated with a bleeding tendency. A decreased fibrinolytic activity, on the other hand, is often associated with thromboembolic diseases. Plasminogen activators like t-PA and u-PA are used as thrombolytic agents in the treatment of myocardial infarction and in other thrombotic diseases.

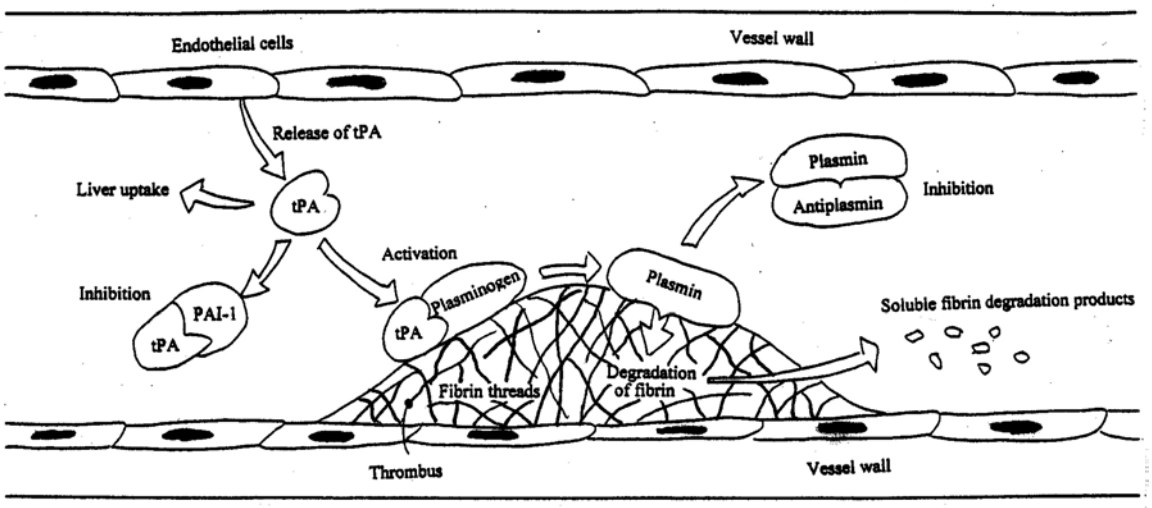

Figure 1. A schematic picture of the fibrinolytic system (adapted from Wiman, MFR informerar 1987)

THE COMPOUNDS OF THE FIBRINOLYTIC SYSTEM

Plasminogen and Plasmin

Human plasminogen (EC 3.4.21.7) is a single-chain glycoprotein of 92 kd and contains approximately 2% carbohydrate (Collen and Maeyer, 1975). The gene for human plasminogen is located on chromosome 6q26-q27; it spans 53.5 kilobases (kb) and is the largest gene among the constituents of the fibrinolytic system (Magnaghi et al, 1994). The molecule consists of 791 amino acids and contains 24 disulfide bonds. It has a concentration of about 1.5 µM in plasma (Ries, 1997). Plasminogen contains five kringles on the A-chain. Each of the five kringles consists of 78 to 80 amino acid residues. The kringles is important in creating the ability for plasminogen to bind to exposed lysyl residues in fibrin (Wiman and Wallen, 1977a; Thorsen et al, 1981; Thewes et al, 1990), in antiplasmin (Wiman et al, 1979a), in tetranectin (Clemmensen et al, 1986), in histidine-rich glycoprotein (Lijnen et al, 1980), in collagen (Kelm et al, 1994), in high-molecular-weight and low-molecular-weight kininogen (Selim et al, 1997), in extra cellular matrix (Kundsen et al, 1986), in immunoglobulin G (Harpel et al, 1989) or in cell surface receptors (Miles et al, 1991; Hajjar et al, 1986; Ullberg et al, 1989). The catalytic domain of plasminogen (B chain) is made up of 230 amino acids. The active site consists of the three amino acids Ser741, His603 and Asp646. Plasminogen can exist in two forms: glu-plasminogen and lys-plasminogen. The glu-plasminogen is the natural form, which can be cleaved at the Arg68-Met69, Lys77-Lys78 or Lys78-Val79 to form lys-plasminogen. Lys-plasminogen is more readily convertible to plasmin than glu-plasminogen (Wallen and Wiman, 1970, 1972; Wiman and Wallen, 1975a, Wiman and Collen, 1978c; Collen and Maeyer, 1975).

The conversion of plasminogen to plasmin is conducted by t-PA or u-PA, by cleaving the peptide bond Arg561-Val562 in plasminogen and thereby generates active two-chain plasmin (Ling et al, 1965).

t-PA and u-PA

One of the two-plasminogen activators in humans is t-PA, a serine protease (EC 3.4.21.68) of 68 kd. The t-PA gene is located on bands p12-p11 on chromosome 8 (Benham et al, 1984). In normal plasma, the antigen concentration of t-PA is about 10 µg/L (Rijken and Collen, 1981). Most of the t-PA in plasma is present in a complex with its primary inhibitor, PAI-1 (Chmielewska et al, 1983) or to some of the other protease inhibitors in plasma (Thorsen and Philips, 1984; Nordenhem and Wiman, 1998). t-PA is mainly expressed in the endothelial cells of the blood vessels. The single-chain t-PA is unique among serine proteases, since it is almost fully active, especially in the presence of fibrin (Wallen et al, 1982). However, it can be converted to two-chain t-PA by cleaving the peptide bond Arg275-Ile276 (Canipari and Strickland, 1985; Saksela and Rifkin, 1988), thereby increasing its activity, especially in the absence of fibrin (Wallen et al, 1983). The t-PA structure contains a finger domain, an epidermal growth factor domain, two kringle domains and a catalytic domain. The tPA-molecule can bind to the surface of fibrin, mainly via kringle 2 and this binding stimulates the activity of t-PA (Kelley et al, 1991). The catalytic domain of t-PA consists of 230 amino acids whose sequence is homologous to that of the other serine proteases. The active site contains the three amino acids: His322, Asp371 and Ser478 (Hoylaerts et al, 1982; Kaneko et al, 1992; Berg et al, 1993; Collen and Lijnen, 1991). Recombinant variant of t-PA is commonly used as thrombolytic agents, especially in connection with the acute phase of myocardial infarction.

Urokinase, or u-PA, (EC 3.4.2.73) is found in relatively high concentration in human urine. It is secreted by various cell types as a single-chain glycoprotein with a molecular mass of 55 kd (Wun et al, 1982; Nielsen et al, 1982). The human u-PA gene is 6.4 kb long

and situated on chromosome 10q24 (Triputti et al, 1985). Like t-PA, it belongs to the serine protease family. Three different domains can be distinguished on the basis of the amino acid sequence. The epidermal growth factor domain interacts with the u-PA receptor. The single kringle domain has no affinity for fibrin. The protease domain contains the His204, Asp255 and Ser356, which form the catalytic triad of the active centre. The principal cleavage site that converts the inactive single chain molecule to the active two-chain form is Lys158-Ile159. As a clinical thrombolytic agent, single-chain u-PA can be used together with t-PA to get thrombolysis (Ichinose et al, 1986; Vassalli et al, 1991).

In tissues, u-PA plays an important role in the degradation of extra cellular matrix that enables cells to migrate. It also plays important role in wound healing, inflammation, embryogenesis, and invasion of tumour cells and metastasization of tumours (Dano et al, 1985; Gyetko et al, 1994; Ossowski, 1988, Ossowski et al, 1991). Thus, it activates plasminogen to plasmin, which in turn may activate matrix metalloproteases. These then degrades the extracellular matrix.

Antiplasmin

Antiplasmin, also called plasmin inhibitor, is the main inhibitor of plamsin in human plasma. It is a single chain 70 kd glycoprotein and its concentration in plasma is about 70 mg/L (Moroi and Aoki, 1976; Collen, 1976). It is synthesized in the liver and reduced in patients with liver disease. Antiplasmin is an important regulator in the fibrinolytic system. It inhibits plamsin rapidly, interferes with the adsorption plasmin(ogen) to fibrin (Collen and Wiman, 1979; Lijnen et al, 1987). It belongs to serpin super family of proteins. Deficiency of antiplasmin leads to a severe bleeding disorder (Sakata and Aoki, 1982). For a more detailed description of antiplasmin structure and function, see below (Serpin chapter).

Plasminogen activator inhibitor-1 (PAI-1)

There are several plasminogen activator inhibitors in plasma, PAI-1, PAI-2 (placental plasminogen activator inhibitor), PAI-3 (protein C inhibitor) and protease nexin (Kruithof, 1988). PAI-1, a 52-kd glycoprotein, is the most important inhibitor of the plasminogen activators (Loskutoff et al, 1983; Chmielewska et al, 1983; Kruithof et al, 1984). A few individuals with total PAI-1 deficiency have been described. They seem to suffer from a mild bleeding tendency (Stankiewicz et al, 1991). An increased plasma level of PAI-1, on the other hand, is connected to a thrombotic tendency, both venous thromboembolism (Paramo et al, 1985; Eriksson et al, 1989; Schulman and Wiman, 1996) and myocardial infarction (Hamsten et al, 1987; Held et al, 1997; Cortellaro et al, 1993). For a more detailed description of PAI-1 structure and function see below (Serpin chapter).

Plasminogen activator inhibitor-2 (PAI-2)

PAI-2 was discovered in human placenta (kawano et al, 1968), later on it was found in macrophages and several other different cell lines. In normal plasma, it is typically not found. However, during pregnancy high levels of PAI-2 are found also in plasma (Kruithof et al, 1987; Lecander and Åstedt 1987). Also in many patients with acute myeloblastic leukaemia PAI-2 could be found in high concentrations (Scherrer et al, 1991). PAI-2 is a single chain protein, consisting of 425 amino acids (Ye et al, 1987; Ny et al, 1989). The gene of PAI-2 spans 16.5 kb and is located on chromosome 18q22.1 (Ye et al, 1987, 1989). PAI-2 is an efficient inhibitor of tcu-PA (Kruithof et al, 1986). It also inhibits tct-PA, but it is a poor inhibitor of sct-PA (Kruithof et al, 1986). The exact physiological role of PAI-2 is not known, but an impaired fibrinolysis could be important in late state pregancy or even more so during delivery.

Thrombin-activatable fibrinolysis inhibitor (TAFI)

TAFI (EC 3.4.17.20) is a 60 kd glycoprotein of 417 amino acids, typically synthesised in the liver. It is also known as procarboxypeptidase U or plasma procar-boxypeptidase B. It can be activated by thrombin, trypsin, kallikrein or plasmin into the active enzyme TAFIa (carboxypeptidase U or plasma carboxypeptidase B). The most efficient activator seems to be the complex of thrombin and thrombomodulin (Redlitz et al, 1995; Bajzar et al, 1995, 1996). Activation occurs by releasing a 92-residue activation peptide and generates the active 35-kd enzyme. TAFIa can potentially inhibit fibrinolysis by removing carboxyterminal lysine residues from partially degraded fibrin, thereby decreasing plasminogen binding to the surface of fibrin (Sakharov et al, 1997; Wang et al, 1998). TAFI is therefore not an inhibitor but an enzyme that may modify fibrinolytic activity.

SERPINS

General introduction about serpins

Serpins (serine protease inhibitors) form a super family of proteins including a large number of members of different species. The membership is based on the presence of a single common core domain consisting of three β-sheets and 8-9 α-helices. Serpins contain more than 40 members that display more than 35% of homology regarding their primary structures. These inhibitors were identified from viruses, insects, plants, animals and human (Gettins, 2002). Because of their importance in regulation of many physiological processes, such as blood coagulation, fibrinolysis and inflammation, human serpins are the best characterized. Of the 34 known human serpins, at least seven have no inhibitory function. Some of the functional serpins are α_1-protease inhibitor, PAI-1, antithrombin and antiplasmin, whereas some non-inhibitory members are ovalbumin or maspin. Each inhibitory serpin has its own specific target proteases.

