

The plasmin–antiplasmin system: structural and functional aspects

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Abstract The plasmin–antiplasmin system plays a key role in blood coagulation and fibrinolysis. Plasmin and α_2 -antiplasmin are primarily responsible for a controlled and regulated dissolution of the fibrin polymers into soluble fragments. However, besides plasmin(ogen) and α_2 -antiplasmin the system contains a series of specific activators and inhibitors. The main physiological activators of plasminogen are tissue-type plasminogen activator, which is mainly involved in the dissolution of the fibrin polymers by plasmin, and urokinase-type plasminogen activator, which is primarily responsible for the generation of plasmin activity in the intercellular space. Both activators are multidomain serine proteases. Besides the main physiological inhibitor α_2 -antiplasmin, the plasmin–antiplasmin system is also regulated by the general protease inhibitor α_2 -macroglobulin, a member of the protease inhibitor I39 family. The activity of the plasminogen activators is primarily regulated by the plasminogen activator inhibitors 1 and 2, members of the serine protease inhibitor superfamily.

Keywords Plasmin(ogen) · α_2 -Antiplasmin · Serine protease inhibitors (serpins) · Plasminogen activators ·

Plasminogen activator inhibitors · α_2 -Macroglobulin · Multidomain serine proteases

Abbreviations

A2PI	α_2 -Antiplasmin, α_2 -Plasmin inhibitor
CHO	Carbohydrate
EGF-like	Epidermal growth factor-like
FN1	Fibronectin type I
K	Kringle
LBS	Lysine binding site
LMW	Low molecular weight
α_2 M	α_2 -Macroglobulin
NTP	N-terminal peptide of Pgn
PAI-1, -2	Plasminogen activator inhibitor 1, 2
Pgn	Plasminogen
Plm	Plasmin
RCL	Reactive centre loop
Serpin	Serine protease inhibitor
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor

Introduction [1]

The plasmin–antiplasmin system holds a key position in blood coagulation and fibrinolysis. A schematic representation of the fibrinolytic system with its main components is given in Fig. 1 [2]. The plasmin–antiplasmin system controls and regulates the dissolution of the fibrin polymers into soluble fragments (for details see later sections). As with every complex system, blood coagulation and fibrinolysis also have to be tightly controlled and regulated. Plasminogen (Pgn) is activated by its two main physiological activators, tissue-type plasminogen activator (tPA)

The recommended names in the UniProt Knowledgebase (SwissProt and TrEMBL) were used. The protein structures are based on the coordinates deposited in the Protein Data Bank (PDB) and were visualized as well as rendered using the software PyMOL. If not stated otherwise, the standard rainbow colour representation was used.

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fibrinolytic system

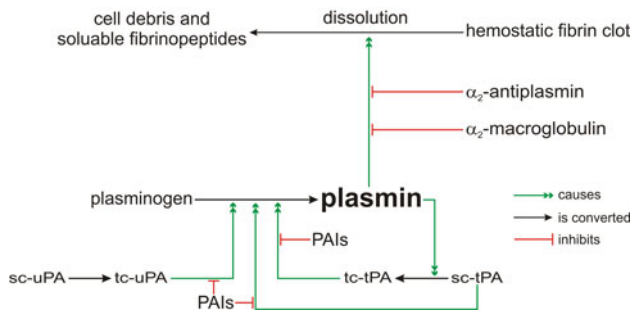


Fig. 1 Schematic representation of the main components of the fibrinolytic system (adapted from reference [2]). *sc-tPA*, *tc-tPA* single-chain, two-chain tPA; *sc-uPA*, *tc-uPA* single-chain, two-chain uPA; *PAIs* plasminogen activator inhibitors

and urokinase-type plasminogen activator (uPA). Plasmin (Plm) is inhibited by its main physiological inhibitor α_2 -antiplasmin (A2PI) and the general protease inhibitor α_2 -macroglobulin (α_2 M). The activities of tPA and uPA are regulated by their two main physiological inhibitors, the plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2).

The structural data for the proteins involved in the plasmin–antiplasmin system discussed in this review article are compiled in Table 1.

Multidomain serine proteases

Many serine proteases in blood plasma are multidomain proteins, especially those involved in blood coagulation and fibrinolysis and those in the complement system. Usually, serine proteases are present in plasma in their inactive proenzyme or zymogen form. The catalytic activity is normally generated by a specific activation process involving limited proteolysis. The 3-D structure of a serine protease domain is usually characterized by two six-stranded antiparallel β -barrels surrounded by α -helical and 3_{10} -helical segments. The active site cleft with the catalytic triad His, Asp, and Ser is located at the interface of two structurally similar subdomains (see sections: [Plasminogen](#), [Tissue-type plasminogen activator](#), and [Urokinase-type plasminogen activator](#)). Besides the serine protease domain, multidomain serine proteases contain specific domains such as kringles, fibronectin (FN), and epidermal growth factor-like (EGF-like) domains, which mediate efficient binding to the target structures thus facilitating an efficient cleavage reaction of the serine protease which is usually strictly limited to the intended site. For instance, efficient binding of plasmin(ogen) to the fibrin blood clot is mediated by the lysine binding sites (LBS) located in the kringle domains of the molecule thus facilitating an efficient cleavage of the fibrin polymer by

Plm into soluble fragments (for details see section [Plasminogen](#)). The most important structural data for the multidomain serine proteases Pgn, tPA, and uPA are summarized in Table 1.

Plasminogen [3–6]

Human Pgn (P00747, EC 3.4.21.7; concentration in plasma 100–200 mg l⁻¹) is a single-chain, multidomain glycoprotein (about 2% CHO) of about 90 kDa (791 amino acids) and is a member of the peptidase S1 family present in blood as zymogen. Pgn is synthesized mainly in the liver as a protein 810 residues long [7], and mature Pgn, Glu-Pgn, comprises 791 amino acids generated during the secretion process by cleaving the 19-residue signal peptide [8]. Glu-Pgn is the proenzyme form of the serine protease Plm, the key component of the fibrinolytic system (Fig. 1). The gene for Pgn (*PLG*, 52.5 kb) is located on chromosome 6q26–6q27 and is organized into 19 exons in the range 75–387 bp [8–10]. In plasma, Pgn is partially bound to histidine-rich glycoprotein (P04196) [11]. Histidine-rich glycoprotein binds at sites of tissue injury and seems to act as a high-affinity receptor to immobilize Pgn on cell surfaces [12].

Pgn is composed of an N-terminal peptide (NTP) [13], five triple-loop structures stabilized by three intrachain disulfide bridges called kringles [14], and the trypsin-like serine protease part carrying the catalytic triad His⁶⁰³, Asp⁶⁴⁶, and Ser⁷⁴¹ [15]. Human Pgn is partially N-glycosylated at Asn²⁸⁹ and O-glycosylated at Ser²⁴⁹ and Thr³⁴⁶ giving rise to Pgn variants I (Asn²⁸⁹, Thr³⁴⁶) and II (only Thr³⁴⁶), which can be readily separated by affinity chromatography [16–20]. In addition, Pgn is partially phosphorylated at Ser⁵⁷⁸ [21] with a so far unassigned function. The schematic organization of the primary structure of human Pgn is shown in Fig. 2 [2]. Two N-terminally different forms of Pgn have been identified, Glu-Pgn and Lys-Pgn. Lys-Pgn is formed by cleavage of the Lys⁷⁷–Lys⁷⁸ peptide bond in Glu-Pgn, releasing the NTP [22].

