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Student Review Article

SERPINS: FORM, FUNCTION, AND DYSFUNCTION

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Abstract. The serpin superfamily of serine protease inhibitors is one of the most ubiquitous and successful classes of inhibitors in the living world. Their unique mechanism of suicide inhibition has led to much research and several important discoveries. They function via rapid incorporation of a reactive centre loop (RCL) within a β-sheet following the former's proteolysis by the target protease: the serpin thus achieves a conformation which is more stable than the native form. Through this conformational change, the target protease structure is distorted and its function disrupted. Alpha-1-antitrypsin (AAT) has often been studied as an archetype for the serpin superfamily, and is discussed in more detail in this review. Of particular interest are the mutant variants of AAT, which have a tendency to polymerise, and thus offer insights into some mechanisms of serpin polymerisation.

Keywords Serpin, RCL, glycosaminoglycan, AAT, loop-sheet polymerisation, serpinopathy

1 The Serpin Superfamily

1.1 Introduction

Serpins are a diverse superfamily of proteins, most of which are serine protease inhibitors - hence their name (Huntington 2011; Khan et al. 2011). The size and ubiquity of this superfamily is testament to the evolutionary success of the serpin structure and function. There are more than 1500 serpin-like genes identified in a wide spectrum of organisms (Law et al. 2006). While the distribution may be vast, it is not even: all multicellular eukaryotes possess serpins (Law et al. 2006), whereas they are found only infrequently in prokaryotes (Irving et al. 2002b). Similarly, serpin structure differs between kingdoms of life. In fact, 'classical' serpins are found in higher eukaryotes and viruses, but not in prokaryotes (Irving et al. 2002b).

Some serpins not only inhibit protease inhibitors, but also cysteine proteases (Irving et al. 2002a) such as the caspases (Lockett et al. 2012), cathepsins (Fluhr et al. 2011; Higgins et al. 2010), and calpains (Luke et al. 2007). Still other serpins have no inhibitory activity, such as chicken ovalbumin (Huntington 2011), and corticosteroid binding globulin (CBG) and thyroxine binding globulin (TBG) in humans (Carrell et al. 2011). HSP47 is another non-inhibitory human serpin, which serves as a collagen-specific molecular chaperone (Nagata, 2003), and has potential as a target for Alzheimer's disease therapy (Bianchi et al. 2011).

So far, 36 serpins have been identified in humans, 27 of which are inhibitory (Law et al. 2006) as shown in Table 1. They serve functions including regulation of inflammation (Horn et al. 2012; Huntington 2011; Khan et al. 2011; Law et al. 2006), coagulation (Huntington 2011; Khan et al. 2011; Law et al. 2006), fibrinolysis (Huntington 2011; Khan et al. 2011), complement system (Khan et al. 2011), apoptosis (Law et al. 2006), and blood pressure (Ricagno et al. 2010). In the clinic, serpins could also serve as biomarkers in the diagnosis and therapy of cancer (Ghazy et al. 2011; Lim et al. 2012). These potential markers include SERPINB11 (Lim et al. 2012) and maspin - a non-inhibitory serpin which is involved in apoptosis and reduces risk of metastasis (Ghazy et al. 2011). This diverse array of functions is

RCL: reactive centre loop

AAT: alpha-1-antitrypsin

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down to the unique biochemistry of serpins.

Table 1. The Human Serpins

Serpin name[a]	Target protease (if inhibitory) Function (if non-inhibitory)	Official gene symbol	Chromosomal location	Source
Alpha-1-antitrypsin	Elastase, plasmin, thrombin, trypsin, chymotrypsin, and plasminogen activator	SERPINA1	14q32.1	NCBI, Gene ID: 5265 (2013)
Alpha-1-antitrypsin-like	Pseudogene	SERPINA2	14q32.1	Seiksas et al. (2006)
Alpha-1-antichymotrypsin	Chymotrypsin	SERPINA3	14q32.1	NCBI, Gene ID: 12 (2013); Rubin et al. (1990)
Kallistatin	Kallikrein	SERPINA4	14q32.13	Chai et al. (1993); NCBI, Gene ID: 5267 (2013)
Protein C inhibitor	Protein C, kallikreins, various plasminogen activators	SERPINA5	14q32.1	NCBI, Gene ID: 5104 (2013)
Corticosteroid binding globulin	Binds corticosteroid hormones	SERPINA6	14q32.1	NCBI, Gene ID: 866 (2013)
Thyroxine-binding globulin	Binds thyroxine	SERPINA7	Xq22.2	NCBI, Gene ID: 6906 (2013)
Angiotensinogen	Precursor of angiotensin I	SERPINA8	1q42.2	NCBI, Gene ID: 183 (2013)
Germinal center B-cell expressed transcript-1	Trypsin, thrombin, plasmin	SERPINA9	14q32.13	NCBI, Gene ID: 327657 (2013); Paterson et al. (2007)
Protein-Z related protease inhibitor	Factor Xa, Factor XIa	SERPINA10	14q32.13	NCBI, Gene ID: 51156 (2013)
Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 11	Serine-type endopeptidases	SERPINA11	14q32.13	NCBI, Gene ID: 256394 (2013); Nextprot BETA (2013)
Vaspin	Insulin-sensitising adipocytokine	SERPINA12	14q32.13	Hida et al. (2005); NCBI, Gene ID: 145264 (2013)
Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 13	Pseudogene	SERPINA13	14q32.13	NCBI, Gene ID: 388007 (2013)
Monocyte neutrophil elastase inhibitor	Neutrophil elastase, cathepsin G, and proteinase-3	SERPINB1	6p5	NCBI, Gene ID: 1992 (2013)
Plasminogen activator inhibitor-2	Urinary plasminogen activator, tissue-type plasminogen activator	SERPINB2	18q21.3	Harrop et al. (1999); NCBI, Gene ID: 5055 (2013)