The structural properties of serpins

The first crystal structure of a serpin was obtained in 1984 and was that of cleaved human α_1-protease inhibitor (Leobermann et al, 1984). Later on several other serpins were crystallized and their structures determined by X-ray crystallography. Thus, data were reported for ovalbumin (Stein et al, 1990, 1991), α_1-antichymotrypsin (Baumann et al, 1991), PAI-1 (Mottonen et al, 1992) and antithrombin (Mourey et al, 1993). They all share similarities in their three-dimensional structures, such as three dominant β-sheets (A, B and C), nine α-helices (A - I) and one RCL (RCL) (Huber and Carrell, 1989; Baumann et al, 1991). A β-sheet is the largest of the three β-sheets and is composed of five strands, the first strand is a short strand of 5-6 residues and the remainder are longer strands of similar length, about 12-15 residues. The central strands of the A β-sheet has a parallel direction, but the others are antiparallel. The B β- and C β-sheets are shorter and composed of four-six strands. In the native serpin conformation, the RCL is well exposed at the surface of the protein. This loop contains the scissible peptide bond in those serpins with an inhibitory function and therefore interacts with the active site of the target proteases. The length of the RCL is typically about 17 residues with very little variation (Stein et al, 1990).

The interaction of serpins and proteases

The typical reaction between serpins (I) and proteases (E) is schematically demonstrated in Fig 2. The first stage is the reversible formation of a non-covalent complex (E•I). At this stage, the serpin molecule is still intact. The second stage involves a proteolytic attack on the scissile peptide bond, resulting first in the formation of a tetrahedral intermediate (O´Malley et al, 1997) and later on in a complex where the P1-P1´ peptide bond is cleaved (Wiman and Collen, 1978a; Olson and Shore, 1982; Lawrence et al, 1995;

Wilczynska et al, 1995; Egelund et al, 1998). After the formation of E•I, there are two possible pathways for the intermediate. Firstly, in the inhibitor pathway, cleavage of the peptide bond, P1-P1´, in the serpin occurs and is followed by the formation of a covalently stabilized complex (E-I*). Secondly in the substrate pathway, the formed complex is hydrolyzed, forming a cleaved inhibitor (I*) and release of the free enzyme (E).

From the structural point of view, serpins undergo major conformational changes upon binding of proteases (Gettins et al, 1993). The RCL of a serpin is cleaved in the final complex (Nilsson and Wiman, 1982). P1´ and P3 residues are separated by a distance of 60 Å in the complex between the serpin and the protease (Wilczynska et al, 1997). Therefore, after cleavage of the scissile peptide bond in RCL part of the RCL rapidly becomes inserted into the A β-sheet of the serpin (Fa et al, 1995; Shore et al, 1995; Stratikos and Gettins, 1997, 1998). This results in that the covalently attached protease is dragged from the initial docking position on the serpin to the opposite side. This destroyes the active site of the enzyme and entraps the enzyme in an inactive complex with the inhibitor (Wilcynska et al, 1997). The progression of the inhibitor pathway depends on the rate of the cleaved RCL insertion into the A β-sheet. If insertion of the RCL is prohibited, then cleavage of the complex

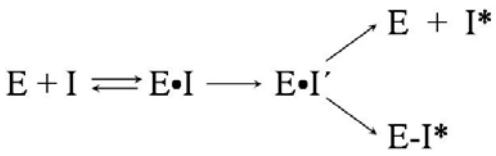

Figure 2. Reaction of a serine protease (E) with a serpin (I)

occurs, resulting in regeneration of the enzyme. Thus, the inhibitor has become a substrate.

Conformational changes: cleaved, "latent" and polymerized

It is known that many proteins have the inherent potential to undergo significant changes in their ordered structure. Serpins also have this potential for conformational change. It has been reported that there are at least four metastable conformational states of serpins: native, cleaved, "latent" and polymeric (Hekman and Loskutoff, 1985; Levin and Santell, 1987; Lomas et al, 1995a; Wardell et al, 1997; Dafforn et al, 1999; Zhou et al, 2001a).

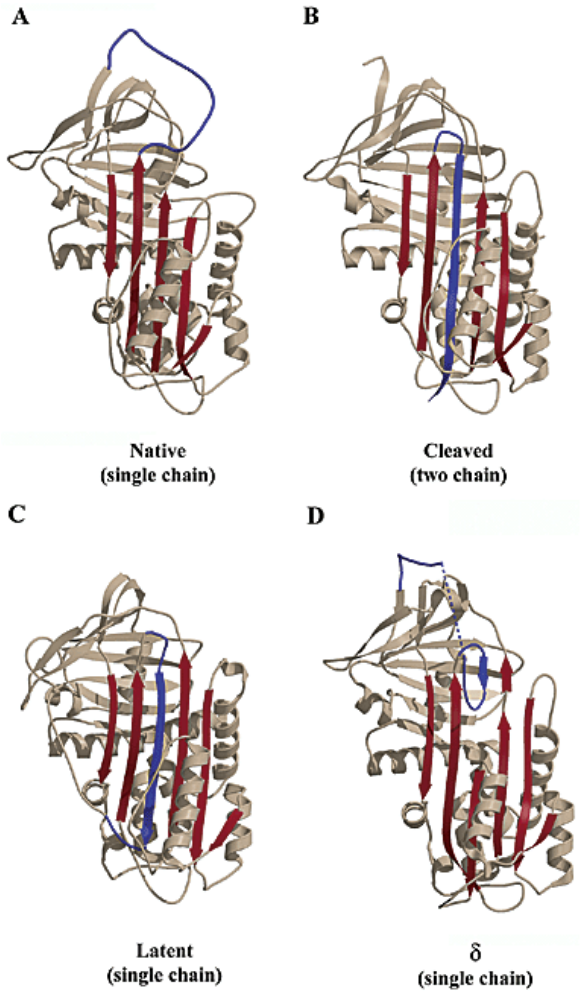

Figure 3. General structure of a Serpin (reproduced with permission, Lomas and coworkers, J Mol Biol 1998; 275: 419-425)

The conformation of the RCL in serpins shows a high variability, so there is no absolute structural requirement for its function. In the native non-inhibitory ovalbumin, the RCL is a 3-turn α-helix. In other serpins it has been found that the RCL typically has no regular secondary structure (Stein et al, 1990; Wei et al, 1994; Elliott et al, 1996, 1998; Li et al, 1999; Kim et al, 2001).

The structures of cleaved forms of inhibitory serpins are very stable and have therefore been quite easy to study by X-ray crystallography (Stein et al, 1991; Kaslik et al, 1997; Boudier and Beith, 2001). All of them show the same remarkable expansion of A β-sheet, due to the insertion of the RCL as a new strand into this ß-sheet. During this energetically favourable process, the newly formed COOH-terminal and NH_2-terminal amino acids become separated by approximately 70 Å. This explains why the transition from uncleaved to cleaved serpin is practically irreversible.

It was found that PAI-1 produced by bovine endothelial cells to a large extent were inactive, but that it could become reactivated by treatment with strong denaturing agents (Hekman and Loskutoff, 1985). Therefore they called this form of PAI-1 for a "latent" molecule. Later it was found that PAI-1 indeed was synthesized as an active molecule, but that it due to stability problems at physiological conditions rapidly was converted to the "latent" form (Chmielewska et al, 1987). Structural analysis of "latent" PAI-1 revealed a conformational change similar to what has been found in the cleaved serpins, that is insertion of the RCL into the A β-sheet (Mottonen et al, 1992). A similar phenomenon was later reported regarding the heterodimeric structure of antithrombin (Carrell et al, 1994).

Lomas reported that also antitrypsin might be transformed into an inactive "latent" conformation by heating at high temperatures in stabilizing concentrations of sodium citrate (Lomas et al, 1995a). In this study, it has been shown that also human antiplasmin might become converted into a "latent" form at low pH (Wang et al, 2004).

The ability of serpins to fold into a metastable state and subsequently undergo insertion of the RCL into the A β-sheet is an essential part of the protease inhibition mechanism. An unwanted consequence is the tendency of serpins to form polymers through RCL-sheet insertion mechanisms involving the loop of one molecule and an A β-sheet of another. This can been seen with most serpins by heating, followed by analysis of the products on a non-denaturing polyacryl amide gel (Paston et al, 1995). A ladder pattern is seen that corresponds to non-covalent dimers, trimers and higher order species. This polymerization can eventually also occur in vivo. The best known phenomenon of such loop-sheet polymers is the Z-variant of antitrypsin (Lomas et al, 1992). This is a frequent natural variant in some populations in which Glu342 had been changed to a Lys (Jeppon and Laurell, 1988; Lomas et al, 1992; Elliott et al, 1998a). Zhou reported that recombinant native PAI-1 spontaneously forms polymers in vitro at low pH although with distinctly different electrophoretic patterns of polymerization (Zhou et al, 2001b).

Antiplasmin

Antiplasmin was found in 1976 by three different groups (Moroi and Aoki, 1976; Collen, 1976; Müllertz and Clemmensen, 1976). Antiplasmin is mainly produced by liver (Saito et al, 1982) and is reduced in liver diseases (Aoki and Yamanaka, 1978). It is a glycoprotein consisting of a single polypeptide with a molecular mass of 70 kd containing approximately 13% carbohydrates (Collen and Wiman, 1978). Holmes had determined the primary structure of mature human alpha 2-antiplasmin by DNA sequencing of overlapping cDNA fragments prepared from human liver mRNA. Mature human antiplasmin contains 452 amino acids as deduced from the cDNA sequence (Holmes et al, 1987) with the Asn in NH_2-terminal. Later on it had been found that antiplasmin

had an additional 12 amino acids at the NH_2-terminal and was composed of 464 amino acids with NH_2-terminal Met (Sumi et al, 1989; Bangert et al, 1993). In plasma Met-antiplasmin converts to Asn-antiplasmin (Koyama et al, 1994). There are four potential asparagine-linked glycosylation sites in the molecule, which comprises 452 amino acids. The plasma concentration of antiplasmin is 70 mg/L, approximately one-half the concentration of plasminogen on a molar basis. The half-life of the native inhibitor is approximately 3 days (Wiman and Collen, 1977). The gene of antiplasmin has been localized to chromosome 17p13 (Kato et al, 1993).

Antiplasmin has three functional properties. But its main physiological activity is inhibition of plasmin by forming an irreversible complex with plasmin (Wiman and Collen, 1978a, 1979). It inhibits plasmin extremely rapidly (Wiman and Collen, 1978a). In addition, it competes with fibrin regarding the affinity to plasmin(ogen), which is a very important regulatory phenomenon in the fibrinolytic process (Wiman and Collen, 1978c; Wiman et al, 1979a). Antiplasmin may also become cross-linked to the α-chains of fibrin during clotting (Aoki and Sakata, 1980; Reed et al, 1992).

Antiplasmin is capable of reacting with and forming stable enzymatically inactive complexes with plasmin. The reaction occurs in several steps (Wiman and Collen, 1978a, 1979; Wiman et al, 1978b; Christensen et al, 1996). The first, which has been proposed to be rate limiting, takes place between one of the lysine-binding sites in the plasmin molecule and a complementary site in the antiplasmin molecule. The second is a non-covalent interaction between the substrate-binding pocket in the plasmin active site and the scissile peptide bond in the RCL of antiplasmin. Subsequently, peptide bond cleavage occurs. After formation of an ester bond between antiplasmin and plasmin, major conformational changes occur both in the plasmin molecule and in the antiplasmin molecule. The lysine-binding site mediated interaction has a major role in regulating the fibrinolytic system. The same site in the plasmin molecules are also involved in the interaction with fibrin. Fibrin-bound plasmin reacts much slower with antiplasmin as compared to free plasmin, thereby keeping the fibrinolytic process localized (Wiman and Collen, 1978a; Wiman et al, 1979a). It has been accepted that the COOH-terminal lysine (Lys452) in antiplasmin is responsible for the lysine-binding site mediate interactions with different proteins (Wiman and Collen, 1979; Wiman et al, 1979b, 1982; Sasaki et al, 1986; Holmes et al, 1987; Lijnen et al, 1987; Hortin et al, 1988; Sugiyama et al, 1988; Miles et al, 1991; Sjöström et al, 1997). In the present investigation it has been found that the COOH-terminal lysine is not at all important in this reaction, but that another site indeed is involved (see below).

Native antiplasmin is typically very stable at neutral pH, but at acidic pH or at elevated temperatures it rapidly becomes inactivated (Wiman and Collen, 1977). The reason for that antiplasmin becomes inactivated at decreased pH, or the mechanisms involved in these processes were not known at the start of the present project. However, now we know that both formation of “latent” molecules and polymerization processes are important parts of this phenomenon (see below).