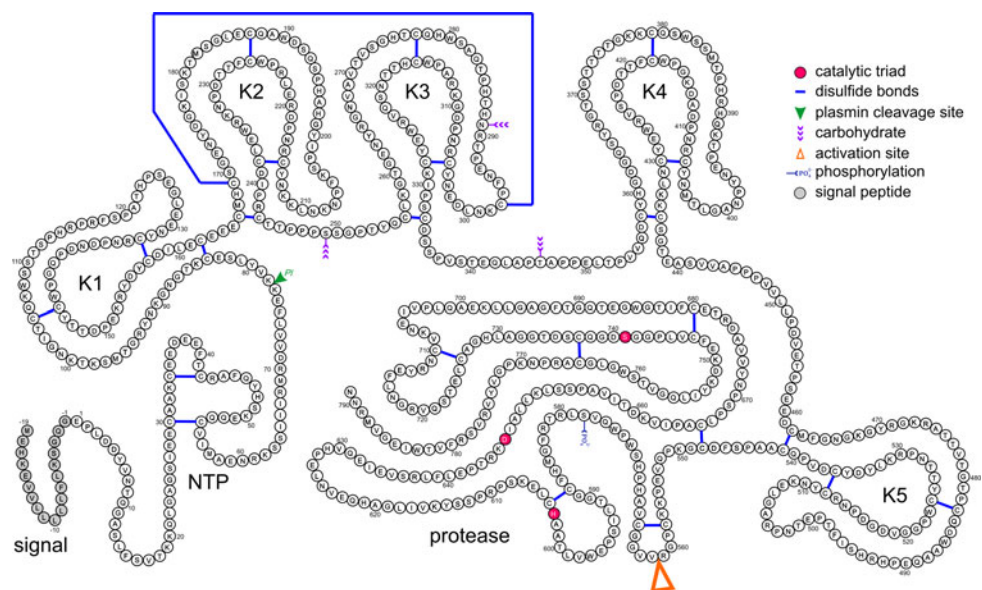
Pgn is activated by the two main physiological plasminogen activators, tPA and uPA [23], to the active, two-chain Plm molecule held together by two interchain disulfide bridges (Cys⁵⁴⁸–Cys⁶⁶⁶, Cys⁵⁵⁸–Cys⁵⁶⁶) by cleavage of the Arg⁵⁶¹–Val⁵⁶² peptide bond and the release of the 77-residue NTP [24, 25]. Upon activation, Pgn undergoes an unusual conformational change usually not observed in proenzyme–enzyme pairs of the trypsin-type family [25]. Native Glu-Pgn exhibits a compact spiral shape, whereas Lys-Pgn and Plm have a more open, elongated structure. In addition to tPA and uPA, two bacteria-derived proteins, streptokinase A or C (A: P10520;

Table 1 Structural data for the main proteins involved in the plasmin–antiplasmin system

Protein (abbreviation)	UniProt entry	Size ^b		Plasma concentration (mg l ⁻¹)	Gene Name	Gene Size (kb)	Domains
		Daltons	Amino acids				
Plasminogen (Pgn)	P00747/EC 3.4.21.7	88,432	791	100–200	<i>PLG</i>	52.5	1 NTP of Pgn, 5 K, serine protease
Tissue-type plasminogen activator (tPA)	P00750/EC 3.4.21.68	59,042	527	0.005–0.01	<i>PLAT</i>	32.4	1 EGF-like, 2 K, 1 FN1, serine protease
Urokinase-type plasminogen activator (uPA)	P00749/EC 3.4.21.73	46,386	411	0.005–0.01	<i>PLAU</i>	6.4	1 EGF-like, 1 K, serine protease
α_2 -Antiplasmin (A2PI)	P08697	50,451	452	~ 70	<i>SERPINF2</i>	~ 16	
α_2 -Macroglobulin (α_2 M)	P01023	160,797	1,451	~ 1,200	<i>A2M</i>	~ 48	
Plasminogen activator inhibitor 1 (PAI-1)	P05121	42,769	379	~ 0.024 (variable)	<i>SERPINE1</i>	12.2	
Plasminogen activator inhibitor 2 (PAI-2)	P05120	46,596	415	<0.01	<i>SERPINB2</i>	16.4	
Neuroserpin	Q99574	44,704	394	not detectable	<i>SERPINI1</i>	89.8	
Glia-derived nexin	P07093	41,865	379		<i>SERPINE2</i>	–	

^b Mass calculated from the amino acid sequence

Fig. 2 Schematic representation of the primary structure of human Pgn (from reference [2]). The catalytic triad (His⁶⁰³, Asp⁶⁴⁶, and Ser⁷⁴¹), the activation site (Arg⁵⁶¹–Val⁵⁶²), the Plm cleavage site (Lys⁷⁷–Lys⁷⁸), the phosphorylation site (Ser⁵⁷⁸), the CHO attachment sites (Asn²⁸⁹, Ser²⁴⁹, and Thr³⁴⁶), and the 24 disulfide bridges as well as the signal peptide are indicated. NTP N-terminal peptide; K1–K5 kringles 1–5

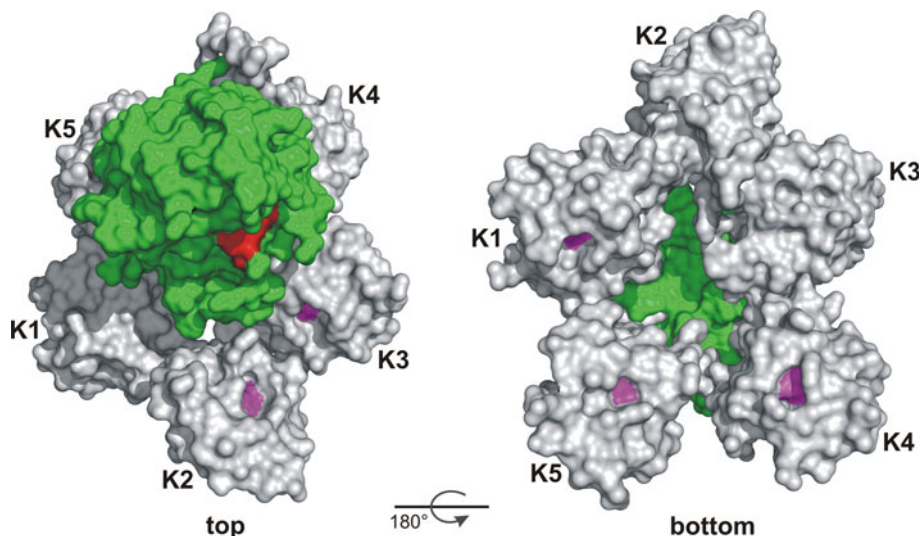


C: P00779) and staphylokinase (P68802), can act as Pgn activators. They form equimolar complexes with Pgn, and this complex is able to activate Pgn [26, 27].

The heavy chain of Plm (483 amino acids) contains five homologous kringle structures, K1 (Cys⁸⁴–Cys¹⁶²), K2 (Cys¹⁶⁶–Cys²⁴³), K3 (Cys²⁵⁶–Cys³³³), K4 (Cys³⁵⁸–Cys⁴³⁵), and K5 (Cys⁴⁶²–Cys⁵⁴¹), with the disulfide bridges arranged in the pattern Cys¹–Cys⁶, Cys²–Cys⁴, Cys³–Cys⁵. Kringles 2 and 3 are linked together in a clamp-like fashion by an additional interkringle disulfide bridge

(Cys¹⁶⁹–Cys²⁹⁷). With the exception of K3, each kringle contains a functional LBS characterized by anionic and cationic centres interspaced by a hydrophobic groove. In the case of human Pgn K1, the anionic and cationic centres contain two Asp and two Arg residues, respectively, and the hydrophobic groove is lined out with aromatic residues (one Trp, three Tyr, and one Phe) [28, 29]. The LBS mediate binding to the substrate fibrin(ogen) [30] and to its main physiological inhibitor A2PI [31–33] and to small molecules of the ω -aminocarboxylic acid type such as

Fig. 3 Top and bottom 3-D structural model of human Pgn based on overlapping 3-D structures of Pgn fragments (form reference [44]). Each kringle is shown in *grey*, the central Trp residue in each LBS is shown in *magenta*, the protease domain is shown in *green*, and the active site residues are shown in *red*



6-aminohexanoic acid [34]. In addition, specific binding via kringle domain(s) to bacteria [35, 36] and to mammalian cell surfaces [37] has been described. K3 is the only kringle with a functionally inactive LBS [38, 39], but substitution of Lys³¹¹ to Asp in the anionic centre of the LBS results in a weak but distinct affinity for ω -amino-carboxylic acids [40]. The affinity of the antifibrinolytic drug 6-aminohexanoic acid for the various kringles decreases in the order K1 > K4 > K5 > K2 >> K3 [41–43].