Squamous cell carcinoma antigen-1	Cathepsin K, cathepsin L, cathepsin S	SERPINB3	18q21.3	NCBI, Gene ID: 6317 (2013); Schick et al. (1998)
Squamous cell carcinoma antigen-2	Cathepin G, mast cell chymase	SERPINB4	18q21.3	NCBI, Gene ID: 6318 (2013); Schick et al. (1998)
Maspin	Tissue-type plasminogen activator	SERPINB5	18q21.33	NCBI, Gene ID: 5268 (2013); Sheng et al. (1998)
PI6	Cathepsin G	SERPINB6	6p25	NCBI, Gene ID: 5269 (2013); Scott et al. (1999);
Megsin	Plasmin, matrix metalloproteinases	SERPINB7	18q21.33	NCBI, Gene ID: 8710 (2013); Ohtomo et al. (2008);
PI8	Furin	SERPINB8	18q21.1	Leblond et al. (2006); NCBI, Gene ID: 5271 (2013)
PI9	Granzyme B	SERPINB9	6p25	NCBI, Gene ID: 5272 (2013)
Bomapin	Thrombin, trypsin	SERPINB10	18q21.3	NCBI, Gene ID: 5273 (2013); Riewald and Schleef (1995)
Serpin peptidase inhibitor, clade B (ovalbumin), member 11	Unclear function in host-pathogen interactions	SERPINB11	18q21 cluster	NCBI, Gene ID: 89778 (2013); Seixas et al. (2012)
Yukopin	Trypsin, plasmin	SERPINB12	18q21 cluster	Askew et al. (2001); NCBI, Gene ID: 89777 (2013)
Headpin	Cathepsin L, cathepsin V	SERPINB13	18q21.33	NCBI, Gene ID: 5275 (2013); Welss et al. (2003)
Antithrombin	Thrombin, factor Xa, chymotrypsin	SERPINC1	1q25.1	NCBI, Gene ID: 462 (2013); Yang et al. (2010)
Heparin cofactor II	Thrombin	SERPIND1	22q11.21	NCBI, Gene ID: 3053 (2013)
Plasminogen activator inhibitor type 1	Urinary plasminogen activator, tissue-type plasminogen activator	SERPINE1	7q22.1	NCBI, Gene ID: 5054 (2013)
Protease nexin-1	Thrombin	SERPINE2	2q36.1	Li et al. (2012); NCBI, Gene ID: 5270 (2013)
Pigment epithelium derived factor	Neurotrophic factor	SERPINF1	17p13.3	NCBI, Gene ID: 5176 (2013)
Alpha-2 antiplasmin	Plasmin	SERPINF2	17p13	NCBI, Gene ID: 5345 (2013)

Complement-1 inhibitor	Activated C1r, activated C1s	SERPING1	11q12.1	NCBI, Gene ID: 710 (2013)
Heat shock protein 47	Chaperone protein	SERPINH1	11q13.5	NCBI, Gene ID: 871 (2013)
Neuroserpin	Tissue-type plasminogen activator	SERPINI1	3q26.1	NCBI, Gene ID: 5274 (2013)
Pancpin	Tissue-type plasminogen activator	SERPINI2	3q26.1	NCBI, Gene ID: 5276 (2013); Silverman et al. (2001)

[a]Serpin name is shaded if it is a known protease inhibitor

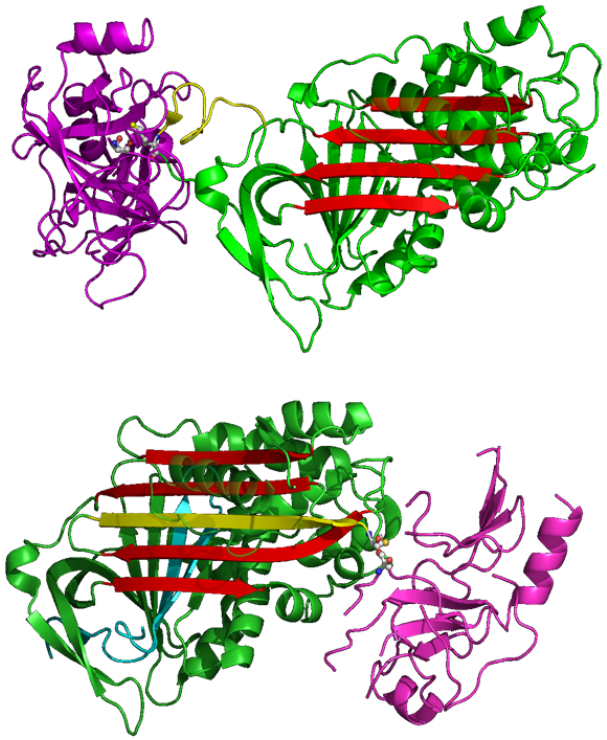

Figure 1: Serpin (AAT) and serine protease (trypsin) interaction
The trap is set (top)
The serpin (green) presents the RCL (yellow) containing the P1 residue (white) for its serine protease target (magenta). The active site serine is white. The 4-stranded β-sheet A in red is a metastable 2° structure.
The serine protease takes the bait (bottom)
After cleavage of the RCL the protease is covalently bound via its reactive serine residue to the serpin (white). The RCL (yellow) now inserts into β-sheet A to form a highly stable conformation. The serine protease is irreversibly inhibited. The cleaved serpin has a new chain (cyan). Note that the serine protease has been dragged over $70, \mathring{A}$ from the original position of the bait residue to the serpin's distal end.
Source: Pymol rendering using PDB entry 1K9O from Ye et al., (2001) (top) and PDB entry 1EZX from Huntington (2000) (bottom)