PAI-1

PAI-1 was first found in conditioned medium from bovine aorthic endothelial cells (Loskutoff et al, 1983). Almost simultaneously it was also found in plasma (Chmielewska et al, 1983; Kruithof et al, 1984). PAI-1 is the most important inhibitor of the plasminogen activators. In spite of that the concentration in plasma is low, it still is physiologically relevant, since it reacts extremely rapidly with both tPA and uPA (Chmielewska et al, 1988; Kruithof et al, 1986). The PAI-1 gene is located on chromosome 7q21.3-q22.3 and spans 12.2kb (Ginsburg et al, 1986; Ny et al, 1986; Bosma

et al, 1988). Increased plasma levels of PAI-1 are associated with the development of thrombotic diseases, both myocardial infarction (Hamsten et al, 1985, 1987; Paramo et al. 1985; Cortellaro et al. 1993) and deep vein thrombosis (Eriksson et al, 1989; Schulman and Wiman, 1996). Decreased levels of PAI-1 are connected with a mild bleeding tendency (Dieval et al, 1991; Carmeliet et al, 1993; Lee et al, 1993; Takahashi et al, 1996; Farrehi et al, 1998). In addition, data have been published suggesting that PAI-1 is able to modulate cell adhesion and cell migration (Pappot et al, 1995; Bajou et al, 1998).

Active PAI-1 in plasma is circulating in a complex with vitronectin (Declerk et al, 1988; Wiman et al, 1988; Sigurdardottir et al, 1990). PAI-1 binds to the NH_2-terminal somatomedin B domain in vitronectin (Seiffert and Loskutoff, 1991; Sigurdardottir and Wiman, 1994; Lawrence et al, 1994; van Meijer et al, 1994). PAI-1 bound to vitronectin is more stable than free PAI-1 (Lindahl et al, 1989a). PAI-1 can also bind to heparin, which has been used for its purification (Lindahl and Wiman, 1989b). Heparin also changes the behaviour of PAI-1. Especially its reaction with thrombin becomes dramatically increased (Ehrlich et al, 1991; Keijer et al, 1991).

Three different conformations have been described for PAI-1, namely active, “latent” and substrate forms (Hekman and Loskutoff, 1985; Chmielewska et al, 1987; Declerck et al, 1992; Urano et al, 1992). At physiological conditions, active PAI-1 spontaneously changes its conformation and converts to the “latent” form with a half-life about 4 h (Wiman et al, 1988; Sigurdardottir and Wiman, 1990). PAI-1 is the only serpin to transform into the “latent” conformation spontaneously under physiological conditions. The crystal structure of “latent” PAI-1 revealed that the RCL is fully inserted into the A β-sheet (Mottonen et al, 1992; Aertgeerts et al, 1994). In the active conformation, the RCL is fully exposed on the surface of the molecule (Aertgeerts et al, 1994). In vitro, “latent” PAI-1 can be reactivated by treatment with denaturing agents, followed by dialysis against a buffer without denaturing agents (Hekman and Loskutoff, 1985). Variants of PAI-1 that preferably works as substrates have been produced (Munch et al, 1993; Gils et al, 1996).

The active PAI-1 interacts rapidly both with tPA and uPA to form stoichiometric 1:1 inactive complexes (Lindahl et al, 1990). The inhibition of t-PA or u-PA by PAI-1 involves an interaction between the RCL of the serpin and the catalytic site of the protease. It has been suggested that also other types of secondary interactions are involved (Chmielewska et al 1988). Otherwise the reactions between PAI-1 and the plasminogen activators follow the same scheme as the other serpins (Lawrence et al, 1995; Wilczynska et al, 1995; Egelund et al, 1998).

Antithrombin

Antithrombin is a plasma protease inhibitor that may inactivate many active coagulation enzymes, but especially thrombin and Factor Xa. The concentration of antithrombin in human plasma is approximately 2 μM (Murano, 1980). Decreased concentrations are connected with venous thromboembo-lism. The reaction rate between coagulation factors and antithrombin is typically quite low. However, in the presence of heparin the reaction rate is increased almost 1000-fold. This results in physiologically relevant reaction rates in the vicinity of healthy vessel wall endothelium in the microcirculation, due to the presence of heparane sulphate at the surface of endothelial cells. Antithrombin exhibits sequence, structural and functional homology to other members of the serpin family (Huber and Carrell, 1989). In addition, antithrombin has a defined binding site for heparin (Desai et al, 1998; Dementiev et al, 2004; Carter et al, 2005).

It has been found that during certain conditions antithrombin loses activity by polymerization processes (Corral et al, 2004). As with many other serpins this occurs by in-

serting the RCL from one molecule into the Aß-sheet of another molecule. It has been reported that a hydrogen-bond between His334 (in strand 5 of the Aß-sheet) and Asn186 (in strand 3 of the Aß-sheet) is important in regulating this phenomenon. If this hydrogen-bond becomes disrupted under some conditions, such as increased temperature or decreased pH, polymerization will occur (Zhou et al, 2003).

Conformational disease

Recently, it has been realized that many diseases are caused by protein with decreased conformational stability (Carrell and Lomas, 1997, 2002; Kopito and Ron, 2000). This conformational instability may result in structures with less solubility, eventually leading to precipitation and intracellular accumulation of these proteins. Subsequently, this may lead to cellular damage. This type of phenomenons might occur in many diseases, such as in Alzheimer´s and Parkinson´s disease (Tran and Miller, 1999; Walker and LeVine, 2000).

Regarding serpins it is known that the RCL from one molecule may be inserted into the A β-sheet of another serpin molecule, resulting in polymerization (Lomas et al, 1992; Stein and Carrell, 1995). One example of such a conformational disease is the relatively common condition of α_1-protease inhibitor deficiency in humans (Berg and Eriksson, 1972; Brantly and Nukiwa, 1988; Brantly et al, 1988; Mahadeva et al, 1998, 1999). The main function of α_1- protease inhibitor is to protect the tissues and against the neutrophil elastase. The lack of α_1- protease inhibitor results in an imbalance between enzymes and inhibitors in the lung, which causes early-onset emphysema (Larsson, 1978). The Z mutation in α_1- protease inhibitor is a glutamate to lysine substitution at residue P17. This amino acid is located at the head of strand five of the A β-sheet and at the base of the mobile RCL (Elliott et al, 1996). As a result of this mutation, the A β-sheet opens up and allows the RCL to become partially inserted into the A β-sheet. The partial insertion in the top of the Aß-sheet leaves the bottom-half open and ready to accept the RCL from another α_1- protease inhibitor molecule (Lomas et al, 1993a,b; Gooptu et al, 2000; Sivasothy et al, 2000; Mahadeva et al, 2002). Sequential inter molecular loop-sheet linkages occur with the formation of long chain polymers. The rate of forming polymers could be accelerated by raising the temperature and could be blocked by competing peptides (Lomas et al, 1992; Skinner et al, 1998). It was demonstrated that purified PAI-2 spontaneously polymerises at room temperature, similar to the Z-form of α_1-proteases inhibitor. The polymerezation may lead to reduced secretion efficiency. The mechanism of PAI-2 polymerisation is inserting RCL into the Aβ-sheet of another molecule (Mikus and Ny, 1996; Wilczynska et al, 2003a, 2003b).

THE PRESENT INVESTIGATION

Aims of the study

a. To increase the knowledge about the antiplasmin function in regulating the fibrinolytic system.
b. To identify the complimentary site in antiplasmin to the lysine-binding site in plasmin(ogen).
c. To understand the mechanisms involved in antiplasmin stability.

METHODOLOGY

Site-directed mutagenesis of antiplasmin cDNA

In vitro site-directed mutagenesis is a useful technique for studying protein structure-function relationships. QuikChange site-directed mutagenesis can easily be used to produce site-specific mutations in a double-stranded plasmid. This method does not need any subcloning mutagenesis of cDNA into vectors. In addition, this method does not require specialized vectors, restriction sites

or multiple transformations. The rapid four-step procedure generates mutants with more than 80% efficiency.

The oligonucleotide primers, each complementary to opposite strands of the plasmid, are extended during temperature cycling by PfuTurbo DNA polymerase. Following temperature cycling, the products are treated with Dpn I, which digest methylated and hemimethylated parental DNA template. The nicked DNA containing the desired mutations is then transformed into XL1-Blue super competent cells. After transformation, these cells repair the nicks in the mutated plasmid. Using the described technique we have constructed about 25 different antiplasmin variants. The nucleotide sequences of all the variants have been confirmed by DNA sequencing.

Expression of antiplasmin variants in S2 cells

The Drosophila Expression System (DES) utilizes a cell line derived from Drosophila melanogaster, Schneider 2 (S2) cells, and a simple plasmid vector for expression of heterologous proteins. S2 cells are easily maintained in loosely adherent or suspension cultures at room temperature and do not require CO_2. The vectors used for expression in S2 cells are very versatile, allowing inducible expression from the metallothionein (MT) promoter or constitutive expression from the actin Ac5 promoter. The expression can either be intracellular or secreted for simplified purification. Many native signal sequences are functional in S2 cells and can be used to secrete proteins. Stable cell lines expressing heterologous proteins can be generated in 3-4 weeks from a single cotransfection of the expression vector and pCOHygro selection vector.

To express the antiplasmin variants, transfected Drosoplhila Schneider S2 cells were cultured and extended in Schneider medium containing L-glutamine, heat-inactivated foetal bovine serum (final concentration 10%), 50U/ml penicillin G and 50µg/ml streptomycin sulfate. After the volume had been expanded to 500 ml with a cell concentration of about 3x 10^9/L, the cells were transferred to the same volume of Drosophila serum-free medium containing L-glutamine and penicillin/streptomycin at the same concentrations as above. Pluronic F68 (final concentration 0.05%) and $CuSO_4$ at a final concentration of 500 µM were also added. The cells were cultured with gentle stirring at room temperature in the darkness for 3 days. The cells were then removed by centrifugation at 2000 g for 30 min, and the supernatant containing antiplasmin was stored frozen at -70 °C.

Purification of antiplasmin variants

The native antiplasmin was purified from plasma as previously described, utilizing affinity chromatography on “kringles” 1-3 from plasminogen bound to Sepharose (Wiman, 1980).

Regarding purification of recombinat antiplasmin variants another method was developed, since we expected some of the variants to bind less good to the lysine-binding sites in plasminogen. Three steps were used for purification of the recombinant antiplasmin variants from the supernatant. First, the supernatant (about 500 ml) was mixed with DEAE-Sepharose CL6B in a batch procedure (about 25ml, equilibrated with 0.05 M Tris buffer, pH 8.0) during slow stirring for 2 hours at 4 ºC. The suspension was then filtered through a Büchner-funnel and washed with about 1 l of 0.05 M Tris buffer, pH 8.0. The washed DEAE-Sepharose CL6B was packed in a column with a diameter of 2.5 cm and then washed with the same Tris buffer until the absorbance at 280 nm was less than 0.1. The elution was performed by a linear gradient from 0 to 0.4 M NaCl in the same Tris buffer. The antiplasmin concentration was determined in the different samples by ELISA. The antiplasmin-containing fractions were pooled and dialysed overnight against 0.04 M sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl. The material was then applied to a Sephacryl S-300 HR

column equilibrated with 0.04 M sodium phosphate buffer, pH7.5, containing 0.1 M NaCl. The fractions containing antiplasmin as determined by ELISA were pooled. The pool was subsequently applied to an anhydrotrypsin-agarose column (column volume about 2 ml), equilibrated with the same phosphate/NaCl buffer as above. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.1. Elution was then performed with the equilibration buffer, also containing 0.3 M arginine-HCl. Fractions containing antiplasmin were dialysed against 0.04 M sodium phosphate buffer, pH 7.3, containing 0.1 M NaCl and then stored frozen at -70 ºC until use.