So far, no 3-D structure of complete human Pgn is available. A 3-D structural model of human Pgn based on known and overlapping 3-D structures of Pgn fragments exhibits the spiral shape shown in Fig. 3 [44], which resembles the known shape of Glu-Pgn visualized by electron microscopy [45, 46]. However, 3-D structural data for all kringle domains and of the catalytic chain are available. As an example, the 3-D structures of the triple-kringle domain K1 + 2 + 3 termed angiostatin (1KI0), determined by X-ray diffraction, is shown in Fig. 4 [47]. The kringle structures are characterized by a central cluster of four Cys residues composed of the two inner disulfide bridges (Cys²–Cys⁴, Cys³–Cys⁵), which are almost perpendicular to each other (as an example, see K2). The kringle domains have a rather low content of secondary structure with some β -strands but usually a low helical content. As an example, K1 is given in the surface representation with negatively and positively charged residues shown in red and in blue, respectively, and all other residues are shown in grey. The central Trp residue of the LBS is marked in magenta (indicated by an arrow). The binding of a ligand has little influence on the conformation but stabilizes the structure. The 3-D structure of the catalytic domain of human Pgn determined by X-ray diffraction (1DDJ) exhibits the expected trypsin-like shape, and is

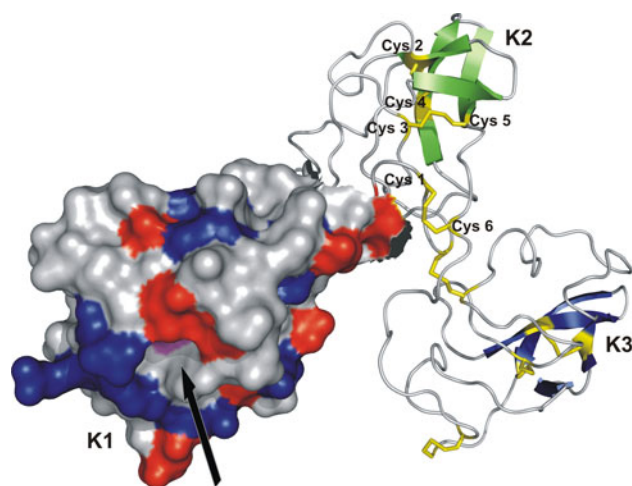


Fig. 4 3-D structure of human Pgn kringles 1 + 2 + 3 (K1, K2, K3) termed angiostatin determined by X-ray diffraction (1KI0, [47]). Each kringle contains three disulfide bridges (yellow) arranged in the pattern Cys¹–Cys⁶, Cys²–Cys⁴, Cys³–Cys⁵ exemplified in kringle 2 (K2). Kringle 1 (K1) is shown as a surface representation with negatively and positively charged residues shown in red and blue, respectively, and all other residues are shown in grey. The central Trp residue of the LBS is shown in magenta (arrow)

shown in Fig. 5 [25]. Each of the two structurally similar subdomains is characterized by an antiparallel β -barrel and the active site cleft is located at the interface of the two subdomains.

The primary function of Plm is the cleavage of insoluble fibrin polymers at specific sites resulting in soluble fragments. A schematic representation of fibrinogen and the pattern of cleavage by Plm is shown in Fig. 6 [48]. The cleavage sites giving rise to the major fragments D and E of fibrin are indicated by black arrows. The degradation of the fibrin polymer by Plm is initiated by the cleavage of the Lys⁵⁸³–Met⁵⁸⁴ peptide bond in the α chain, followed by

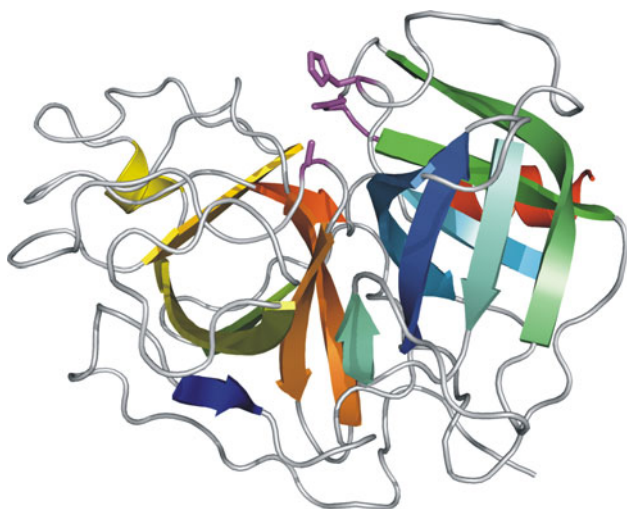


Fig. 5 3-D structure of the catalytic domain of human Pgn determined by X-ray diffraction (1DDJ, [25]). The active site residues located at the interface of the two structurally similar subdomains are shown in magenta

the cleavage of the peptide bonds Lys²⁰⁶-Met²⁰⁷ and Lys²³⁰-Ala²³¹, also in the A α chain, thus releasing a C-terminal 40-kDa fragment and generating fragment X (260 kDa). Cleavage of fragment X in all three chains results in one fragment Y (160 kDa) and one fragment D (100 kDa), and further cleavage of fragment Y produces a second fragment D and fragment E (60 kDa) [48].

In addition, Plm acts as a proteolytic factor in many other physiological processes such as mediation of cell migration by degrading the extracellular matrix, wound healing, tissue remodelling, angiogenesis, embryogenesis, and pathogen and tumour cell invasion [23, 49-54]. The rather broad specificity of Plm in vivo results in the

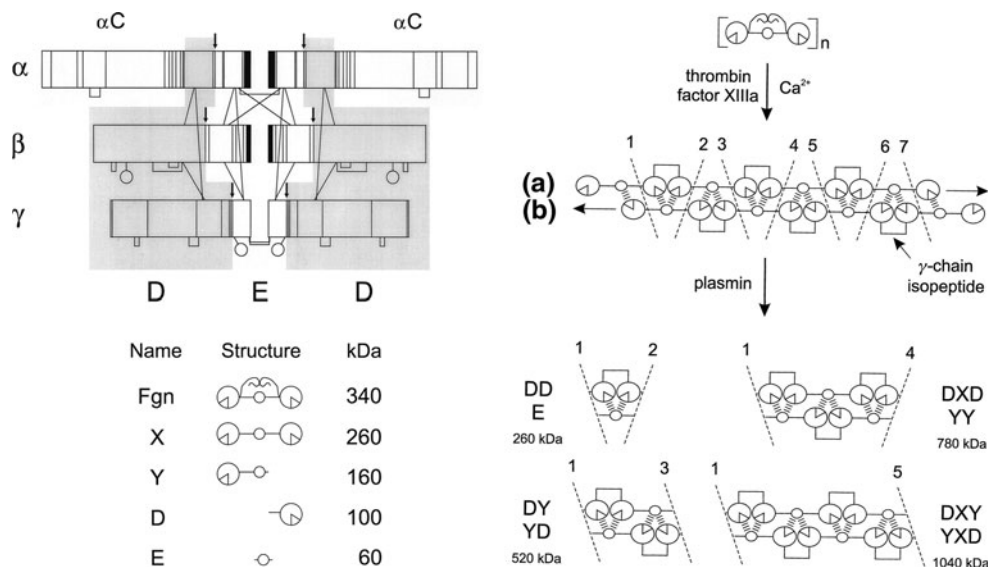
inactivation and degradation of matrix proteins such as collagens, fibronectin (P02751), and laminins [55-57], and components of the blood coagulation cascade such as coagulation factor FVa, von Willebrand factor, and thrombospondin [56, 58, 59]. In vitro, Plm has a similar specificity to trypsin cleaving primarily peptide bonds after basic residues. The main physiological inhibitor of Plm is A2PI, a serpin, which is discussed in section α_2 -Antiplasmin or α_2 -plasmin inhibitor.