1.2 Serpin Structure and Conformational Changes

Serpins are approximately $350 - 400$ amino acids long (Patson 2000) and with a relative molecular weight roughly $40 - 60$ kDa (Gettins, 2002). They are globular (Ricagno et al. 2010) glycoproteins in their native conformation (Hopkins et al. 1997). Their secondary structure consists of helical (N-terminal) and β-barrel (C-terminal) domains (Huntington 2011). There are a total of nine α-helices (Patschull et al. 2011) as well as three β-sheets (Patschull et al. 2011).

Serpins also possess a reactive centre loop or RCL (Huntington 2011), exposed for the initial interaction with the protease to be inhibited (Lawrence et al. 1994). This peptide chain can be between $20 - 24$ residues in length (Huntington 2011). In the case of inhibitory serpins, the RCL is characterised by an electrically neutral residue at position P14, separated from the N-terminal end of the 'bait' amino acid (P1) by 12 residues (Lawrence et al. 1994). The specificity of the serpin-protease reaction is heavily dependent on the RCL , making RCL sequences a target for molecular engineering (Bottomley and Stone, 1998) and a source of serpin mutational dysfunctions (Yamasaki et al. 2010).

The mechanism of inhibitory serpins involves a significant change in their structure (Huntington 2000) as well as that of the target protease (Huntington 2011). This change from native to inhibiting state is associated with an increase in the stability of the serpin structure (Singh and Jairajpuri, 2011). In the native metastable state, the serpin has a 5 stranded β-sheet A and an exposed RCL; in the hyperstable state (following serpin-protease interaction), the RCL has been cleaved and becomes inserted in the β-sheet A as the new fourth strand (Figure 1).

Serpins can also take on the hyperstable inactive conformation without protease interaction. This mechanism involves incorporating the RCL into β-sheet A of the same serpin molecule following disruption of the intermolecular bonds between the first chain in β-sheet C and the rest of the sheet (Na and Im, 2007). The likelihood of taking this 'latent' form affects the serpin's half-life (Thompson et al. 2011).

Research by Seo et al. (2000) showed that the strain associated with the metastable structure is not localised to one structure of the serpin, but is more likely to

be diffuse. They identified several surface hydrophobic regions on α-1-antitrypsin (AAT) as contributors to this global tension. Other reasons include overclustered side-chains, thermodynamically unfavourable polar-nonpolar non-covalent bonds, and surface cavities (Im et al. 1999). However, Seo et al. (2000) point out that there is little, if any, effect on serpin inhibitory activity following stabilising mutations in regions of tension which are not vital to serpin mechanism and which are not involved in conformational change. One residue which seems central to conformational change in AAT and other serpins is lysine 335 (Im and Yu, 2000), which interacts with helix F (hF) and the loop between hF and β-sheet A's third strand (thFs3A) (Seo et al. 2000).

A study by Baek et al. (2007) introduced disulfide bonds into AAT in order to investigate the conformational changes occurring in serpins during inhibition. Their study showed that in order for RCL incorporation as the fourth strand of β-sheet A, hF and thFs3A must be able to move away from the fifth strand. Krishnan et al. (2011) also studied AAT and confirmed that there is dissociation of hF from β-sheet A. They identified other conformational changes in the serpin preceding RCL incorporation, such as dissociation of strands 5 and 6 from the rest of β-sheet A, and disruption of the intermolecular bonds between RCL and β-sheets B and C.

1.3 Protease Conformational Change

Serpins have a profound effect on target serine proteases, not only by inhibiting the active site but also by distorting the structure of the protease (Huntington 2000).

Serpins and their targets interact in a 1 to 1 ratio, forming an SDS-stable complex (Egelund et al. 1998). The resistance of this complex to SDS is explained by the covalent bond formed between serpin and protease (O'Malley et al. 1997). This, coupled with the ratio of interaction, has led to serpins being called 'suicide' inhibitors (Lawrence et al. 1995).

The P1 − P1′ bond of the serpin RCL serves as 'bait' for the attacking protease, P1′ being the amino acid closer to the C-terminal end (O'Malley et al. 1997). The protease cleaves the bond to form an acyl-intermediate with the serpin (Lawrence et al. 1995). Normally, protease action is completed by hydrolysis of the acyl-intermediate to form a tetrahedral intermediate which then disintegrates, resulting in separation of the protease-product complex (Hedstrom 2002). However, the formation of the acyl-intermediate is followed by rapid burying of the RCL into β-sheet A as the new fourth strand (Lawrence et al. 1995). This prevents hydrolysis either by preventing entry of water into the active site (Lawrence et al. 1995) or by disturbing the conformation of the active site (Dementiev et al. 2006; Huntington 2011), or both (O'Malley et al. 1997). Thus, serpin-protease complexes are trapped in an acyl-intermediate stage (Egelund et al. 1998).