Determination of antiplasmin activity and antigen concentration

Antiplasmin activity was determined by a titration method against plasmin of known concentration, essentially as described (Wiman, 1981). Antiplasmin antigen concentration was determined by an ELISA method. For this purpose, Maxisorp microtiter plates were coated for 2 h at room temperature with goat anti-antiplasmin IgG, diluted in 0.1 M $NaHCO_3$ buffer, pH 9.6. The plates were then incubated at room temperature for 30 min with 0.04 M sodium phosphate buffer, pH 7.3, containing 0.1 M NaCl and 1 g/L BSA and washed three times with the phosphate/NaCl buffer, but without BSA. The samples (200 µL) to be analysed were compared with an antiplasmin standard (in the beginning native antiplasmin from human plasma was used, but later a wt-antiplasmin standard was prepared). The microtiter plates were incubated for 2 h at room temperature and then washed four times with the phosphate/NaCl buffer. Then, the anti-antiplasmin-IgG, conjugated with horseradish peroxidase, was added to the samples. After incubation for 1 h and washing the plates four times with phosphate/NaCl buffer, the horseradish peroxidase substrate o-phenylenediamine in the presence of H_2O_2 was added. After a new incubation for 10 minutes, 50 µL stop solution (3 M H_2SO_4) was added to each well, and the A_{492} recorded in a microtiter plate reader.

Polyacrylamide gel electrophoresis (PAGE)

The non-denaturing PAGE system used is essentially that described by Jovin (1973). The separating gel contained 10 % (w/v) acrylamide in 0.38 M Tris-phosphate buffer, pH 8.8, whereas the stacking gel contained 4.5 % acrylamide in 0.12 M Tris-phosphate buffer, pH 6.9. The cathode buffer in the upper chamber contained 0.0529 M Tris buffer, pH 8.2, containing 0.0684 M glycine, while the anodic buffer in the lower chamber contained 0.1 M Tris buffer, pH 7.8. All the gel electrophoresis was performed in Bio-Rad Mini Protean II apparatus at room temperature. SDS/PAGE was performed in a Mini-protean II electrophoresis apparatus (Bio-Rad, Stockholm, Sweden) as described by Laemmli (1970). Proteins were separated in 10 % (w/v) polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

Determination of rate constants in the reaction between plasmin and the antiplasmin variants

In order to study the reaction between plasmin and the different antiplasmin variants, the two reactants were mixed in 0.1 M sodium phosphate buffer, pH 7.3, or the same buffer containing 1.0 mM 6-aminohexanoic acid. The final plasmin concentration (active site titrated) used in these experiments was 0.6 nM, whereas the antiplasmin concentration varied between 1 and 5 nM. After specified times of reaction (0–300 s), samples were withdrawn into tubes containing 0.6 mM Flavigen Pli (final concentration), 20 mM 6-aminohexanoic acid and polyclonal rabbit anti-human antiplasmin IgG (1 mg/mL). By this procedure further inhibition of plasmin was rapidly and efficiently decreased, allowing long incubation times with the plasmin substrate, which is necessary to accurately measure the low plasmin concentrations. After incubation for 1.5 h, plasmin cleavage of the chromogenic substrate was

stopped by addition of acetic acid (final concentration 1%, v/v) and the A_{405} recorded. A_{405} is thus a reliable measure of the residual plasmin concentration at the time of sampling. Then, the reaction rate constants were calculated from the results using the classic formula for second-order reactions, using data obtained before 50% of the plasmin was inhibited. In the experiments performed in the presence of 6-aminohexanoic acids, in which the antiplasmin concentration was almost 10-fold higher than the plasmin concentration, pseudo-first-order conditions were assumed and the reaction rate constants were calculated from the half-lives of plasmin in these experiments (also before 50% of the plasmin activity was inhibited).

Binding of antiplasmin variants to plasminogen or "kringles" 1-3 measured by surface plasmon resonance

The interaction between plasminogen or "kringles" 1-3 in plasminogen and each one of the antiplasmin variants were analysed by surface plasmon resonance in a BIAcore 2000 (BIAcore AB, Uppsala, Sweden). Plasminogen or "kringles" 1-3 was immobilised at the carboxymethyl surface of a CM5 chip (BIAcore AB) using amine coupling according to the manufacturers instructions. For the immobilization procedure, the proteins were diluted to 2.5 µg/ml in an acetate buffer, pH 5.5 (BIAcore AB) and coupled to the surface in one of the flow cells, at a flow rate of 10µl/min for 7 minutes. At the same time, a reference surface was prepared in one of the other flow cells. For the kinetic measurements, 5 different concentrations of each antiplasmin variant were injected for 3 minutes followed by a 10 minutes dissociation phase. At the end of each cycle, the surface was regenerated by a one-minute injection of 1 M NaCl, followed by a one-minute injection of 20 mM 6-aminohexanoic acid. The experiments were carried out in 40 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl, at a flow rate of 30 µl/min. Assuming a 1:1 stoichiometry in a bimolecular reaction model, the association and dissociation phases of the obtained sensorgrams were analysed by curve fitting, using the BIAevaluation software (version 3.1). The experimental procedure with wt-antiplasmin was carried out at several different occasions, during quite a long period of time. Deviating results were obtained, if analyses were performed several days after the coupling procedure. For this reason, in the experiments where all the different antiplasmin variants (including wt-antiplasmin) were included, the chips were used within 24 hours after coupling.

Computer modelling of the COOH-terminal 25 or 40 amino acid residues in antiplasmin

A computer model of the C-terminal 25 or 40 amino acids in antiplasmin was constructed by CS Chem3d Ultra, version 7.0 (Cambridge Soft, Cambridge, MA, USA), followed by energy minimization utilizing the MM2 protocol. Modelling was performed on different lengths of the C-terminal portion of the antiplasmin molecule, ranging from 25 to 50 residues from Lys452. However, energy minimization did not work well on structures with more than 40 amino acids.

RESULTS AND DISCUSSION

The site in antiplasmin interacting with lysine-binding sites in plasmin (Papers I and II)

The reactions between plasmin and different antiplasmin variants

In order to study the lysine-binding-site mediated interaction between plasmin and antiplasmin and possibly identify the amino acids in antiplasmin of importance for this interaction we constructed 7 different single site mutants in COOH-terminal portion of antiplasmin. Two mutants were produced in which the COOH-terminal lysine (Lys452) was replaced by amino acids without a positive charge (Glu or Thr). We also produced variants in which other charged amino acids in

this portion of the molecule were changed to either uncharged residues or residues of opposite charge (Figure 4). All the antiplasmin variants were expressed in insect cells (*Drosophila* S2 cells), purified, and characterized with regard to their reactions with plasmin. The rate constants for the reactions between plasmin and the antiplasmin variants, in the absence or presence, of 6-aminohexanoic acids, are demonstrated in table1. The rate constants for the reactions between 'native' human antiplasmin and plasmin in the presence or absence of 6-aminohexanoic acids are also shown for comparison.

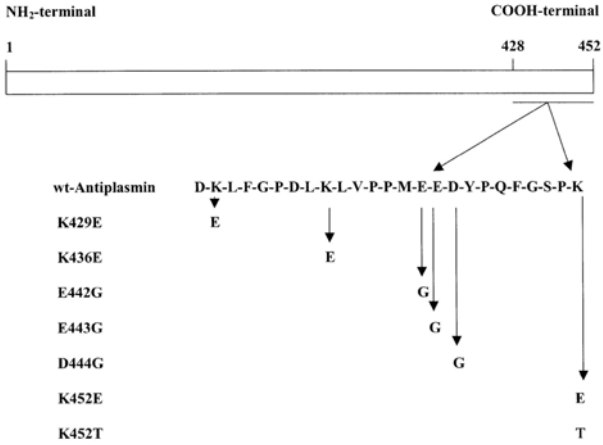

Figure 4. Mutations in the antiplasmin COOH-terminal portion

All variants of antiplasmin except for K436E had a rate constant higher than 10^7 $m^{-1} \cdot s^{-1}$. This is not far from the rate constant obtained with native antiplasmin. In addition, the method used here gave very similar results regarding the rate constants as those reported earlier (Wiman and Collen, 1978a; Christensen et al, 1996). Interestingly, the mutants K452E and K452T did not differ in behaviour from wt-antiplasmin, suggesting that the COOH-terminal lysine is of little importance in the lysine-binding-site mediated interaction between plasmin and antiplasmin. On the other hand, the variant K436E reacted much more slowly (about five fold) than the other variants, rather suggesting that this residue is important.

In the presence of 6-aminohexanoic acids, the reaction rate decreased 10-fold or more for most variants. Also in this case the results with wt-antiplasmin and the mutants K452E and K452T did not differ. Only the variant K436E was less affected by 6-aminohexanoic acids (2.5-fold decrease in reaction rate), again suggesting that this residue is involved in the lysine-binding-site mediated interaction between plasmin and antiplasmin.

The Interaction between Plasminogen and Antiplasmin Variants as Studied by Surface Plasmon Resonance

To further elucidate this process we studied the interaction beween these antiplasmin variants and immobilized plasminogen or an elastase fragment from plasminogen constituting "kringles" 1-3, using surface plasmon resonance. The antiplasmin variants studied were wt-antiplasmin, K429E, K436E, E443G, D444G, K452E and K452T. Clearly, the two mutants K452E and K452T reacted quite similarly to wt-antiplasmin. The K_D is in fact quite similar for all these variants, again suggesting that Lys 452 is not involved in the lysine-binding site interaction between plasminogen and antiplasmin. On the other hand the mutant K436E displays a much lower association rate constant, both to intact plasminogen and to "kringles" 1-3. In fact, the affinity was very poor, making it difficult to get reliable values for the K_D using the present conditions. Nevertheless, the K_D values for the interaction between the K436E mutant and either plasminogen or the "kringles" 1-3 fragment were estimated as about 40-fold higher as compared to wt-antiplasmin. Thus, our data show that K436 is indeed very important in the lysine-binding site interaction between plasminogen and antiplasmin. Taken together with our previous investigation, using completely different technology, our data proves that Lys436 but not Lys452 in antiplasmin is responsible for the interaction through the lysin-binding sites in plasmin. The mutant E443G displays somewhat lower affinity for both plasminogen and "kringles" 1-3, as compared to wt-antiplasmin. It is possible that this residue is also involved in the lysine-binding-site mediated interaction.

Table 1. Rate constants (in 10^6 $M^{-1}s^{-1}$) in the reactions between plasmin and the different antiplasmin variants in the absence (No 6-AHA) or the presence (6-AHA) of 1.0 mM 6-AHA

Variant	No 6-AHA	6-AHA
Native	25.3±1.7	2.5
Wt-AP	10.9±0.3	1.1
K429E	27.3±2.5	2.7
K436E	2.1±0.3	0.8
E442G	19.5±1.0	1.5
E443G	24.3±1.2	1.6
D444G	21.6±0.9	1.6
K452E	11.5±0.7	0.8
K452T	12.7±1.0	0.9

Molecular modelling of the COOH-terminal portion of antiplasmin

The amino-acid sequence of the COOH-terminal 40 residues in antiplasmin is GNKDFLQSLKGFPRGDKLFGPDLKLVPPMEEDYPQFGSPK-OH (Holmes et al, 1987). The COOH-terminal lysine is residue 452 in the antiplasmin molecule. Molecular modelling resulted in the structures shown in figure 5. We constructed a number of models with different lengths, ranging from 25 to 50 residues from the COOH-terminal Lys452. All models were similar around the two sites Lys452 and Lys436. In these models, the side chain of the COOH-terminal lysine residue (K452) seems to be in the close vicinity to the side chain of Phe448, perhaps explaining why it is not involved in the lysine-binding-site mediated interaction. Lys436, on the other hand, is clearly exposed at the surface of the molecule. In addition the negatively charged side-chain of Glu443 is within the expected range from the positively charged side-chain of Lys436 to form a potentially complementary site to a