Defects or mutations in the PLG gene are the cause of thrombophilia (MIM 188050) [60], a form of recurrent thrombosis, and type I plasminogen deficiency (MIM 173350). Ligneous conjunctivitis (MIM 217090) is usually the most common and initial form of type I plasminogen deficiency and is a rare form of chronic conjunctivitis characterized by chronic tearing and redness of the conjunctivae [61, 62].

Plasminogen activators

There are two main physiological activators of Pgn, tPA and uPA, which are both multidomain serine proteases previously mentioned (see Table 1). Although tPA and uPA both catalyse the same reaction, namely the activation of Pgn to Plm by cleavage of the Arg⁵⁶¹-Val⁵⁶² peptide bond, and thus clearly have the same basic biological function at the molecular level, tPA and uPA have different biological roles. Whereas tPA is primarily responsible for the dissolution of the fibrin polymers by Plm and thus helps to maintain vascular haemostasis, uPA is predominantly involved in the generation of pericellular Plm activity for the degradation of the extracellular matrix and for other intercellular processes where Plm activity is required.

Fig. 6 Cleavage of human fibrinogen by Plm (from reference [48]). Schematic representation of fibrinogen, the fibrin polymer, the pattern of cleavage by Plm, and the main fragments generated. The main Plm cleavage sites are indicated by arrows. Cleavage of fibrin by Plm leads to the main fragments X (260 kDa), Y (160 kDa), D (100 kDa), and E (60 kDa)



Tissue-type plasminogen activator

Human tPA (P00750, EC 3.4.21.68; concentration in plasma 5–10 $\mu\text{g l}^{-1}$) is synthesized by various cell types such as endothelial cells and keratinocytes and also in the brain [63, 64]. It is a single-chain multidomain glycoprotein (about 7% CHO) of 70 kDa (527 amino acids) containing one O-glycosylation site at Thr⁶¹ and three N-glycosylation sites at Asn¹¹⁷, Asn¹⁸⁴ (partial), and Asn⁴⁴⁸ [65, 66] and belongs to the peptidase S1 family [67]. The tPA gene (*PLAT*, 32.4 kb) is located on chromosome 8p12 and is organized into 14 exons in the range 43–914 bp [68, 69]. The single-chain form (proenzyme) itself exhibits a very high enzymatic activity compared with the fully active two-chain form of tPA, which is a unique property of the proenzyme form of serine proteases. The single-chain form is converted to the completely active, two-chain form held together by a single interchain disulfide bridge (Cys²⁶⁴–Cys³⁹⁵) upon cleavage of the Arg²⁷⁵–Ile²⁷⁶ peptide bond by Plm, kallikrein, or coagulation factor Xa [70]. The A-chain (275 amino acids) contains one FN1, one EGF-like, and two kringle domains and the B-chain (252 amino acids) comprises the serine protease part [71]. The second kringle in tPA carries an active LBS as do the kringles in Pgn [72], but kringle 1 is devoid of an active LBS. This seems to be due to the replacement of the usual Trp residue by a Ser in the hydrophobic cleft of the LBS in tPA kringle 1 [73].

The 3-D structure of the double module FN1+EGF-like was determined by NMR spectroscopy (ITPG) and is shown in Fig. 7a [74]. The FN1 domain (43 amino acids) is characterized by two antiparallel β -sheets, a double stranded sheet and a triple-stranded sheet (shown in red), stabilized by two intrachain disulfide bridges arranged in the pattern Cys¹–Cys³, Cys²–Cys⁴. The first disulfide bridge links the two β -sheets, the second disulfide bridge is located within the triple-stranded β -sheet. The EGF-like domain (39 amino acids) is characterized by two antiparallel, two-stranded β -sheets (shown in blue) interconnected by a loop structure. The structure is stabilized by three intrachain disulfide bridges arranged in the pattern Cys¹–Cys³, Cys²–Cys⁴, Cys⁵–Cys⁶. It appears that the FN1+EGF-like double module, the two kringles, and the serine protease domain are involved in the interaction of tPA with fibrin [75, 76]. tPA binds with high affinity to fibrin, resulting in an enhanced activation of Pgn by tPA. Exposed C-terminal Lys and Arg residues in fibrin generated by proteolysis with Plm bind to the LBS in kringle 2 of tPA [77]. The 3-D structure of the catalytic domain of human tPA in complex with a low molecular weight (LMW) inhibitor (dansyl-EGR-chloromethyl ketone) was determined by X-ray diffraction (1RTF) and exhibits the expected trypsin-like fold (Fig. 7b) [78]. The inhibitor is

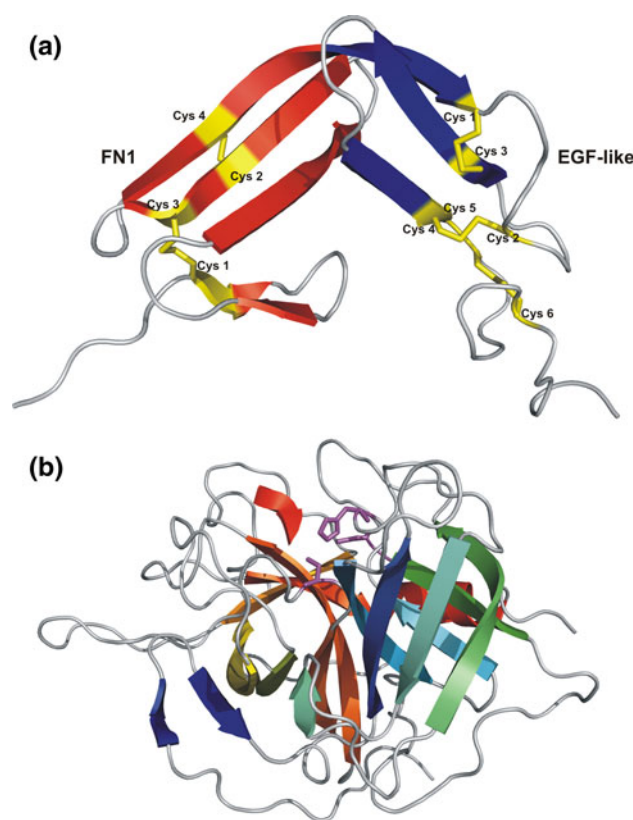


Fig. 7 **a** 3-D structure of the double module of human tPA comprising the fibronectin type I (FN1) domain and the epidermal growth factor-like (EGF-like) domain determined by NMR spectroscopy (ITPG, [74]). The disulfide bridges shown in yellow exhibit the following patterns: in FN1 (red) Cys¹–Cys³, Cys²–Cys⁴, and in EGF-like (blue) Cys¹–Cys³, Cys²–Cys⁴, Cys⁵–Cys⁶. **b** 3-D structure of the catalytic domain of human tPA in complex with the LMW inhibitor dansyl-EGR-chloromethyl ketone determined by X-ray diffraction (1BDA, [78]). The inhibitor shown in red is covalently bound to His³²² and Ser⁴⁷⁸ in the active site cleft (magenta)

covalently bound to His³²² and Ser⁴⁷⁸ in the active site cleft of tPA located at the interface of the two subdomains. Lys⁴²⁹ forms a salt bridge with Asp⁴⁷⁷ promoting an active conformation in single-chain tPA. The single-chain, proenzyme form of tPA exhibits only a five to tenfold decreased activity compared with fully active, two-chain tPA [79]. Both forms seem to exhibit the same biological function.