The incorporation of the RCL into β-sheet A also drags the attached protease over 70$\mathring{A}$ from the original position of the bait residue to the distal end of the serpin and results in close approximation between serpin and protease (Huntington 2000) as shown in Figure 1. This could explain the disturbance of active site geometry (Stratikos et al. 1999).

The active site is not the only portion of the protease which is affected. Another study on AAT-trypsin complex (Huntington 2000) showed that approximately 37% of the protease becomes disordered as a result of the dragging force exerted on Ser195 (in the active site) as the RCL is incorporated into β-sheet A. This action also destroys a salt-bridge between Ile16 and Asp194 of the protease, formed during zymogen activation (Huntington 2000). The distortion of serine protease structure has been proposed as another facet to the serpin-inhibition mechanism (Huntington 2000), in view of the fact that such disordered proteases are more prone to proteolytic attack (Egelund et al. 2001) and decreased stability (Kaslik et al. 1997).

The degree of serine protease distortion varies from one serpin-protease complex to another, possibly dependent on factors including RCL length, the presence of protease ligands such as Ca^{2+}, and particular characteristics of serpin and protease loops such as location, length, and sequence (Huntington 2011).

1.4 Serpin Modulation

The glycosaminoglycans heparin and heparin sulphate are modulators of many serpins' function, activating most of the serpins involved in haemostasis (Huntington 2003). However, heparin can also inhibit serpins, such as kallistatin (Chen et al. 2001).

The majority of glycosaminoglycan-modulated serpins possess a sequence neighbouring or involving helix D for interaction with the glycosaminoglycan; the same is true of helix H for protein C inhibitor (PCI) (Rein et al. 2011). The mechanism of activation often involves heparin binding to the serpin and the target protease, bringing them closer and facilitating serpin-protease interaction. Complexes falling under this category include those between PCI and thrombin or activated protein C (Li et al. 2008); antithrombin and thrombin, fIXa or fXa (Olson et al. 2010); glia-derived nexin and thrombin (Baker et al. 1980); heparin cofactor II (HCII) and thrombin (Verhamme, 2012); as well as protein Z inhibitor (PZI) and fXa or fXIa (Huang et al. 2011). The bridging effect of heparin on PZI enables it to inhibit free factors Xa and XIa, whereas other activating cofac-

tors such as lipid, Ca^{2+}, and protein Z promote PZI's inactivation of membrane-bound factor Xa (Huang et al. 2011).

Interestingly, the 'bridging effect' that heparin has on serpins could have therapeutic relevance in oncology. A study by Higgins et al. (2010) showed that heparin improves the inhibition of the papain-like cathepsin L by squamous cell carcinoma antigen-1 and -2 (SCCA-1, SCCA-2), both of which are serpins. This find is interesting in that not only could it explain heparin's anti-metastatic properties, but it is also the first report of heparin promoting serpin inhibition of cysteine protease (Higgins et al. 2010).

Glycosaminoglycans can also enhance serpin function through allosteric alterations (Rein et al. 2011), such as with antithrombin and HCII. Heparin and heparan sulphate cause allosteric activating changes in antithrombin mostly through a mutual pentasaccharide sequence (Olson et al. 2010). This allosteric change does not increase antithrombin's inhibition of thrombin but of factors IXa and Xa (Olson et al. 2010) as well as plasma kallikrein (Olson and Björk, 1991). Ca^{2+} also increases antithrombin's inhibition of factor IXa by allosterically activating the latter (Bedsted et al. 2003).

HCII's inhibition of thrombin is also enhanced by heparin through allosteric modifications (Baglin et al. 2002). Similarly to heparin, dermatan sulphate is another glycosaminoglycan that activates HCII through bridging and allosteric activation mechanisms (Verhamme et al. 2004).

Alternatively, allosteric modification of the target protease can enhance serpin action. For example, interaction of thrombomodulin with thrombin causes the protease configuration to alter, providing a binding-site for the serpin PCI (Yange et al. 2003).

Other modulators of serpin function include vitronectin and cations. The serpin plasminogen activator inhibitor-1 (PAI-1) inhibits β-trypsin, tissue-type (tPA) and urokinase-type (uPA) plasminogen activator (Komissarov et al. 2007) hence playing a crucial role in the regulation of fibrinolysis. The half-life of PAI-1 is normally $1-2$ hours, however this can be altered by modulating factors like vitronectin and cations (Thompson et al. 2011). Vitronectin alone can prolong PAI-1 half-life by approximately 1.5 times (Thompson et al. 2011), through binding of vitronectin's somatomedin B domain to α-helix F of PAI-1 (Komissarov et al. 2007). Cations such as Mg^{2+} and Ca^{2+} prolong half-life slightly, whereas Cu^{2+} and Co^{2+} without vitronectin reduce half-life significantly but prolong it in the presence of vitronectin (Thompson et al. 2011). Calcium can also inhibit the activity of AAT [discussed below].