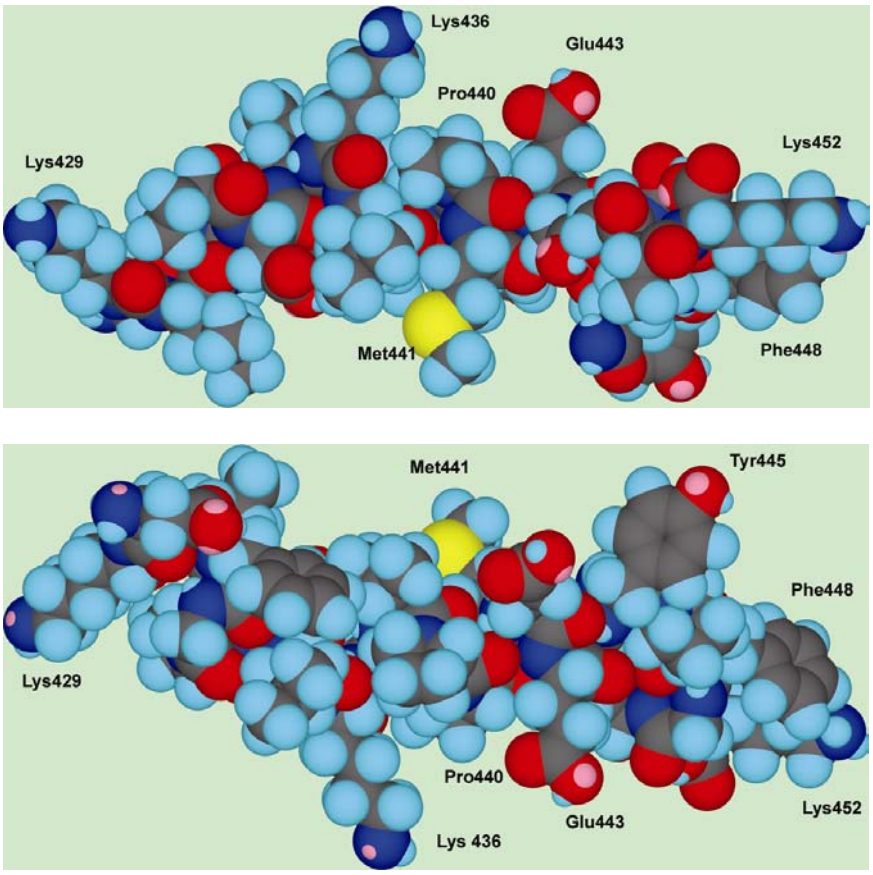

Figure 5. Computer model of the COOH-terminal 25 amino acids in antiplasmin viewed from two different angels. Some of the residues are numbered to facilitate viewing.

lysine-binding site. Also these two charged side-chains seem to be separated by the hydrophobic part of the side chain in Pro439.

Antiplasmin stability (Papers III and IV)

Inactivation of antiplasmin

Previously, it has been demonstrated that human antiplasmin is quite stable at physiological conditions, but that activity is rapidly lost at acidic conditions (Wiman, 1977). Here we have studied that process in more detail. Antiplasmin was purified from plasma as previously described (Wiman, 1980, 1981) using affinity chromatography on "kringles" 1-3 in plasminogen, insolubilised to Sepharose 4B, followed by gel filtration on Sephacryl S-200. The antiplasmin preparation appeared as a single band on SDS/PAGE, with an estimated molecular weight of about 70,000. It was almost completely active as judges from activity measurements and from its ability to form a SDS stable complex with plasmin.

The purified antiplasmin was inactivated at decreased pH (pH 4.9-6.1) and at different temperatures (22 and 37 °C). The decline in activity typically followed first-order kinetics. After plotting the logarithm of antiplasmin activity against time, the half-lives were calculated. In addition to this, we also performed inactivation experiments at elevated temperatures (45 and 55 °C), but only at pH 5.5. The results are summarised in table 2. Almost no inactivation occurred at pH above 6.1 at temperatures below 37 °C. However, at elevated temperatures (above 45 °C), a rapid inactivation was observed. In order to further study the mechanism for inactivation of antiplasmin at different pH and at different temperatures, we also followed these processes by PAGE at non-denaturing conditions. The results of the PAGE were also analysed by running Western blots, in order to confirm that the "polymerized" bands seen really constituted antiplasmin. At elevated temperatures, our data indicated that polymerization rapidly occurred, which most likely is responsible for the rapid inactivation under these conditions. In contrast, inactivation of antiplasmin at pH 5.5 and 22 °C occurred mostly without any visible polymerization tendency. Thus the mechanism for inactivation at normal temperatures is different than that at elevated temperatures. One possible explanation for this is the formation of "latent" molecules. Our data have demonstrated that inactivation of antiplasmin at slightly decreased pH is a quite complicated process, involving both polymerisation and perhaps also formation of "latent" molecules.

Reactivation of antiplasmin

Antiplasmin, which has been inactivated at pH 4.9 for 24 h at 22 °C, does not have any measurable activity. If such material was incubated at neutral pH, by dilution with 0.1 M sodium phosphate buffer, pH 7.3, a gradual increase in antiplasmin activity was observed. The maximal activity obtained was still low, about 5% of the theoretical value. Antiplasmin, inactivated in the same way as above was also treated with different concentrations of guanidinium chloride (1-4 M), followed by dialysis and measurement of antiplasmin activity. In this way much higher antiplasmin activity was obtained. The maximal activity obtained corresponds to a specific activity of about 37% during 0.5-2 h incubation with 4 M guanidinium chloride. Antiplasmin, inactivated at pH 6.2 at 55 °C, which mainly yielded polymerized material, was incubated at 3.2 M guanidinium chloride, followed by dialysis against 0.1 M sodium phosphate buffer, pH 7.3. In this case, activity measurements revealed that very little antiplasmin activity was recovered (<3%).

Structures of importance for the stability of antiplasmin as studied by site-directed mutagenesis

We decide to study the inactivation of antiplasmin at dereased pH or slightly increased tempertatures by investigating the stability of single-site mutants of antiplasmin. The rationale for chosing the specific variants were as follows: The amino acid sequences of PAI-1 and antiplasmin were compared, since PAI-1 has a reversed pH-dependent stability as

compared to antiplasmin (Hekman and Loskutoff, 1985; Lindahl et al, 1989a; Lawrence et al, 1994; Mångs et al, 2000; Wang et al, 2004).

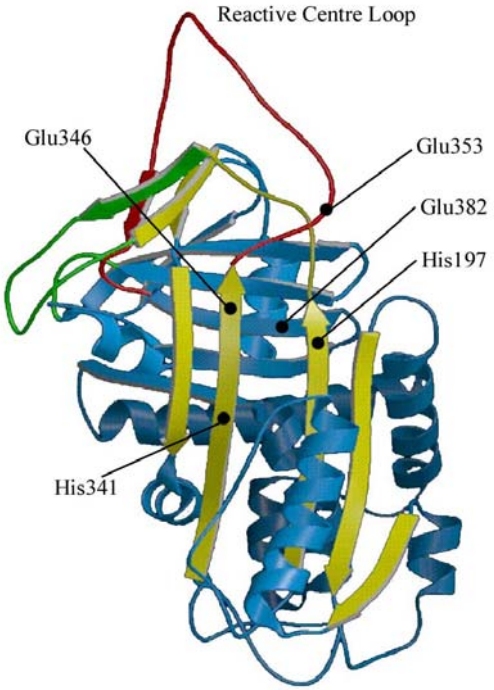

Figure 6. A schematic picture of PAI-1 (adapted from Aertgeerts *et al* 1994, *J.Struct.Biol.*, 113:239-245), with the most important mutations marked. Since antiplasmin has not been crystallized, we rather show a model of PAI-1.

Indeed, this revealed some important sequence differences in some of the interesting parts in antiplasmin, which might be involved in the stability mechanism (RCL, the A β-sheet or the B β-sheet). Some of these differences may be the reason for why PAI-1 and antiplasmin are stable at different pH. With these differences in mind, we used site-directed mutagenesis to produce 11 single-site mutants (His197Thr; Gln212His; Ile246His; His250Asp; His250Thr; His266Glu; His341Asp; His341Thr; Glu346Thr; Glu353Ala; Glu382His) within these regions.
The inactivation of wt-antiplasmin was studied at different pH (pH 4.7-5.6) at room temperature and the decline in activity was found to follow first-order kinetics. The lower the pH, the faster inactivation occurred. The half-life of wt-antiplasmin is very similar to that of "native" antiplasmin at pH 4.9 (Wang et al, 2004). The half-lives of 4 of these antiplasmin variants (His250Asp; His250Thr; His266Glu; His341Asp) did not deviate much from the result obtained with wt-antiplasmin. Two of the mutants (Gln212His and Ile246His) had a slight increased half-life. However, four of the mutants (His197Thr, His341Thr, Glu353Ala and Glu382His) displayed a significantly increased stability. The mutant His341Thr was found to have the longest half-life at pH 4.9, about 7 fold increased stability as compared to wt-antiplasmin. One mutant, Glu346Thr, had a shorter half-life than wt-antiplsmin.

Table 2. Inactivation of antiplasmin as a function of pH and temperature with time. The decline in antiplasmin activity followed first-order kinetics. The data shown (mean of three independent experiments) represent the half-lives in the transition from active to inactive inhibitor.

pH	22 °C	37 °C	45 °C	55 °C
4.9	14 min	< 5 min		
5.2	47 min	9 min		
5.5	24 h	9 h	~1 h	~10 min
5.8	> 30 h	27 h		
6.1	> 30 h	> 30 h		

The mutants (His197Thr; His341Thr; Glu346Thr; Glu353Ala; Glu382His) (Figure 6), which have deviating properties as compared to wt-antiplasmin, have been studied more extensively. These variants were also analysed by PAGE under non-denaturing conditions after incubation both at pH 4.9 at room temperature for 2 hours, and at 45 °C at pH 5.5, for 2 hours. After incubation at pH 4.9 for 2 hours at room temperature, it was obvious that several of the variants had polymerised to different extent. Interestingly, Glu346Thr, which has a shorter activity half-life than wt-antiplasmin, only have a very small tendency to polymerise under these conditions. Therefore, inactivation of this mutant must occur by other means than polymerisation, e.g. by formation of "latent" molecules. Also, regarding the mutants His197Thr and Glu382His, the results are in favor of formation of "latent" molecules, as the main pathway of inactivation. Treatment of the antiplasmin variants at slightly increased temperature (45 °C) at pH 5.5 for 2 hours did not display any major differences on PAGE, as compared to the untreated samples, in spite of that the activity had decreased to less than 40% (10-40%). Even if the decline in antiplasmin activity at decreased pH is similar, it seems that the recombinant wt-antiplasmin is more prone to polymerise as compared to the "native" antiplasmin. On the other hand at elevated temperature wt-antiplasmin rather transforms into a "latent" conformation, which is different from the "native" antiplasmin. This is a clear difference in behaviour between "native" antiplasmin and recombinant wt-antiplasmin.

Table 3. Inactivation of antiplasmin variants at pH 4.9 and 22 ºC. The decline in antiplasmin activity typically followed first-order kinetics. The data shown represent the half-lives in the transition from active to inactive inhibitor. The result for "native" antiplasmin is included for comparison.

Variants	half-life (min)
"native"	14.0
wt-AP	12.4
His197Thr	38.9
Gln212His	20.5
Ile246His	20.5
His250Asp	14.0
His250Thr	11.2
His266Glu	10.0
His341Asp	11.8
His341Thr	99.0
Glu346Thr	6.5
Glu353Ala	24.2
Glu382His	23.7

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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: 239-45.

Aoki N and Yamanaka T. (1978) The alpha2-plasmin inhibitor levels in liver diseases. *Clin Chim Acta*. 84: 99-105.

Aoki N and Sakata Y. (1980) Influence of alpha 2-plasmin inhibitor on adsorption of plasminogen to fibrin. *Thromb Res*.19: 149-155.

Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D and Foidart JM. (1998) Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med*. 4: 923-8.

Bajzar, L. Manuel R. and Neisheim, M.E. (1995) Purification and characterization of TAFI, a thrombin activable fibrinolysis inhibitor. *J.Biol.Chem*. 270: 1477–1484.

Bajzar, L, Morser J. and Neisheim, M.E. (1996) TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin–thrombomodulin complex. *J.Biol.Chem*. 271: 16603–16608.

Bangert K, Johnsen AH, Christensen U and Thorsen S. (1993) Different N-terminal forms of alpha 2-plasmin inhibitor in human plasma. *Biochem J*. 291: 623-5.

Baumann U, Huber R, Bode W, Grosse D, Lesjak M and Laurell CB. (1991) Crystal structure of cleaved human alpha 1-antichymotrypsin at 2.7 A resolution and its comparison with other serpins. *J.Mol.Biol*. 218: 595-606.