Interactions of tPA with endothelial cells and smooth muscle vascular cells lead to an increased activation of Pgn. In the case of endothelial cells, the Ca²⁺/phospholipid-binding protein annexin A2 (P07755) binds via its exposed C-terminal Lys and Arg residues to the kringle 2 domain of tPA [80, 81].

The main physiological inhibitors of tPA are PAI-1 and PAI-2, which are discussed in section Plasminogen activator inhibitor 1 and Plasminogen activator inhibitor 2, respectively. An increased activity of tPA leads to hyperfibrinolysis (MIM 173370), an excessive bleeding disorder

[82]. There are several forms of tPA in therapeutic use, among them alteplase (Activase, Genentech) and reteplase (Retavase, Centocor, and Roche), which are used to initiate fibrinolysis in the case of acute myocardial infarction, acute ischaemic stroke, and pulmonary embolism.

Urokinase-type plasminogen activator

Human uPA (P00749, EC 3.4.21.73; concentration in plasma 5–10 $\mu\text{g l}^{-1}$) is synthesized in the lung and the kidney, but also in keratinocytes and endothelial cells [83, 84]. It is a single-chain multidomain glycoprotein (about 2% CHO) of 55 kDa (411 amino acids) [85, 86] containing one O-glycosylation site at Thr¹⁸ and one N-glycosylation site at Asn³⁰² and two phosphorylation sites at Ser¹³⁸ and Ser³⁰³ [87, 88]. The phosphorylation of Ser¹³⁸/Ser³⁰³ seems to modulate the urokinase receptor (uPAR) transducing ability [88]. The uPA gene (*PLAU*, 6.4 kb) is located on chromosome 10q24 and is organized into 11 exons [89]. Human uPA is converted to its active, two-chain high molecular weight form held together by a single interchain disulfide bridge (Cys¹⁴⁸–Cys²⁷⁹) upon cleavage of the Lys¹⁵⁸–Ile¹⁵⁹ peptide bond by Plm, kallikrein, coagulation factor XIIa, or cathepsin [90]. The A-chain (157 amino acids) contains one EGF-like and one kringle domain and the B-chain (253 amino acids) comprises the serine protease part. In addition to the high molecular weight form, there is also a LMW form of uPA, the major form found in urine. The LMW form of uPA is generated by Plm or by uPA itself cleaving the Lys¹³⁵–Lys¹³⁶ peptide bond. Thus LMW uPA is devoid of the EGF-like and kringle domains, and the former A-chain consists only of a minichain (22 amino acids) linked to the catalytic domain by a single interchain disulfide bridge [91].

The 3-D structure of the double domain EGF-like+kringle determined by NMR spectroscopy (1URK) is shown in Fig. 8a [92]. The EGF-like module exhibits the same structural fold as the corresponding domain in tPA (Fig. 7a) and the kringle domain resembles the kringles in other kringle-containing proteins, e.g. human Pgn (Fig. 4). The kringle structure in uPA is devoid of an active LBS due to the replacement of essential residues in the cationic (Lys→Tyr) and anionic (Asp→Arg) centres and two aromatic residues in the hydrophobic groove of the LBS. Three consecutive Arg residues (Arg¹⁰⁸–Arg¹¹⁰) and two His residues (His⁸⁵ and His⁸⁷) at the opposite ends of the kringle domain are involved in heparin binding [93, 94]. The EGF-like module mediates binding to its specific cellular receptor uPAR [95, 96]. The 3-D structure of human LMW uPA was determined by X-ray diffraction (1GJA) and is shown in Fig. 8b [97]. The serine protease part exhibits the expected trypsin-like fold with two subdomains each containing one antiparallel β -barrel and the

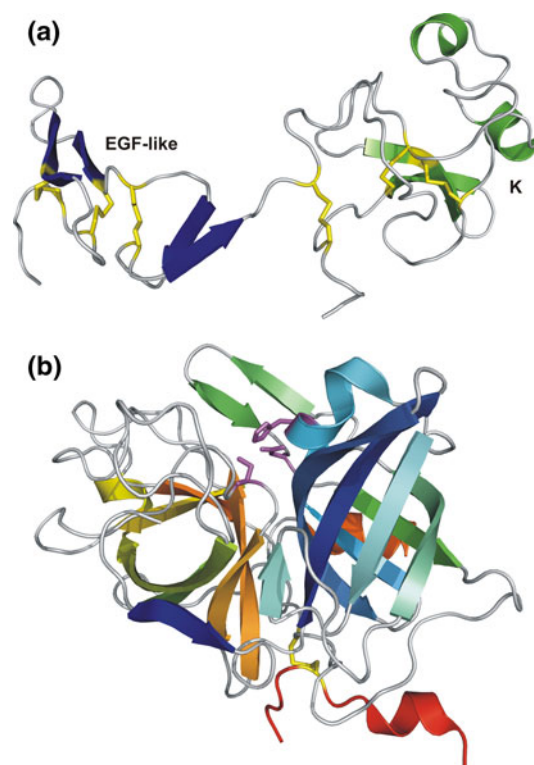


Fig. 8 **a** 3-D structure of the EGF-like (blue) and kringle (green) double domain of human uPA determined by NMR spectroscopy (1URK, [92]). The disulfide bridges are shown in yellow and are arranged as in Fig. 7a (EGF-like) and Fig. 4 (kringle). **b** 3-D structure of human LMW uPA determined by X-ray diffraction (1GJA, [96]). The minichain (red) is linked to the catalytic domain by a single interchain disulfide bridge (yellow)

active site cleft with the catalytic triad is located at the interface of the two subdomains. The minichain (in red) is linked to the catalytic domain by a single interchain disulfide bridge (in yellow).

The main physiological inhibitors of uPA are PAI-1 and PAI-2, which are discussed in section [Plasminogen activator inhibitor 1](#) and [Plasminogen activator inhibitor 2](#), respectively. The uPA system consisting of the serine proteases Plm and uPA, the serpins A2PI, PAI-1, and PAI-2, and uPAR seems to play an important role in cancer metastasis [98]. uPA is available under the name Abbotkinase (Abbott) and is used clinically in the treatment of thrombolytic disorders (pulmonary embolism).

Serine protease inhibitors (serpins) [99–102]

The regulation of proteolytic activity is a fundamental process in all living organisms. Serine protease inhibitors (serpins) are inhibitors (mass range 40–70 kDa) of serine proteases and more than 1,500 serpin-like genes have been identified so far in all three kingdoms of life as well as in

viruses [101, 103, 104]. Serpins regulate very diverse physiological processes such as coagulation and fibrinolysis, complement activation, inflammation, angiogenesis, apoptosis, neoplasia, and viral pathogenesis.

Inhibitory serpins are different from other inhibitors in blood plasma in the sense that they are consumed in a one-to-one reaction known as ‘suicide inhibition’. The standard serpin structure is characterized by a conserved core structure usually built of three β -sheets (A, B, and C in red, green, and yellow, respectively), nine α -helices (hA–hI in grey), and the reactive centre loop (RCL in blue) containing the serine protease recognition site with the scissile peptide bond (P1–P1’). Serpins exist in different conformational states, some of which have been characterized in detail as can be seen in Fig. 9 [100]. The various conformational states carry characteristic features:

1. The native state: the native state is usually characterized by three β -sheets A, B, and C, nine α -helices hA–hI, and an RCL of approximately 20 amino acids located on the surface of the molecule.
2. The latent, inserted state: in the latent state the RCL is inserted into β -sheet A. This uncleaved, stressed state is metastable and is often compared with a spring under tension.
3. The cleaved state: in the cleaved state the stress is released, the scissile peptide bond P1–P1’ in the RCL is cleaved, and the N- and C-terminal ends of the cleaved peptide bond are now on either side of the molecule, often as far apart as 70 Å.
4. The serpin–serine protease complex: in the first step of the inhibition reaction a noncovalent Michaelis-like

complex is formed between the serpin and the serine protease characterized by an interaction between the RCL of the serpin and the catalytic triad of the serine protease.