Serpin modulation can also occur at the level of the nucleus. Serpinin, released from neuroendocrine cells during exocytosis of dense core granules (DCGs), interacts with extracellular receptors to increase transcription of protease nexin-1 (PN-1) (Koshimizu et al. 2010). PN-1 is a serpin which prevents the proteolysis of DCG proteins in the Golgi complex, thus favouring the replacement of the exocytosed DCGs (Kim et al. 2006). On the other hand, PAI-1 transcription is increased by transforming growth factor (TGF)-β, a mechanism which has been implicated in vascular disease associated with non-insulin dependent diabetes (Nakayama et al. 2011). PAI-2 transcription is inhibited by heparin (Pepe et al. 1997), which contrasts with the activation of PAI-1 by heparin. Maspin can also be regulated through the rate of its transcription, either inhibited, such as by protease activated receptor-1 (Villares et al. 2011), or activated, such as by nitric oxide (Khalkhali-Ellis et al. 2003).

2 ALPHA-1-ANTITRYPSIN (AAT)

2.1 The relevance of AAT

AAT has often been studied as an archetype for the structure, function, and dysfunction of the serpin superfamily (Baek et al. 2007; Ekeowa et al. 2010; Huntington 2000; Krishnan et al. 2011; Mushero et al. 2011; Sengupta et al. 2009; Seo et al. 2000), thus emphasising its importance in understanding serpins.

2.2 Structure and function

Plasma AAT is 394 residues long (Janciauskiene et al. 1998), with a relative molecular weight of 52 kDa (Dickens et al. 2011).

AAT has three glycans and eight alpha-helices, the latter of which consist mostly of the most N-terminal 150 residues (Loebermann et al. 1984). AAT inhibits neutrophil elastase, and is produced mainly in the liver at a rate of 2 g/day (Greene and McElvaney, 2010) but also in other sites (Dickens and Lomas 2011), particularly the lungs (van't Wout et al. 2011). In the lungs, AAT production is higher in pro-inflammatory macrophages than in anti-inflammatory macrophages and immature dendritic cells: in all cases, lipopolysaccharide stimulates an increase in AAT release (van't Wout et al. 2011). The main role of AAT is to limit the damage inflicted by neutrophil elastase on tissues at sites of inflammation (Dickens and Lomas, 2011).

The 'bait' residue for elastase is methionine 358: in fact, oxidation of methionine at this position can block inhibitory function (Taggart et al. 2000). Replacement of this residue with arginine can result in AAT inhibiting thrombin, causing heparin-independent anticoagulant activity and a subsequent bleeding disorder (Owen et al. 1983).

AAT not only prevents lung tissue damage by inhibiting neutrophil elastase, but also by inhibiting lung endothelial cell apoptosis (Petrarche et al. 2006). This is an example of cross-class inhibition, since RCL-intact AAT is internalised by endothelial cells to directly inhibit caspase-3, a cysteine protease involved in apoptosis (Petrarche et al. 2006). The Z-variant of AAT [discussed below] also has direct caspase-3 inhibitory activity (Greene et al. 2010).

Another serine protease inhibited by AAT is matriptase, an enzyme which spans the plasma membrane and whose catalytic activity is extracellular (Janciauskiene et al. 2008). Given the role of matriptase in activating prostatin which then modulates epithelial sodium channels, inhibition of matriptase by AAT offers therapeutic potential for patients with cystic fibrosis (characterised by a defect in sodium absorption) (Janciauskiene et al. 2008).

2.3 AAT fragments

Elastase cleaves AAT to release a C-terminal product of 4kDA (Schulze et al. 1992), corresponding to residues $358-394$ (Janciauskiene et al. 1998). Tryspin gives the same product: AAT-trypsin complex is composed of two peptides which can be separated by SDS-page, showing that the C-terminal product is bound to the complex by non-covalent forces (Boswell et al. 1983).

The cleaved form of AAT has been shown to increase LDL capture, internalisation, and breakdown in HepG2 cells (Janciauskiene et al. 1997). The cellular response is likely initiated through binding of the C-terminal fragment of cleaved AAT (Janciauskiene et al. 1998). Since AAT is an acute-phase protein, this observation could provide an explanation for hypocholesterolemia succeeding inflammation (Janciauskiene et al. 1997).

The 36 residue C-terminal segment of cleaved AAT also confers chemoattractant properties to elastase-AAT complex, and thus mediates inflammation in the absence of bacteria or complement activation (Banda et al. 1988).

Another truncated form of AAT is SPAAT. SPAAT (short peptide from AAT) is composed of the 44 most C-terminal residues of AAT and can be found bound to the extracellular matrix in humans (Niemann et al. 1997*a*). Here, it could serve a protective role from excess tissue degradation since it is a competitive reversible inhibitor of neutrophil elastase (Niemann et al. 1997b). This contrasts with the irreversible inhibition of elastase by full-length AAT. SPAAT can also be cleaved to release an octapeptide sequence (Wright et al. 2000).

This octapeptide (MFLEAIPM), formed from residues P8-P1 of AAT RCL, was shown to inhibit elastase in a study by Wright et al. 2000. Further kinetic analysis showed that this was uncompetitive inhibition through non-covalent interactions, mostly attributable to the four most N-terminal residues. The study showed that the octapeptide can also form an acyl-enzyme intermediate with elastase, and the uncompetitive inhibition is possibly through stabilisation of this intermediate. Taken together, SPAAT and MFLEAIPM present a possible 'cascade' of protease inhibition by AAT (Wright et al. 2000).