Beauchamp NJ, Pike RN, Daly M, Butler L, Makris M, Dafforn TR, Zhou A, Fitton HL, Preston FE, Peake IR and Carrell RW. (1998) Antithrombins Wibble and Wobble (T85M/K): archetypal conformational diseases with in vivo ”latent”-transition, thrombosis, and heparin activation. *Blood.* 92: 2696-706.

Benham FJ, Spurr N, Povey S, Brinton BT, Goodfellow PN, Solomon E and Harris TJ. (1984) Assignment of tissue-type plasminogen activator to chromosome 8 in man and identification of a common restriction length polymorphism within the gene. *Mol.Biol.Med*. 2: 251-259.

Berg DT, Burck PJ, Berg DH and Grinnell BW. (1993) Kringle glycosylation in a modified human tissue plasminogen activator improves functional properties. *Blood*. 81: 1312-22.

Berg NO and Eriksson S. (1972) Liver disease in adults with alpha$_1$-antitrypsin deficiency. *N.Engl.J.Med* 287: 1264-1267.

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.

Boudier C and Bieth JG. (2001) The reaction of serpins with proteinases involves important enthalpy changes. *Biochemistry*. 40: 9962-9967.

Brantly M. and Nukiwa T. (1988) Crystal RG. Molecular basis of alpha-1-antitrypsin deficiency. *Am.J.Med* 84: 13-31.

Brantly M, Courtney M and Crystal RG. (1988) Repair of the secretion defect in the Z form of α1-antitrypsin by addition of a second mutation. *Science.* 242: 1700-1702.

Burrows JAJ, Willis LK and Perlmutter DH. (2000) Chemical chaperones mediate increased secretion of mutant α_1-antitrypsin (α_1-AT) Z: a potential pharmacological strategy for prevention of liver injury and emphysema in α_1-AT deficiency. *Proc.Natl.Acad.Sci USA* 97: 1796-1801.

Bykowska K, Rijken DC and Collen D. (1981) Purification and characterization of the plasminogen activator secreted by a rat brain tumor cell line in culture. *Thromb. Haemost*. 46: 642-644.

Canipari R and Strickland S. (1985) Plasminogen activator in the rat ovary. Production and gonadotropin regulation of the enzyme in granulosa and thecal cells. *J.Biol.Chem*. 260: 5121-5125.

Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC and Collen D. (1993) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J.Clin.Invest*. 92: 2756-2760.

Carrell RW, Stein PE, Fermi G and Wardell MR. (1994) Biological implications of a 3 A structure of dimeric antithrombin. *Structure*. 2: 257-270.

Carrell RW and Lomas DA. (1997) Conforma-tional disease. *Lancet* 350:134-138.

Carrell RW. (1999) How serpins are shaping up. *Science*. 285: 1861

Carrell RW, Huntington JA, Mushunje A and Zhou A. (2001) The conformational basis of thrombosis. *Thromb Haemost*. 86: 14-22.

Carrell RW and Lomas DA. (2002) Alpha1-antitrypsin deficiency--a model for conformational diseases. *N.ngl.J.Med*. 346: 45-53.

Carter WJ, Cama E and Huntington JA. (2005) Crystal structure of thrombin bound to heparin. *J.Biol.Chem*. 280: 2745-2749.

Chang W.S.W. and Lomas D.A. (1998) "latent" alpha1-antichymotrypsin.A molecular explanation for the inactivation of alpha1-antichymotrypsin in chronic bronchitis and emphysema. *J.Biol.Chem*. 273: 3695–3701.

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, Carlsson T, Urden G. and Wiman B. (1987) On the relationship between different molecular forms of the fast inhibitor of tissue plasminogen activator. *Fibrinolysis* 1: 67–73.

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: 327-32.

Christensen U, Bangert K,and Thorsen S. (1996) Reaction of human alpha2-antiplasmin and plasmin stopped flow fluorescence kinetics. *FEBS Lett*. 387: 58–62.

Clemmensen I, Petersen LC and Kluft C. (1986) Purification and characterization of a novel, oligomeric, plasminogen kringle 4 binding protein from human plasma: tetranectin. *Eur.J.Biochem*. 156: 327-333.

Collen D and de Maeyer L. (1975) Molecular biology of human plasminogen. I. Physiochemical properties and microheterogeneity. *Thromb.Diath.Haemorrh*. 34: 396-402.

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

Collen D. and Wiman B. (1978) Fast-acting plasmin inhibitor in human plasma. *Blood*. 51: 563-569.

Collen D and Wiman B. (1979) Turnover of antiplasmin, the fast-acting plasmin inhibitor of plasma. Blood. 53: 313-324.

Collen D. and Lijnen HR. (1991) Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood*. 78: 3114-3124.

Corral J, Huntington JA, Gonzalez-Conejero R, Mushunje A, Navarro M, Marco P, Vicente V, and Carrell RW. (2004) Mutations in the shutter region of antithrombin result in formation of disulfide-linked dimers and severe venous thrombosis. *J.Thromb. Haemost*. 2: 931-939.

Cortellaro M, Cofrancesco E, Boschetti C, Mussoni L, Donati MB, Cardillo M, Catalano M, Gabrielli L, Lombardi B, Specchia G, 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.

Dafforn TR, Mahadeva R, Elliott PR, Sivasothy P and Lomas DA. (1999) A kinetic mechanism for the polymerization of α_1-antitrypsin. *J.Biol.Chem*. 274: 9548-9555.

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS and Skriver L. (1985) Plasminogen activators, tissue degradation, and cancer. *Adv Cancer Res. 44*: 139-266.

Davis RL, Shrimpton AE, Holohan PD, et al. (1999) Familial dementia caused by polymerization of mutant neuroserpin. *Nature* 401: 376-379.

Declerck, P.J. Mol, M. De Alessi, M.C. Baudner, S. Paques, E.P. Preissner K.T. *et al.*, (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.

Dementiev A, Petitou M, Herbert JM and Gettins PG (2004) The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. *Nat Struct Mol Biol*. 11: 863-867.

De Taeye B, Compernolle G, Dewilde M, Biesemans W and Declerck PJ. (2003) Immobilization of the distal hinge in the labile serpin plasminogen activator inhibitor 1: identification of a transition state with distinct conformational and functional properties. *J.Biol.Chem*. 278: 23899-23905.

De Taeye B, Verbeke K, Compernolle G, Biesemans W, Gils A and Declerck PJ. (2003) Structural determinants in the stability of the serpin/proteinase complex. *Biochem Biophys Res Commun.* 307: 529-534.

Desai UR, Petitou M, Bjork I and Olson ST. (1998) Mechanism of heparin activation of antithrombin. Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. *J.Biol.Chem*. 273: 7478-7487.

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.

Dobo J and Gettins PG. (2004) alpha1-Proteinase inhibitor forms initial non-covalent and final covalent complexes with elastase analogously to other serpin-proteinase pairs, suggesting a common mechanism of inhibition. *J.Biol.Chem*. 279: 9264-9.

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.

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.

Elliott PR, Lomas DA, Carrell RW and Abrahams JP. (1996) Inhibitory conformation of the reactive loop of alpha 1-antitrypsin. *Nat Struct Biol.* 3: 676-681.

Elliott PR, Stein PE, Bilton D, Carrell RW and Lomas DA. (1996) Structural explanation for the dysfunction of S α_1-antitrypsin. *Nat Struct Biol* 3: 910-911.

Elliott PR, Abrahams JP and Lomas DA. (1998) Wild-type alpha 1-antitrypsin is in the canonical inhibitory conformation. *J.Mol.Biol*. 275: 419-425.

Elliott PR, Bilton D and Lomas DA. (1998a) Lung polymers in Z alpha1-antitrypsin deficiency-related emphysema. *Am J Respir Cell Mol Biol*. 18: 670-674.

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 S, Carlson J and Velez R. (1986) Risk of cirrhosis and primary liver cancer in alpha$_1$-antitrypsin deficiency. *N.Engl.J.Med*. 314: 736-739.

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*. 1995 34: 13833-13840.

Farrehi PM, Ozaki CK, Carmeliet P and Fay WP. (1998) Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice. *Circulation*. 97: 1002-1008.

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

Gettins PG. (2002) The F-helix of serpins plays an essential, active role in the proteinase inhibition mechanism. *FEBS Lett*. 523: 2-6.

Gettins PG. (2002) Serpin structure, mechanism, and function. *Chem Rev.* 102: 751-804

Gils A, Knockaert I and Declerck PJ. (1996) Substrate behavior of plasminogen activator inhibitor-1 is not associated with a lack of insertion of the reactive site loop. *Biochemistry*. 35: 7474-7481.

Ginsburg D, Zeheb R, Yang AY, Rafferty UM, Andreasen PA, Nielsen L, Dano K, Lebo RV and Gelehrter TD. (1986) cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. *J.Clin.Invest*. 78: 1673-1680.

Gooptu B, Hazes B, Chang WS, Dafforn TR, Carrell RW, Read RJ and Lomas DA. (2000) Inactive conformation of the serpin alpha(1)-antichymotrypsin indicates two-stage insertion of the reactive loop: implications for inhibitory function and conformational disease. *Proc Natl Acad Sci USA*. 97: 67-72.

Gyetko MR, Todd RF 3rd, Wilkinson CC and Sitrin RG. (1994) The urokinase receptor is required for human monocyte chemotaxis in vitro. *J.Clin.Invest*. 93: 1380-1387.

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

Hajjar, K.A., Harpel, P.C., Jaffe, E.A. and Nachman, R.L. (1986) Binding of plasminogen to cultured human endothelial cells. *J.Biol.Chem*. 261: 11656–11662.

Hamsten A, Wiman B, de Faire U and Blomback M. (1985) Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. *N.Engl.J.Med*. 313: 1557-1563.

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

Harpel PC, Sullivan R and Chang TS. (1989) Binding and activation of plasminogen on immobilized immunoglobulin G. Identification of the plasmin-derived Fab as the plasminogen-binding fragment. *J.Biol.Chem*. 264: 616-624.

Hekman C.M. and Loskutoff, D.J. (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, Bjorkander 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.

Holmes WE, Lijnen HR, Nelles L, et al. (1987) α_2-Antiplasmin Enschede: alanine insertion and abolition of plasmin inhibitory activity. *Science* 238: 209-211.

Holmes WE, Nelles L, Lijnen HR and Collen D. (1987) Primary structure of human α_2-antiplasmin, a serine protease inhibitor (Serpin). *J.Biol.Chem*. 262: 1659–1664.

Hortin GL, Gibson BL. and Fok KF. (1988) α_2-Antiplasmin's carboxy-terminal lysine residue is a major site of interaction with plasmin. *Biochem.Biophys.Res.Commun*. 155: 591–596.

Hoylaerts M, Rijken DC, Lijnen HR and Collen D. (1982) Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J.Biol.Chem*. 257: 2912-2919.

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

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

Huntington JA, Pannu NS, Hazes B, Read RJ, Lomas DA and Carrell RW. (1999) A 2.6Å structure of a serpin polymer and implications for conformational disease. *J.Mol.Biol*. 293: 449-455.

Huntington JA, McCoy A, Belzar KJ, Pei XY, Gettings PG and Carrell RW. (2000) The conformational activation of antithrombin. A 2.85-A structure of a fluorescein derivative reveals an electrostatic link between the hinge and heparin binding regions. *J.Biol.Chem*. 275: 15377-15383.

Huntington JA, Read RJ and Carrell RW. (2000) Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. 407: 923-926.

Ichinose A, Fujikawa K and Suyama T. (1986) The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. *J.Biol.Chem*. 261: 3486-3489.

Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, Erdreich-Epstein A and DeClerck YA. (2001) Plasminogen activator inhibitor-1 promotes angiogenesis by stimulating endothelial cell migration toward fibronectin. *Cancer Res.* 61: 5587-5594.

Janciauskiene S, Dominaitiene R, Sternby NH, Piitulainen E and Eriksson S. (2002) Detection of circulating and endothelial cell polymers of Z and wild type alpha 1-antitrypsin by a monoclonal antibody. *J.Biol.Chem*. 277: 26540-26546.