5. Cleaved serpin with inactivated serine protease: upon insertion of the RCL into β -sheet A the serine protease is drawn from one side of the serpin to the opposite side and the cleavage of the reactive peptide bond releases the tension. The serpin is now covalently linked to the side-chain of the serine residue in the catalytic triad of the serine protease.

The inhibition of a serine protease by a serpin is a two-stage process [105]:

1. The first step is a fast and reversible second-order reaction leading to a noncovalent 1:1 Michaelis-like complex of the serpin with the serine protease.
2. The second step is a slower and irreversible first-order reaction with the insertion of the RCL into β -sheet A and the subsequent cleavage of the reactive peptide bond P1–P1’ in the RCL, resulting in the covalent attachment of the serpin to the side-chain of the Ser residue in the catalytic triad of the serine protease.

Plasmin inhibitors

The main physiological inhibitor of Plm is the serpin A2PI which has unique N- and C-terminal extensions and as a

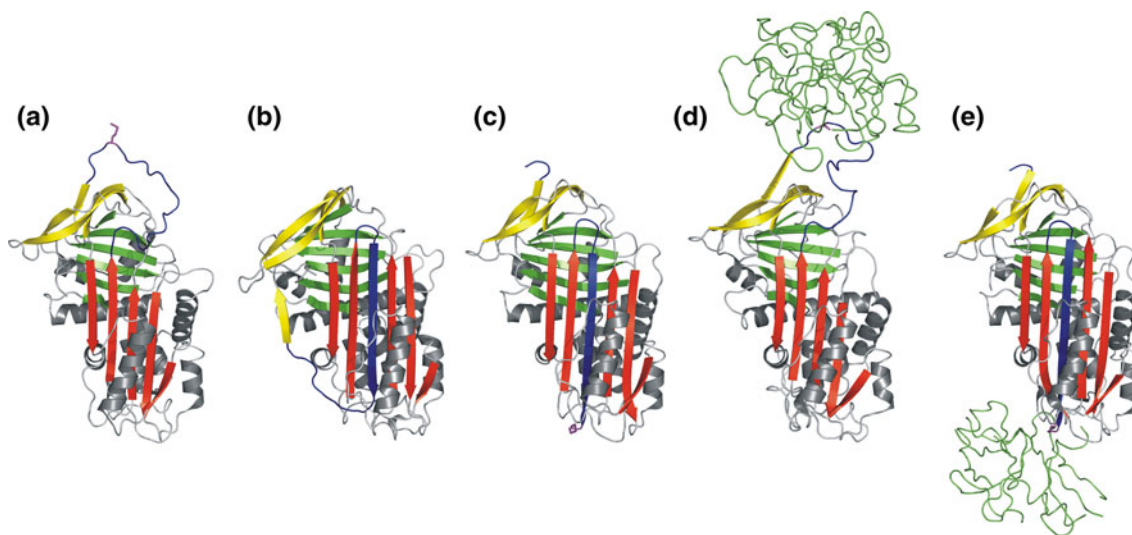


Fig. 9 Various conformational states of serpins [100]. **a** Native state of human α_1 -antitrypsin (IQLP). **b** Latent, inserted state of human antithrombin III (2ANT). **c** Cleaved state of human α_1 -antitrypsin (7API). **d** Noncovalent, Michaelis-like complex of alaserpin (from

Manduca sexta) in complex with rat trypsin (1I99). **e** Cleaved serpin with inactivated serine protease of human α_1 -antitrypsin with bovine trypsin (1EZS)

consequence also has special biological properties. In addition, Plm activity is also regulated by the general protease inhibitor α_2 M.

α_2 -Antiplasmin or α_2 -plasmin inhibitor [106, 107]

Human A2PI, also termed α_2 -plasmin inhibitor (P08697; concentration in plasma: about 70 mg l⁻¹), a member of the serpin superfamily, is a single-chain plasma glycoprotein (14% CHO) of about 67 kDa primarily synthesized in the liver [108, 109]. It contains four fully glycosylated N-glycosylation sites (Asn⁸⁷, Asn²⁵⁰, Asn²⁷⁰, Asn²⁷⁷) [110, 111], a single disulfide bridge (Cys³¹–Cys¹⁰⁴), two Cys residues (Cys⁶⁴ and Cys¹¹³) of undefined state [112], and a sulfated Tyr residue (Tyr⁴⁴⁵) of unknown function [113]. The A2PI gene (*SERPINF2*, about 16 kb) is located on chromosome 17p13.3 and is organized into ten exons [114]. In human plasma, two N-terminally different forms are in circulation: (1) a form with 464 residues with Met as N-terminus (Met-A2PI), and (2) a form with 452 residues with Asn as N-terminus (Asn-A2PI) [115, 116]. Approximately 30% of mature A2PI circulates in human plasma in a C-terminally truncated form lacking at least 26 amino acids [117]. This truncated form inhibits Plm less rapidly than mature A2PI [118, 119]. The RCL contains the scissile peptide bond P1–P1' at Arg³⁶⁴–Met³⁶⁵ [120]. So far, no 3-D structure of human A2PI is available. However, the 3-D structure of a N-terminally truncated form of murine A2PI (Q61247) sharing 71% sequence identity with human A2PI was determined by X-ray diffraction (2R9Y) [121]. As expected, murine A2PI exhibits the typical fold of the serpin core structure with three β -sheets and nine α -helices. However, the 3-D structures of the unique N- and C-terminal extensions of A2PI are still unsolved. In the N-terminal segment of A2PI Gln² forms an isopeptide bond with Lys³⁰³ in the A α chain of fibrin(ogen) catalysed by activated coagulation factor XIIIa [122, 123]. The unique C-terminal extension of A2PI (about 55 amino acids) containing the plasmin(ogen) binding site reveals a remarkable sequence identity between human and other species in the range 58–70%. The C-terminal extension of A2PI is characterized by four totally and two partially conserved Lys residues, of which the C-terminal Lys⁴⁵² is most likely the main binding partner to the LBS in the Pgn kringle domains and Lys⁴³⁶ seems to exhibit a cooperative effect [32, 44]. In contrast, Wang et al. [33, 124] emphasized that Lys⁴³⁶ is the main binding partner and Lys⁴⁵² has no significant influence on the binding to the LBS in the Pgn kringle domains.

A2PI is the main physiological inhibitor of Plm, the main component of the fibrinolytic system, but A2PI also inhibits chymotrypsin and trypsin. Kinetic data show a two-step reaction for the rapid inhibition of Plm: a

reversible second order reaction (k_1 about $3.8 \pm 0.4 \times 10^7$ M⁻¹s⁻¹) followed by an irreversible first-order reaction (k_2 about $4.2 \pm 0.4 \times 10^6$ M⁻¹s⁻¹) [31, 125–128]. In the case of chymotrypsin the reactive bond in the RCL is moved one position further down to the C-terminus of A2PI, to Met³⁶⁵–Ser³⁶⁶, and the association constant (K_a) for inhibiting bovine chymotrypsin is 6.7×10^5 M⁻¹s⁻¹ [129].

Defects in the *SERPINF2* gene result in A2PI deficiency (MIM 262850), an autosomal recessive disorder causing severe haemorrhagic diathesis, an unusual susceptibility to bleeding ([130, 131].