2.4 Regulation of AAT

In the lungs, the activity of AAT can be regulated by surfactant A, a normal component of lung secretions (Sarker et al. 2011). Surfactant A has been shown to bind to AAT to limit its inhibition of elastase in a calcium-dependent manner, involving the carbohydrate side-chains of one or both of the glycoproteins (Gorrini et al. 2005).

Elastase directly promotes transcription of AAT mRNA in monocytes and bronchoalveolar macrophages (Perlmutter et al. 1988). This is the case even in individuals homozygous for the Z variant of the AAT gene; elastase has no effect on AAT secretion however, resulting in intracellular accumulation of AAT in these patients (Perlmutter et al. 1988).

Cations can also modulate the inhibition of trypsin. Inactive trypsin (in an AAT-trypsin complex) exists in equilibrium with the active form: the equilibrium can be shifted towards formation of the latter by stabilising it with Ca^{2+} ions (Calugaru et al. 2001).

2.5 AAT in immunity and inflammation

As an acute-phase protein, AAT serum levels can be used as a marker of inflammatory response (Ziakas et al. 2011). Elastase-AAT complex also corresponds with inflammatory activity: neutrophil degranulation releases neutrophil elastase, which is then inhibited by AAT, forming the complex. For this reason, elastase-AAT can be used as an indirect indication of reperfusion injury following kidney transplant (Zynek-Litwin et al. 2010) or of decreased survival chances in cystic fibrosis patients colonised with Burkholderia cenocepacia (Downey et al. 2007).

AAT dampens inflammation in islet cells and other tissues (Kalis et al. 2010), possibly due to impairment of nuclear factor-kappaB (NFκB) function (Churg et al. 2001; Kalis et al. 2010). The mechanism of AAT's interference with NFκB is unclear: however, it is associated with increased levels of inhibitor of NF-κB (IκB), not due to AAT's protease inhibitory function (Churg et al. 2001).

Interestingly, AAT has shown promise as an adjunct to immunosuppressive therapy to prolong graft viability in insulin-dependent diabetic patients who have received an islet transplant (Lewis et al. 2005). AAT directly

inhibits a mediator of β-cell apoptosis, the cysteine protease caspase-3 (Zhang et al. 2007). AAT can also prevent TNF-αmediated apoptosis in islet β-cells (Zhang et al. 2007), as well as inhibiting other pro-inflammatory cytokines (Pott et al. 2009); the mechanisms remain elusive.

2.6 AAT in vascular disease

The lungs are not the only sites susceptible to damage by imbalance between elastase and AAT levels. Patients with ruptured and unruptured cerebral aneurysms have been shown to have a serum elastase to AAT ratio almost double that of controls, implicating skewed elastase:AAT as a cause of vessel wall damage (Baker et al. 1995).

AAT can protect against vascular disease (through its elastase inhibition function) when associated with HDL (Ortiz-Muñoz et al. 2009). AAT complexed with LDLs (AAT-LDL) could also protect against vascular disease, in women without metabolic syndrome (Kotani et al. 2010). However, oxidative stress due to smoking increases AAT-LDL levels, suggesting that AAT-LDL might have a role to play in cardiovascular disease associated with smoking (Wada et al. 2012).

2.7 AAT variants

Over 95% of AAT deficient individuals are homozygous or heterozygous for the Z-allele (Greene and McElvaney, 2010) on chromosome 14 (Elzouki 1999). The Z-variant of AAT (ZAAT) is characterised by a replacement of Glu342 with Lys (Lomas et al. 1995). ZAAT is not secreted efficiently, hence the AAT deficiency: homozygotes (PiZZ) for the mutant allele have $15-20\%$ of normal circulating AAT levels (Elzouki, 1999). Thus, PiZZ is characterised by emphysema (due to circulating AAT deficiency) and chronic liver disease (due to inclusion of ZAAT in hepatocyte endoplasmic reticulum) (Elzouki 1999). The hepatic inclusion bodies are periodic acid-Schiff diastase (PASD)-resistant positive (Francalanci et al. 2009) since ZAAT is a glycoprotein. The buildup of ZAAT in endoplasmic reticulum (ER) causes ER stress and deranged function (Greene et al. 2010). The accumulation of ZAAT is associated with its ability to form polymers: in fact, reduced polymerisation results in increased circulating ZAAT (Parfrey et al. 2003).

ZAAT polymerises by a mechanism known as loop-A-sheet polymerisation (refer to Figure 2), whereby the RCL of one ZAAT molecule is inserted into the β-sheet A of another ZAAT (Wilczynska et al. 2003). ZAAT loop-sheet polymerisation is due to abnormal opening of β-sheet A: a mutation of phenylalanine (position 51, within the hydrophobic core) to leucine was shown to inhibit β-sheet A opening, thus interfering with formation of ZAAT aggregates (Kim et al. 1995).