Jeppsson JO and Laurell CB. (1988) The amino acid substitutions of human alpha 1-antitrypsin M3, X and Z. *FEBS Lett*. 231: 327-330.

Jovin TM. (1973) Multiphasic zone electrophoresis. I. Steady-state moving-boundary systems formed by different electrolyte combinations. *Biochemistry*. 12: 871-879.

Juhan-Vague I, Valadier J, Alessi MC, Aillaud MF, Ansaldi J, Philip-Joet C, Holvoet P, Serradimigni A and Collen D. (1987) Deficient t-PA release and elevated PA inhibitor levels in patients with spontaneous or recurrent deep venous thrombosis. *Thromb Haemost*. 57: 67-72.

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.

Kaslik G, Kardos J, Szabo E, Szilagyi L, Zavodszky P, Westler WM, Markley JL and Graf L. (1997) Effects of serpin binding on the target proteinase: global stabilization, localized increased structural flexibility, and conserved hydrogen bonding at the active site. *Biochemistry* 36: 5455-5464.

Kato A, Hirosawa S, Toyota S, Nakamura Y, Nishi H, Kimura A, Sasazuki T and Aoki N. (1993) Localization of the human alpha-2-plasmin inhibitor gene (PLI) to 17p13. *Cytogenet Cell Genet.* 62: 190-191.

Kawano T, Morimoto K and Uemura Y. (1968) Urokinase inhibitor in human placenta. *Nature*. 217: 253-254.

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.

Kelley RF, DeVos AM and Cleary S. (1991) Thermodynamics of ligand binding and denaturation for His64 mutants of tissue plasminogen activator kringle-2 domain. *Proteins*. 11: 35-44.

Kelm RJ Jr, Swords NA, Orfeo T and Mann KG. (1994) Osteonectin in matrix remodeling. A plasminogen-osteonectin-collagen complex. *J.Biol.Chem*. 269: 30147-30153.

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

Kopito RR and Ron D. (2000) Conformational disease. *Nat Cell Biol* 2: 207-209.

Koyama T, Koike Y, Toyota S, Miyagi F, Suzuki N and Aoki N. (1994) Different NH2-terminal form with 12 additional residues of alpha 2-plasmin inhibitor from human plasma and culture media of Hep G2 cells. *Biochem.Biophys.Res.Commun*. 200: 417-422.

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.

Kruithof EK. (1988) Plasminogen activator inhibitors--a review. *Enzyme*. 40: 113-121.

Knudsen BS, Silverstein RL, Leung LL, Harpel PC and Nachman RL. (1986) Binding of plasminogen to extracellular matrix. *J.Biol.Chem*. 261: 10765-10771.

Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriphage T4. *Nature* 227: 680-685.

Larsson C. (1978) Natural history and life expectancy in severe alpha1-antitrypsin deficiency, Pi Z. *Acta Med Scand*. 204:345-351.

Lawrence DA, Olson ST, Palaniappan S and Ginsburg D. (1994) Engineering plasminogen activator inhibitor 1 mutants with increased functional stability. *Biochemistry*.33: 3643-3648.

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, 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.

Lecander I and Astedt B. (1987) Specific plasminogen activator inhibitor of placental type PAI 2 occurring in amniotic fluid and cord blood. *J.Lab.Clin.Med*. 110: 602-605.

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.

Loebermann H, Tokuoka R, Deisenhofer J and Huber R. (1984) Human alpha 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.

Leonardsson G, Peng XR, Liu K, Nordstrom L, Carmeliet P, Mulligan R, Collen D and Ny T. (1995) Ovulation efficiency is reduced in mice that lack plasminogen activator gene function: functional redundancy among physiological plasminogen activators. *Proc Natl Acad Sci USA*. 92: 12446-12450.

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

Li J, Wang Z, Canagarajah B, Jiang H, Kanost M and Goldsmith EJ. (1999) The structure of active serpin 1K from Manduca sexta. *Structure Fold Des*. 7: 103-109.

Li W, Johnson DJ, Esmon CT and Huntington JA. (2004) Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat Struct Mol Biol*. 11: 857-862.

Lijnen HR, Hoylaerts M and Collen D. (1980) Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. Role in the regulation of fibrinolysis and identification as histidine-rich glycoprotein. *J.Biol.Chem*. 255: 10214-10222.

Lijnen, H.R., Holmes, W.E., Van Hoef, B., Wiman, B., Rodriguez, H. and Collen, D. (1987) Amino-acid sequence of human α_2-antiplasmin. *Eur.J.Biochem*. 166: 565–574.

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

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.

Ling CM, Summaria L and Robbins KC. (1965) Mechanism of formation of bovine plasminogen activator from human plasmin. *J.Biol.Chem*. 240: 4213-4218.

Lobov S, Wilczynska M, Bergstrom F, Johansson LB and Ny T. (2004) Structural bases of the redox-dependent conformational switch in the serpin PAI-2. *J Mol Biol*. 344: 1359-1368.

Lomas DA, Evans DL, Finch JT and Carrell RW. (1992) The mechanism of $Z\alpha_1$-antitrypsin accumulation in the liver. *Nature* 357: 605-607.

Lomas DA, Evans DL, Stone SR, Chang W-SW and Carrell RW. (1993a) Effect of the Z mutation on the physical and inhibitory properties of α_1-antitrypsin. *Biochemistry* 32: 500-508.

Lomas DA, Finch JT, Seyama K, Nukiwa T and Carrell RW. (1993b) α_1-Antitrypsin S_{iiyama} ($Ser^{53}\rightarrow Phe$): further evidence for intracellular loop-sheet polymerization. *J.Biol.Chem*. 268: 15333-15335.

Lomas, D.A. Elliott, P.R. Chang, W.S.W. Wardell M.R. and Carrell, R.W. (1995a) Preparation and characterization of ”latent” alpha 1-antitrypsin. *J.Biol.Chem*. 270: 5282–5288.

Lomas DA, Elliott PR, Sidhar SK, et al. (1995b) Alpha$_1$-antitrypsin Mmalton (Phe^{52} deleted) forms loop sheet polymers in vivo: evidence for the C sheet mechanism of polymerization. *J.Biol.Chem*. 270: 16864-16870.

Loskutoff DJ, van Mourik JA, Erickson LA and Lawrence D. (1983) Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc Natl Acad Sci USA*. 80: 2956-2960.

Magnaghi P, Citterio E, Malgaretti N, Acquati F, Ottolenghi S and Taramelli R. (1994) Molecular characterisation of the human apo(a)-plasminogen gene family clustered on the telomeric region of chromosome 6 (6q26-27). *Hum.Mol.Genet*. 3: 437-442.

Mahadeva R and Lomas DA. (1998) Genetics and respiratory disease. 2. Alpha$_1$-antitrypsin deficiency, cirrhosis and emphysema. *Thorax* 53: 501-505.

Mahadeva R, Chang W-SW, Dafforn TR, et al. (1999) Heteropolymerization of S, I and Z α_1-antitrypsin and liver cirrhosis. *J Clin Invest* 103: 999-1006.

Mahadeva R, Dafforn TR, Carrell RW and Lomas DA. (2002) 6-mer peptide selectively anneals to a pathogenic serpin conformation and blocks polymerization. Implications for the prevention of Z alpha(1)-antitrypsin-related cirrhosis. *J.Biol.Chem*. 277: 6771-6774.

Mångs, H. Sui G.C. and Wiman, B. (2000) PAI-1 stability: the role of histidine residues. *FEBS Lett*. 475: 192–196.

McCracken AA, Kruse KB and Brown JL. (1989) Molecular basis for defective secretion of the Z variant of human alpha-1-proteinase inhibitor: secretion of variants having altered potential for salt bridge formation between amino acids 290 and 342. *Mol.Cell.Biol*. 9: 1406-1414.

Mikus P and Ny T. (1996) Intracellular polymerization of the serpin plasminogen activator inhibitor type 2. *J.Biol.Chem*. 271: 10048-10053.

Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K. and Plow, E.F. (1991) Role of cell-surface lysines in plasminogen binding to cells: identification of α-enolase as a candidate plasminogen receptor. *Biochemistry* 30: 1682–1691.

Morgan D, Diamond DM, Gottschall PE, et al. (2000) A β-peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 408: 982-985.

Moroi M and Aoki N. (1976) Isolation and characterization of alpha2-plasmin inhibitor from human plasma. A novel proteinase inhibitor which inhibits activator-induced clot lysis. *J.Biol.Chem*. 251: 5956-5965.

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 A 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.

Murano G, Williams L, Miller-Andersson M, Aronson DL and King C. (1980) Some properties of antithrombin-III and its concentration in human plasma. *Thromb Res.* 18: 259-262.

Mushunje A, Zhou A, Huntington JA, Conard J and Carrell RW. (2002) Antithrombin 'DREUX' (Lys 114Glu): a variant with complete loss of heparin affinity. *Thromb Haemost*. 88: 436-443.

Mushunje A, Evans G, Brennan SO, Carrell RW and Zhou A. (2004) "latent" antithrombin and its detection, formation and turnover in the circulation. *J.Thromb. Haemost*. 2: 2170-2177.

Ngo TH, Zhou Y, Stassen JM and Declerck PJ. (2002) Importance of N-terminal residues in plasminogen activator inhibitor 1 on its antibody induced latency transition. *Thromb Haemost*. 88: 288-293.

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.

Nordenhem A and Wiman B. (1998) Tissue plasminogen activator (tPA) antigen in plasma: correlation with different tPA/inhibitor complexes. *Scand.J.Clin.Lab. Invest.* 58: 475-483.

Ny T, Bjersing L, Hsueh AJ and Loskutoff DJ. (1985) Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. *Endocrinology*. 116: 1666-1668.

Ny T, Sawdey M, Lawrence D, Millan JL and Loskutoff DJ. (1986) Cloning and sequence of a cDNA coding for the human beta-migrating endothelial-cell-type plasminogen activator inhibitor. *Proc Natl Acad Sci USA*. 83: 6776-6780.

Ny T, Hansson L, Lawrence D, Leonardsson G and Astedt B. (1989) Plasminogen activator inhibitor type 2 cDNA transfected into Chinese hamster ovary cells in stably expressed but not secreted. *Fibrinolysis*. 3: 189-196.

Ny A, Leonardsson G, Hagglund AC, Hagglof P, Ploplis VA, Carmeliet P and Ny T. (1999) Ovulation in plasminogen-deficient mice. *Endocrinology*. 140: 5030-5035.

Olson ST and Shore JD. (1982) Demonstration of a two-step reaction mechanism for inhibition of alpha-thrombin by antithrombin III and identification of the step affected by heparin. *J.Biol.Chem*. 257: 14891-14895.

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.

Ossowski L. (1988) In vivo invasion of modified chorioallantoic membrane by tumor cells: the role of cell surface-bound urokinase. *J.Cell.Biol*. 107: 2437-2445.

Ossowski L, Clunie G, Masucci MT and Blasi F. (1991) In vivo paracrine interaction between urokinase and its receptor: effect on tumor cell invasion. *J.Cell.Biol*. 115: 1107-1112.

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

Pannekoek H, De Vries C and van Zonneveld AJ. (1988) Mutants of human tissue-type plamsinogen activator (t-PA): structural and functional properties. *Fibrinolysis*. 2: 123132.

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. *Biol.Chem.Hoppe.Seyler*. 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, Hauert J, Michaud M and Schapira M. (1995) Formation and properties of C1-inhibitor polymers. *FEBS Lett*. 368: 401-404.

Patston PA and Gettins PG. (1996) Significance of secondary structure predictions on the reactive center loop region of serpins: a model for the folding of serpins into a metastable state. *FEBS Lett*. 383: 87-92.

Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV and Collen D. (1983) Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature*. 301: 214-221.

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

Redlitz, A. Tan, A.K. Eaton D.L. and Plow, E.F. (1995) Plasma carboxypeptidases as regulators of the plasminogen system. *J.Clin.Invest*. 96: 2534–2538.

Reed GL, Matsueda GR and Haber E. (1992) Platelet factor XIII increases the fibrinolytic resistance of platelet-rich clots by accelerating the crosslinking of alpha 2-antiplasmin to fibrin. *Thromb Haemost*. 68: 315-320.

Ries M. (1997) Molecular and functional properties of fetal plasminogen and its possible influence on clot lysis in the neonatal period. *Semin.Thromb.Hemost*. 23: 247-252.