α_2 -Macroglobulin [132]

Human α_2 M (P01023; concentration in plasma about 1,200 mg l⁻¹) is a member of the protease inhibitor I39 family. It is primarily synthesized in the liver [133]. The α_2 M gene (*A2M*, about 48 kb) is located on chromosome 12p13.3–p12.3 and consists of 36 exons in the range 21–229 bp [134]. Mature α_2 M is a large single-chain plasma glycoprotein (about 10% CHO) of 1,451 amino acids containing eight N-glycosylation sites at Asn³², Asn⁴⁷, Asn²²⁴, Asn³⁷³, Asn³⁸⁷, Asn⁸⁴⁶, Asn⁹⁶⁸, and Asn¹⁴⁰¹ [135]. In plasma, α_2 M is present as a tetramer (about 720 kDa) composed of two noncovalently associated dimers oriented in an antiparallel fashion, and the two monomers in the dimers are held together covalently by two interchain disulfide bridges (Cys²⁵⁵–Cys⁴⁰⁸, Cys⁴⁰⁸–Cys²⁵⁵) [136]. Gln⁶⁷⁰ and Gln⁶⁷¹ are potential crosslinking sites, with the side-chains of Lys residues in other proteins forming isopeptide bonds comparable to Gln² in Asn-A2PI (see section α_2 -Antiplasmin or α_2 -plasmin inhibitor). Like the structurally related complement components C3 (P01024) and C4 (P0C0L4, P0C0L5), α_2 M contains a reactive isoglutamyl cysteine thioester bond Cys⁹⁴⁹–Gly–Glu–Gln⁹⁵². α_2 M contains a 'bait' region of 39 amino acids (Pro⁶⁶⁷–Thr⁷⁰⁵) with three short inhibitory sequence segments (Arg⁶⁸¹–Glu⁶⁸⁶, Arg⁶⁹⁶–Val⁷⁰⁰, Thr⁷⁰⁷–Phe⁷¹²) [137].

α_2 M is a general inhibitor of all four types of proteases and acts as a scavenger. Limited proteolysis in the 'bait' region of α_2 M at specific cleavage sites by the active protease induces a large conformational change in α_2 M, resulting in the trapping of the protease in a large central cavity [135]. Concomitantly, the internal thioester bond is cleaved, which mediates the covalent binding of α_2 M to the protease. In the case of Plm, it appears that proteolysis and conformational changes in α_2 M are limited to one of the two subunits and the association constant K_a for Plm is 1.3×10^5 M⁻¹s⁻¹ [138].

α_2 M seems to be associated with Alzheimer disease (MIM 103950) and a deletion in exon 18 seems to be the cause of an increased risk of Alzheimer disease [139].

Plasminogen activator inhibitors

There are two main physiological plasminogen activator inhibitors, PAI-1 and PAI-2, which are more or less directly involved in the inhibition process of the main plasminogen activators, tPA and uPA. In addition, neuroserpin (predominantly expressed in the brain) mainly inhibits tPA, uPA, and Plm, whereas glia-derived nexin has a broader specificity, but also inhibits uPA and Plm.

Plasminogen activator inhibitor 1

Human PAI-1 (P05121; concentration in plasma about $20 \mu\text{g l}^{-1}$, variable) is a member of the serpin superfamily. It is produced by a variety of cells such as endothelial cells, smooth muscle cells, and liver cells [140]. PAI-1 is a single-chain plasma glycoprotein (13% CHO) of 50 kDa (379 amino acids) containing three N-glycosylation sites at Asn²⁰⁹, Asn²⁶⁵, and Asn³²⁹ (potential) [141]. The PAI-1 gene (*SERPINE1*, 12.2 kb) is located on chromosome 7q21.3-q22 and consists of nine exons [142]. The scissile peptide bond P1–P1' is located at Arg³⁴⁶–Met³⁴⁷ [141, 143]. The 3-D structure of PAI-1 in complex with two inhibitory RCL pentapeptides (Ac-TVASS-NH₂) was determined by X-ray diffraction (1A7C) and is shown in Fig. 10 [144]. The

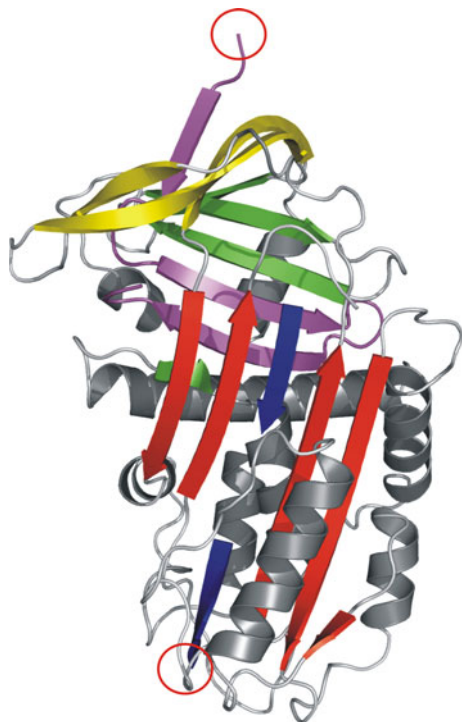


Fig. 10 3-D structure of human PAI-1 in complex with two inhibitory RCL pentapeptides (Ac-TVASS-NH₂) determined by X-ray diffraction (1A7C, [144]). The two pentapeptides (blue) bind between β -strands 3 and 5 in β -sheet A (red). The cleaved C-terminal region is shown in magenta and the cleaved ends of the reactive peptide bond are located on either side of the molecule (red circles)

two pentapeptides (in blue) bind between the space of β -strands 3 and 5 in β -sheet A (in red). This prevents the insertion of the RCL into β -sheet A and as a consequence abolishes the inhibitory reaction with the target serine protease. This form of PAI-1 exhibits the typical structural features of cleaved serpins with the cleaved regions of the RCL on either side of the molecule (red circles) and the cleaved C-terminal region is shown in magenta.

PAI-1 primarily inhibits tPA and uPA with second-order rate constants of $2.5\text{--}4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for two-chain tPA and $1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for uPA, thus regulating fibrinolysis by limiting the Plm production. In addition, PAI-1 also inhibits other serine proteases, but at much slower rates, e.g. thrombin ($1.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$), Plm ($6.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, bovine PAI-1), trypsin ($7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$), and activated protein C [145–148]. PAI-1 is bound to vitronectin (P04004) in plasma and in the extracellular matrix [149]. Vitronectin and also heparin enhance the inhibition rate constant of PAI-1 for thrombin by a factor of up to 200-fold [150]. In the case of a vascular injury, activated platelets can increase the low plasma concentration of PAI-1 by a factor of 10 [151, 152].

Defects in the *SERPINE1* gene are the cause of PAI-1 deficiency (MIM 173360), characterized by an abnormal bleeding tendency [153]. High levels of PAI-1 seem to be associated with myocardial infarction [154].