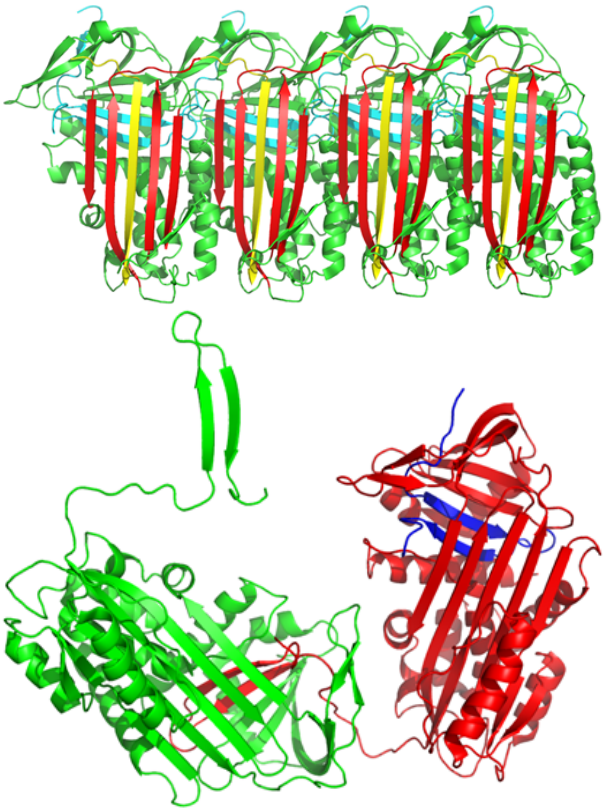

Figure 2: Hypothetical serpin polymerisation schemes
Top: Illustrates loop-A-sheet serpin (AAT) polymerisation. One way that serpin subunits might form a polymeric structure is by inserting the RCL as a fifth β-strand into a neighbouring subunit's β-sheet, in a loop-A-sheet polymerisation mechanism. The distances and links between subunits is exaggerated for clarity. It is envisaged that the close proximity of the β sheets in a serpin polymer would encourage the formation of amyloid-like interactions between subunits.
Bottom: Recent discoveries of dimers and trimers of the serpin antithrombin III suggest a novel interaction through domain swapping, whereby two β-strands are contributed by one subunit to a neighbouring subunit's β-sheet. Two complete subunits are shown (green and red) while only the β-strand contribution is shown of a third (blue). Continuation of the polymer would involve more subunits using similar interactions.
Source: Pymol rendering using PDB entry 1EZX from Huntington (2000) (top) and PDB entry 3T1P, Yamasaki et al. unpublished.

As mentioned previously, there are many sites on a serpin which reduce the stability of its native state. One AAT residue which marks such a site (a hydrophobic surface cavity) is glycine 117 (Lee et al. 2000). This pocket - formed by helix D, helix E, and β-sheet A strand 2 - is obliterated in polymerisation of AAT (Elliott et al. 2000). However, it can be 'filled' by replacing glycine 117 with phenylalanine (bulky side-chain) to increase the stability of native AAT and limit polymerisation without eliminating AAT's inhibitory action (Parfrey et al. 2003). Similar results were observed in ZAAT, where filling the pocket by replacing threonine 114 with phenylalanine resulted in decreased polymerisation and increased ZAAT extracellular release (Parfrey et al. 2003).

Elliott et al. (2000) also studied the Gly117 cavity as well as another four cavities, and compared the sizes of these cavities between four serpins (AAT, alpha-1-antichymotrypsin, PAI-1, and antithrombin). The design of small drugs which can occupy pockets such as these without inhibiting serpin function can have an important role in limiting the pathological conditions associated with intracellular accumulation of polymerised AAT and other serpins (Patschull et al. 2011; Elliott et al. 2000).

Another variant of AAT which exhibits loop-A-sheet polymerisation and accumulation in the endoplasmic reticulum is Siiyama (S53F) (Lomas et al. 1995). The Siiyama variant of AAT (SAAT) is prone to polymerisation due to a propensity for opening of β-sheet A, and impedance of its polymerisation results in increased secretion (Sidhar et al. 1995). Also similar to ZAAT, this variant results in hepatic disease and deficient serum AAT (Janciauskiene et al. 2004).

Siiyama and ZAAT are the most frequent mutant forms of AAT, and result in AAT deficiency in individuals homozygous for the alleles (PiSS and PiZZ genotypes respectively) or possessing both alleles (PiSZ genotype) (Ringenbach et al. 2011). Other variants exist though, such as Mmalton, in which there is deletion of Phe52 (Curiel et al. 1989). Its frequency even exceeds that of SAAT and ZAAT alleles in parts of the Southern Mediterranean (Denden et al. 2010). Like SAAT and ZAAT, Mmalton results in AAT deficiency and polymerises to form hepatic inclusions (Francalanci et al. 2009). However, plasma short-chain polymers of Mmalton were found to be formed by insertion of RCL of one Mmalton molecule into the β-sheet C of another (Lomas et al. 1995). The exposed C-termini of these polymers are more likely to attack by proteases, possibly explaining why Mmalton extracted from blood contains RCL-cleaved AAT (Yamasaki et al. 2011)

3 Serpin polymerisation

The loop-C-sheet polymerisation described above for Mmalton can also be observed in C1 inhibitor (Eldering et al. 1995) and antithrombin dimers (Carrell et al. 1994). In the case of antithrombin dimers, one molecule (in the latent form) has the first strand of β-sheet C separated from the rest of the sheet to permit insertion of the other molecule's RCL (Devlin and Bottomley, 2005). Loop-C-sheet polymers have also been observed in in vitro studies on typical AAT and antithrombin when heated with citrate (Devlin and Bottomley, 2005). Zhang et al. (2008) propose that loop-C-sheet interactions could also account for the polymerisation of the latent forms of some serpins. Their crystallography study of the latent form of tengpinDelta42 (a bacterial serpin) showed hyperinsertion of the RCL into β-sheet A, causing full exposure of β-sheet C. This then allows for hydrogen-bonding between the exposed part of the RCL of one latent serpin molecule with the second strand of β-sheet C of another (Zhang et al. 2008).