Rijken DC and Collen D. (1981) Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. *J.Biol.Chem*. 256: 7035-7041.

Saito H, Goodnough LT, Knowles BB and Aden DP. (1982) Synthesis and secretion of alpha 2-plasmin inhibitor by established human liver cell lines. *Proc Natl Acad Sci USA*. 79: 5684-5687.

Sakata Y and Aoki N. (1982) Significance of cross-linking of alpha 2-plasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis. *J.Clin.Invest*. 69: 536-542.

Sakharov, D.V. Plow E.F. and Rijken, D.C. (1997) On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. *J.Biol.Chem*. 272: 14477–14482.

Saksela O and Rifkin DB. (1988) Cell-associated plasminogen activation: regulation and physiological functions. *Annu Rev Cell Biol*. 4: 93-126.

Sasaki, T., Morita, T. and Iwanaga, S. (1986) Identification of the plasminogen-binding site of human alpha 2-plasmin inhibitor. *J.Biochem*. 99: 1699–1705.

Scherrer A, Kruithof EK and Grob JP. (1991) Plasminogen activator inhibitor-2 in patients with monocytic leukemia. *Leukemia*. 5: 479-486.

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 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.

Selkoe DJ. (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399: 23-31.

Selim TE, Ghoneim HR, Uknis AB, Colman RW and DeLa Cadena RA. (1997) High-molecular-mass and low-molecular-mass kininogens block plasmin-induced platelet aggregation by forming a complex with kringle 5 of plasminogen/plasmin. *Eur.J.Biochem*. 250: 532-538.

Shore J.D., Day, D.E., Francis-Chmura, A.M., Verhamme, I., Kvassman, J.,. Lawrence D.A *et al* .(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.

Sifers RN, Hardick CP and Woo SLC. (1989) Disruption of the 290-342 salt bridge is not responsible for the secretory defect of the PiZ α_1-antitrypsin variant. *J.Biol.Chem*. 264: 2997-3001.

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.

Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J and Whisstock JC. (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J.Biol.Chem*. 276: 33293-33296.

Sivasothy P, Dafforn TR, Gettins PG and Lomas DA. (2000) Pathogenic alpha 1-antitrypsin polymers are formed by reactive loop-beta-sheet A linkage. *J.Biol.Chem*. 275: 33663-33668.

Sjöström, I., Gröndahl, H., Falk, G., Kronvall, G. and Ullberg, M. (1997) Purification and characterisation of a plasminogen-binding protein from *Haemophilus influenzae*. Sequence determination reveals identity with aspartase. *Biochim.Biophys.Acta*. 1324: 182–190.

Skinner R, Chang WS, Jin L, Pei X, Huntington JA, Abrahams JP, Carrell RW and Lomas DA. (1998) Implications for function and therapy of a 2.9 A structure of binary-complexed antithrombin. *J.Mol.Biol*. 283: 9-14.

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. *Am.J.Hematol*. 38: 226-229.

Stein P and Chothia C. (1991) Serpin tertiary structure transformation. *J.Mol.Biol*.221: 615-621.

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.

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

Stein PE and Carrell RW. (1995) What do dysfunctional serpins tell us about molecular mobility and disease? *Nat.Struct.Biol*. 2: 96-113.

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 Getting, P.G.W. (1998) Mapping the serpin–proteinase complex using single cysteine variants of alpha1-proteinase inhibitor Pittsburgh. *J.Biol.Chem*. 273: 15582–15589.

Sugiyama, N., Sasaki, T., Iwamoto, M. and Abiko, Y. (1988) Binding site of α_2-plasmin inhibitor to plasminogen. *Biochim.Biophys.Acta*. 952: 1–7.

Sumi Y, Ichikawa Y, Nakamura Y, Miura O and Aoki N. (1989) Expression and characterization of pro alpha 2-plasmin inhibitor. *J Biochem*.106: 703-707.

Takahashi Y, Tanaka T, Minowa H, Ookubo Y, Sugimoto M, Nakajima M, Miyauchi Y and Yoshioka A. (1996) Hereditary partial deficiency of plasminogen activator inhibitor-1 associated with a lifelong bleeding tendency. *Int.J.Hematol*. 64: 61-8.

Thewes T, Constantine K, Byeon IJ and Llinas M. (1990) Ligand interactions with the kringle 5 domain of plasminogen. A study by 1H NMR spectroscopy. *J.Biol.Chem*. 265: 3906-3915.

Thorsen S, Clemmensen I, Sottrup-Jensen L and Magnusson S. (1981) Adsorption to fibrin of native fragments of known primary structure from human plasminogen. *Biochim.Biophys.Acta*. 668: 377-387.

Thorsen S and Philips M. (1984) Isolation of tissue-type plasminogen activator-inhibitor complexes from human plasma. Evidence for a rapid plasminogen activator inhibitor. *Biochim.Biophys.Acta*. 802: 111-118.

Tran PB and Miller RJ. (1999) Aggregates in neurodegenerative disease: crowds and power? *Trends Neurosci* 22: 194-197.

Tripputi P, Blasi F, Verde P, Cannizzaro LA, Emanuel BS and Croce CM. (1985) Human urokinase gene is located on the long arm of chromosome 10. *Proc.Natl.Acad.Sci.USA*. 82: 4448-4452.

Ullberg M, Kronvall B and Wiman B (1989) New receptor for human plasminogen on gram positive cocci. *APMIS*. 97: 996–1002.

Ullberg M, Karlsson I, Wiman B and Kronvall G. (1992) Two types of receptors for human plasminogen on group G streptococci. *APMIS*. 100: 21–28.

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.

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.

Vassalli JD, Sappino AP and Belin D.(1991) The plasminogen activator/plasmin system. *J.Clin.Invest*. 88: 1067-1072.

Walker LC and LeVine H. (2000) 3rd. The cerebral proteopathies. *Neurobiol Aging*. 21: 559-561.

Wallen P and Wiman B. (1970) Characteri-zation of human plasminogen. I. On the relationship between different molecular forms of plasminogen demonstrated in plasma and found in purified preparations. *Biochim.Biophys.Acta*. 221: 20-30.

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

Wallen P, Bergsdorf N and Ranby M. (1982) Purification and identification of two structural variants of porcine tissue plasminogen activator by affinity adsorption on fibrin. *Biochim.Biophys.Acta*.719: 318-328.

Wallen P, Pohl G, Bergsdorf N, Ranby M, Ny T and Jornvall H. (1983) Purification and characterization of a melanoma cell plasminogen activator. *Eur.J.Biochem*. 132: 681-686.

Wang H, Yu A, Wiman B and Pap S. (2003) Identification of amino acids in antiplasmin involved in its non-covalent 'lysine-binding-site'-dependent interaction with plasmin. *Eur.J.Biochem*. 270: 2023-2029.

Wang H, Pap S and Wiman B. (2004) Inactivation of antiplasmin at low pH: evidence for the formation of ”latent” molecules. *Thromb.Res*. 114: 301-306.

Wang W, Boffa MB, Bajzar L, Walker JB and Neisheim ME. (1998) A study of the mechanism of inhibition of fibrinolysis by activated thrombin activable fibrinolysis inhibitor. *J.Biol.Chem*. 273 27176–27181.

Wardell MR, Chang WS, 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.

Wei A, Rubin H, Cooperman BS and Christianson DW. (1994) Crystal structure of an uncleaved serpin reveals the conformation of an inhibitory reactive loop. *Nat.Struct.Biol*. 1: 251-258.

Wilczynska M, Fa M, Ohlsson PI and Ny T. (1995) The inhibition mechanism of serpins. Evidence that the mobile reactive center loop is cleaved in the native protease-inhibitor complex. *J.Biol.Chem*. 270: 29652-29655.

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. *Nat.Struct.Biol*. 4: 354-357.

Wilczynska M, Lobov S and Ny T. (2003a) The spontaneous polymerization of plasminogen activator inhibitor type-2 and Z-antitrypsin are due to different molecular aberrations. *FEBS Lett*. 537: 11-16.

Wilczynska M, Lobov S, Ohlsson PI and Ny T. (2003b) A redox-sensitive loop regulates plasminogen activator inhibitor type 2 (PAI-2) polymerization. *EMBO J.* 22: 1753-1761.

Wiman B and Wallen P. (1973) Activation of human plasminogen by an insoluble derivative of urokinase. Structural changes of plasminogen in the course of activation to plasmin and demonstration of a possible intermediate compound. *Eur.J.Biochem.* 36: 25-31.

Wiman B and Wallen P. (1975a) Structural relationship between "glutamic acid" and "lysine" forms of human plasminogen and their interaction with the NH2-terminal activation peptide as studied by affinity chromatography. *Eur.J.Biochem*. 50: 489-494.

Wiman B and Wallen P. (1975b) 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.

Wiman B and Wallen P. (1975c) On the primary structure of human plasminogen and plasmin. Purification and characterization of cyanogen-bromide fragments. *Eur.J.Biochem*. 57: 387-394.

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 Wallen P. (1977a) The specific interaction between plasminogen and fibrin. A physiological role of the lysine binding site in plasminogen. *Thromb.Res*. 10: 213-222.

Wiman B and Collen D. (1978a) On the kinetics of reaction between human antiplas-min and plasmin. *Eur. J.Biochem*. 84: 573–568.

Wiman B, Boman L and Collen D. (1978b) On the kinetics of the reaction between human antiplasmin and a low-molecular-weight form of plasmin. *Eur.J.Biochem*. 87: 143–146.

Wiman B and Collen D. (1978c) Molecular mechanisms of physiological fibrinolysis. *Nature.* 272: 549–550.

Wiman B and Collen D. (1979) On the mechanism of the reaction between human α_2-antiplasmin and plasmin. *J.Biol.Chem*. 254: 9291–9197.

Wiman B, Lijnen HR and Collen D. (1979a) On the specific interaction between the lysine-binding sites in plasmin and complementary sites in α_2-antiplasmin and in fibrinogen. *Biochim.Biophys.Acta*. 579: 142–154.

Wiman B, Almqvist Å and Rånby M. (1979b) The non-covalent interaction between human plasmin and α_2-antiplasmin. *Fibrinolysis* 3: 231–235.

Wiman B. (1980) Affinity-chromatographic purification of human α_2-antiplasmin. *Biochem.J.* 191: 229–232

Wiman B. (1981) *Methods in Enzymology: Human A_2-Antiplasmin*, pp. 395–408. Academic Press, New York.

Wiman B, Nilsson T and Cedergren B. (1982) Studies on a form of α_2-antiplasmin in plasma which does not interact with the lysine-binding sites in plasminogen. *Thromb. Res*. 28: 193–199.

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

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

Ye RD, Wun TC and Sadler JE. (1987) cDNA cloning and expression in Escherichia coli of a plasminogen activator inhibitor from human placenta. *J.Biol.Chem*. 262: 3718-3725.

Ye RD, Ahern SM, Le Beau MM, Lebo RV and Sadler JE. (1989) Structure of the gene for human plasminogen activator inhibitor-2. The nearest mammalian homologue of chicken ovalbumin. *J.Biol.Chem*. 264: 5495-5502.

Zhou A, Huntington JA and Carrell RW. (1999) Formation of the antithrombin heterodimer in vivo and the onset of thrombosis. *Blood.* 94: 3388-3396.

Zhou A, Faint R, Charlton P, Dafforn TR, Carrell RW and Lomas DA. (2001) Polymerization of plasminogen activator inhibitor-1. *J.Biol.Chem*. 276: 9115-922.

Zhou A, Carrell RW and Huntington JA. (2001) The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop. *J.Biol.Chem*. 276: 27541-27547.

Zhou A, Stein PE, Huntington JA and Carrell RW. (2003) Serpin polymerization is prevented by a hydrogen bond network that is centered on his-334 and stabilized by glycerol. *J.Biol.Chem*. 278: 15116-15122.

Zhou A, Huntington JA, Pannu NS, Carrell RW and Read RJ. (2003) How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat.Struct.Biol*. 10: 541-544.

Zhou A, Stein PE, Huntington JA, Sivasothy P, Lomas DA and Carrell RW. (2004) How small peptides block and reverse serpin polymerisation. *J.Mol.Biol*. 342: 931-941.