Plasminogen activator inhibitor 2 [155, 156]

Human PAI-2 (P05120; concentration in plasma $<5 \mu\text{g l}^{-1}$, only detectable during pregnancy [157]) is a member of the serpin superfamily. It is produced by epithelial cells, monocytes and macrophages, and keratinocytes [158–162]. Due to the lack of a proper signal sequence (it contains an inefficient internal signal sequence [163]) PAI-2 is not efficiently secreted and therefore accumulates intracellularly as a non-glycosylated 47-kDa protein [164]. A small portion of single-chain PAI-2 is secreted via a facultative translocation pathway [165] as a 60-kDa (415 amino acids) plasma glycoprotein containing three potential N-glycosylation sites at Asn⁷⁵, Asn¹¹⁵, and Asn³³⁹, and one assigned disulfide bridge (Cys⁵–Cys⁴⁰⁵) [156]. The PAI-2 gene (*SERPINB2*, 16.4 kb) is located on chromosome 18q21.2-q22 and contains eight exons [166, 167]. The scissile peptide bond P1–P1' is located at Arg³⁸⁰–Thr³⁸¹ [168]. As Gln² in A2PI, Gln⁸³, Gln⁸⁴, and Gln⁸⁶ located in the C–D interhelical domain in PAI-2 (in other serpins corresponding to the C–D loop) can form isopeptide bonds with Lys residues in the A α chain of fibrinogen, i.e. Lys¹⁴⁸, Lys¹⁷⁶, Lys¹⁸³, Lys²³⁰, Lys⁴¹³, and Lys⁴⁵⁷ catalysed by coagulation factor XIIIa [169, 170]. The 3-D structure of a deletion mutant (deletion of loop Asn⁶⁶–Gln⁹⁸) of PAI-2 was determined by X-ray diffraction (1BY7) and is shown in Fig. 11 [171]. This deletion mutant represents the stressed state of a serpin with a disordered structure of the

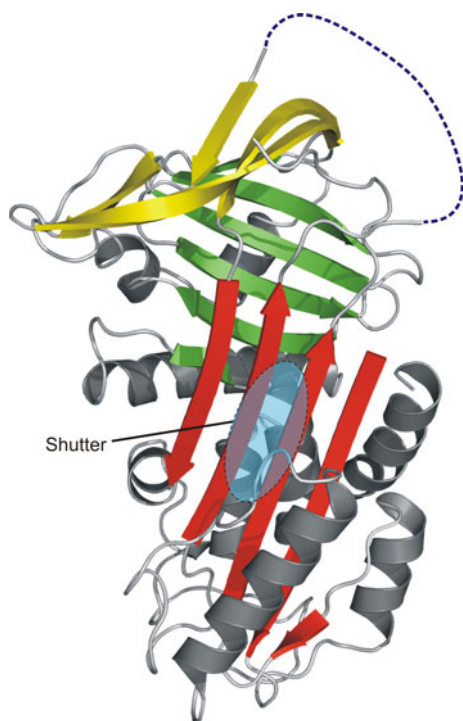


Fig. 11 3-D structure of a deletion mutant of human PAI-2 determined by X-ray diffraction (1BY7, [171]). The reconstructed RCL is shown as a *dashed blue line* and the shutter region located beneath β -sheet A (red) is shown as a *blue-shaded oval*

RCL (the reconstructed RCL is represented as dashed, blue line). A buried cluster of polar amino acids beneath β -sheet A (in red), the so-called shutter region (indicated by a blue-shaded oval) stabilizes the stressed as well as the relaxed forms of serpins. As expected, the insertion of the RCL into β -sheet A is essential for the inhibition reaction.

PAI-2 is an efficient inhibitor of tPA and uPA with second-order rate constants of $0.8\text{--}1.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for two-chain tPA and $2.4\text{--}2.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for uPA [172]. PAI-2 is only detectable in plasma during pregnancy and probably has a role in maintaining the placenta or in embryonic development [157]. As mentioned above, only a small portion of PAI-2 is secreted. Intracellular PAI-2 exhibits other functions than inhibition of uPA and tPA, e.g. PAI-2 influences cell proliferation and cell differentiation, inhibits apoptosis, and alters gene expression.

Little is known of precisely described diseases related to PAI-2 defects (MIM 173390).

Neuroserpin [173–175]

Neuroserpin (Q99574; usually not detectable in plasma) is a member of the serpin superfamily. It is predominantly expressed in the brain [176, 177]. Neuroserpin is a single-chain glycoprotein of 55 kDa (394 amino acids) containing three potential N-glycosylation sites at Asn¹⁴¹, Asn³⁰⁵, and

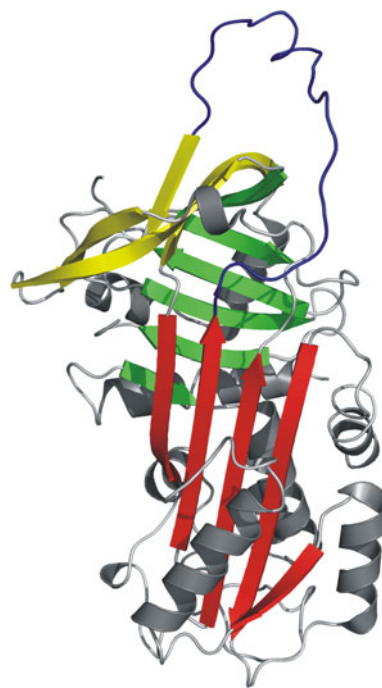


Fig. 12 3-D structure of uncleaved, native human neuroserpin determined by X-ray diffraction (3F5N, 3F02, [180]). Neuroserpin contains the typical structural elements of uncleaved, native serpins: three β -sheets (red, green, yellow), nine α -helices (grey), and the exposed RCL (blue)

Asn³⁸⁵ [178, 179]. The neuroserpin gene (*SERPINI1*, 89.8 kb) is located on chromosome 3q26.1 and consists of nine exons [176, 177]. The scissile peptide bond P1–P1' is located at Arg³⁴⁶–Met³⁴⁷ [176]. The 3-D structure of uncleaved, native neuroserpin was determined by X-ray diffraction (3F5N, 3F02) and is shown in Fig. 12 [180]. It contains the expected elements of the core structure of serpins: three β -sheets (shown in red, green, and yellow), nine α -helices (in grey), and the exposed RCL (in blue).

tPA, uPA, and Plm have been identified as the main targets of neuroserpin activity [176, 180]. The corresponding association constants K_a for recombinant neuroserpin (chicken) are: (1) $1.5 \pm 0.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (sc-tPA), (2) $4.7 \pm 0.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (uPA), and (3) $1.1 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Plm) [181].

Neuroserpin seems to be involved in the formation and reorganization of synaptic connections and may be involved in the protection of neurons from cell damage by tPA. Defects in the *SERPINI1* gene are the cause of familial encephalopathy characterized by neuroserpin inclusion bodies [177, 182].

Glia-derived nexin

Human glia-derived nexin (P07093) is a member of the serpin superfamily. It is synthesized by fibroblasts, heart

muscle cells, and kidney epithelial cells, and also in the brain [183]. Glia-derived nexin is a single-chain plasma glycoprotein (about 6% CHO) of 43 kDa (379 amino acids) containing two potential N-glycosylation sites at Asn⁹⁹ and Asn¹⁴⁰ [184]. The scissile peptide bond P1–P1' is located at Arg³⁴⁶–Ser³⁴⁷ [185]. The glia-derived nexin gene (*SERPINE2*) is located on chromosome 2q33-q35 containing nine exons [186, 187].

Glia-derived nexin has a broad specificity but it primarily inhibits trypsin, thrombin, uPA, and Plm. It seems to be the most important physiological regulator of α -thrombin in tissues [183]. The association constants K_a are: (1) $4.2 \pm 0.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (trypsin), (2) $6.0 \pm 1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (thrombin), (3) $1.5 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (uPA), and (4) $1.3 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Plm) [184, 185].

Diseases related to defects in the *SERPINE2* gene are not very well understood (MIM 177010). However, the *SERPINE2* gene seems to be associated with chronic obstructive pulmonary disease [187].

Concluding remarks

The plasmin–antiplasmin system contains two main protein families, namely multidomain serine proteases represented by Pgn, tPA, and uPA, and serpins represented primarily by A2PI as well as PAI-1 and PAI-2. The data provide evidence that the noncatalytic domains in multidomain serine proteases such as kringle, FN1, or EGF-like domains mediate efficient binding to the target structures, facilitating an efficient cleavage reaction of the corresponding serine protease which is usually strictly limited to the intended site. Thus, the described multidomain serine proteases in the plasmin–antiplasmin system are well-adapted proteins which efficiently fulfil their tasks. The data provided show that the plasmin–antiplasmin system as a very complex system is tightly regulated by a series of physiological inhibitors, in this case primarily by serpins such as A2PI, PAI-1, and PAI-2. Also these inhibitors are very well adapted to their regulatory task.

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