Loop-A-sheet polymerisation occurs in AAT, neuroserpins (Santangelo et al. 2012), and α-1-antichymotrypsin (Crowther et al. 2003). Tsutsui et al. (2008) - using wild-type AAT as a paradigm for other serpins - proposed that the mechanism of loop-A-sheet polymerisation begins by disruption of β-sheet C. This then leads to movement of the first strand from the rest of the sheet via serpin-serpin interaction, causing conformational changes. One such change is the opening of β-sheet A, which allows insertion of another serpin molecule's RCL into the sheet for polymerisation to occur (Tsutsui et al. 2008). Krishnan and Gierasch (2011) point out that even under normal conditions, an equilibrium exists between native serpin and an intermediate with an open β-sheet A. Although normally low in concentration, this intermediate's formation is increased in certain AAT variants (e.g. ZAAT) due to a lower thermodynamic barrier, explaining ZAAT's tendency to polymerise after release from hepatocytes (Krishnan et al. 2011). However, the polymerisation of ZAAT and other AAT mutants within hepatocytes is mostly due to delayed folding to the native serpin state, giving intermediates more opportunity chance to polymerise (Yu et al. 1995).

'S7A' polymerisation can be considered another loop-sheet mechanism. The RCL of one molecule forms hydrogen-bonds with the sixth strand of another molecule's β-sheet A, acting as a seventh strand (S7A) (McGowan et al. 2006). Serpins which exhibit such polymerisation include myeloid and erythroid nuclear termination stage-specific protein (MENT) (McGowan et al. 2006) and PAI-1 (Sharp et al. 1999). A mechanism of 'S5A' polymerisation was also proposed by Yamasaki et al. (2008), wherein both the RCL and the fifth strand of the β-sheet A of one molecule are inserted into the β-sheet A of the other. This mechanism might explain the highly chemically stable polymer which human neuroserpin forms when incubated at 85°C (Ricagno et al. 2010).

Yamasaki et al. (2011) propose that polymerisation via RCL insertion occurs via an intermediate which can return to native state or form a polymer. If it is more likely that the RCL is inserted into another molecule, a polymer forms this intermediate state. However, RCL insertion competes with the inclusion of the C-terminus in the folded serpin, in which case the intermediate form returns to native state. In fact, if the RCL insertion process is slowed down, there is reduced polymerisation and increased functional secretion in ZAAT (Yamasaki et al. 2010).

The loop-sheet mechanisms are the best described for serpin polymerisation, but they are not exclusive. Marszal et al. (2003) described the polymerisation of disulfide-linked dimers of wild-type AAT. The dimers were obtained in vitro, using a mild denaturing buffer without reducing agents, and polymerised through intermolecular interactions on the surface with β-sheet A. The relevance of this find is unclear; however, the similarity in structure (under the electron microscope) of dimer polymers to loop-sheet polymers suggests that the latter may involve disulfide bonds (Marszal et al. 2003).

Not all serpin multimers are pathological. For example, S7a polymerisation of MENT could actually participate in normal chromatin condensation (McGowan et al. 2006). However, the vast majority of serpin polymers are linked to disease states, such as those described above for AAT variants.

Diseases may be due to deficiency of the serpin, which is not secreted but is trapped as polymers in the endoplasmic reticulum (ER) of the secretory cell. This is true for individuals homozygous for the mutant alleles of AAT (as described above). Mutations of antithrombin, α1-antichymotrypsin, and C1-inhibitor can also result in intrahepatocyte polymer formation and subsequent deficiency disease (Belorgey et al. 2007). Deficiency disease can also occur with spontaneous polymerisation following secretion, hence limiting the amount of available serpin: for example, for individuals heterozygous and homozygous for the F229L mutant allele of antithrombin (Picard et al. 2003). Serpins need not necessarily be mutant to polymerise and cause deficiency: wild-type PAI-2 can undergo loop-sheet polymerisation within the cytosol to eventually limit its own secretion (Mikus et al. 1996).

Gain-of-function toxicity is another cause of disease. One such case is that of mutant neuroserpin polymers within ER, resulting in familial encephalopathy with neuroserpin inclusion bodies (FENIB) (Miranda et al. 2008). One possible mediator of this disease is nuclear factor kappa B (NF-ϰB), which is activated by the intraendoplasmic accumulation of neuroserpin polymers (Davies et al. 2009). The pro-inflammatory mediator NF-κB is also elevated with intraendoplasmic deposition of ZAAT polymers: inhibiting NF-ϰB's actions (and subsequent inflammation) may prove to be a line of therapy for this genetic disease (Lawless et al. 2004).

Toxicity can also be a result of serpin oligomers, rather than polymers. Carrell et al. (2008) used AAT and antithrombin to demonstrate that in the initial stages of serpin oligomer formation, the opening of the A-sheet creates a β-acceptor site which can potentially bind to physiologically significant peptides such as neurotransmitters, resulting in toxicity. Hence, extension of the oligomer to form a serpin auto-polymer is actually protective in that auto-polymerisation sequesters otherwise toxic oligomers.

4 Conclusion

To conclude, although serpins' roles in physiology and disease are varied, they share a common structure which allows great versatility and has proven to be an evolutionary success. Understanding serpin structure and their mechanism of inhibition is crucial to developing treatments for their dysfunctions.

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