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POLYMERISATION UNDERLIES ALPHA$_1$-ANTITRYPSIN DEFICIENCY, DEMENTIA AND OTHER SERPINOPATHIES

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1. ABSTRACT

We review here the molecular mechanisms that underlie α_1-antitrypsin deficiency and show how an understanding of this mechanism has allowed us to explain the deficiency of other members of the serine proteinase inhibitor or serpin superfamily. These include the deficiency of antithrombin, C1-inhibitor and α_1-antichymotrypsin in association with thrombosis, angio-oedema and emphysema respectively. Moreover the accumulation of mutant neuroserpin within neurones causes the novel dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB). We have grouped these conditions together as the serpinopathies as recognition of their common pathophysiology provides a platform to develop strategies to treat the associated clinical syndromes.

2. ALPHA-1-ANTITRYPSIN

2.1. Structure and function of α_1-antitrypsin

Alpha-1-antitrypsin is a 394 amino acid (1), 52 kDa, acute phase glycoprotein (2) encoded on chromosome 14q31-32.1 (3). It is synthesised by hepatocytes (4, 5) and secreted into the plasma at a concentration of 1.9-3.5 mg/ml. Alpha-1-antitrypsin is also synthesised by, and secreted from, macrophages (6) and intestinal (7) and bronchial epithelial cells (8). The protein was originally named because of its ability to inhibit pancreatic trypsin (9). Subsequently it has been found to be an effective inhibitor of a variety of other proteinases including neutrophil elastase (10), cathepsin G (10) and proteinase 3 (11). The broad spectrum of proteinase inhibition gave rise to its alternative name of α_1-proteinase inhibitor (12) although this too is inaccurate as other proteins in the α_1 band of serum (such as α_1-antichymotrypsin) are also proteinase inhibitors. More recently the serine proteinase inhibitor (or serpin) superfamily have been classified into phylogenetic clades with α_1-antitrypsin being named SERPINA1 (13). For historical reasons, and as the term is widely used in clinical practice, we will use α_1-antitrypsin in this article.

Crystal structures have demonstrated that α_1-antitrypsin is composed of three β-sheets (A-C) and an exposed mobile reactive loop (Figure 1) that presents a peptide sequence as a pseudosubstrate for the target proteinase (14-18). The critical amino acids within this loop are the P1-P1' residues, methionine-serine, as these act as a 'bait' for neutrophil elastase (19). After docking, the

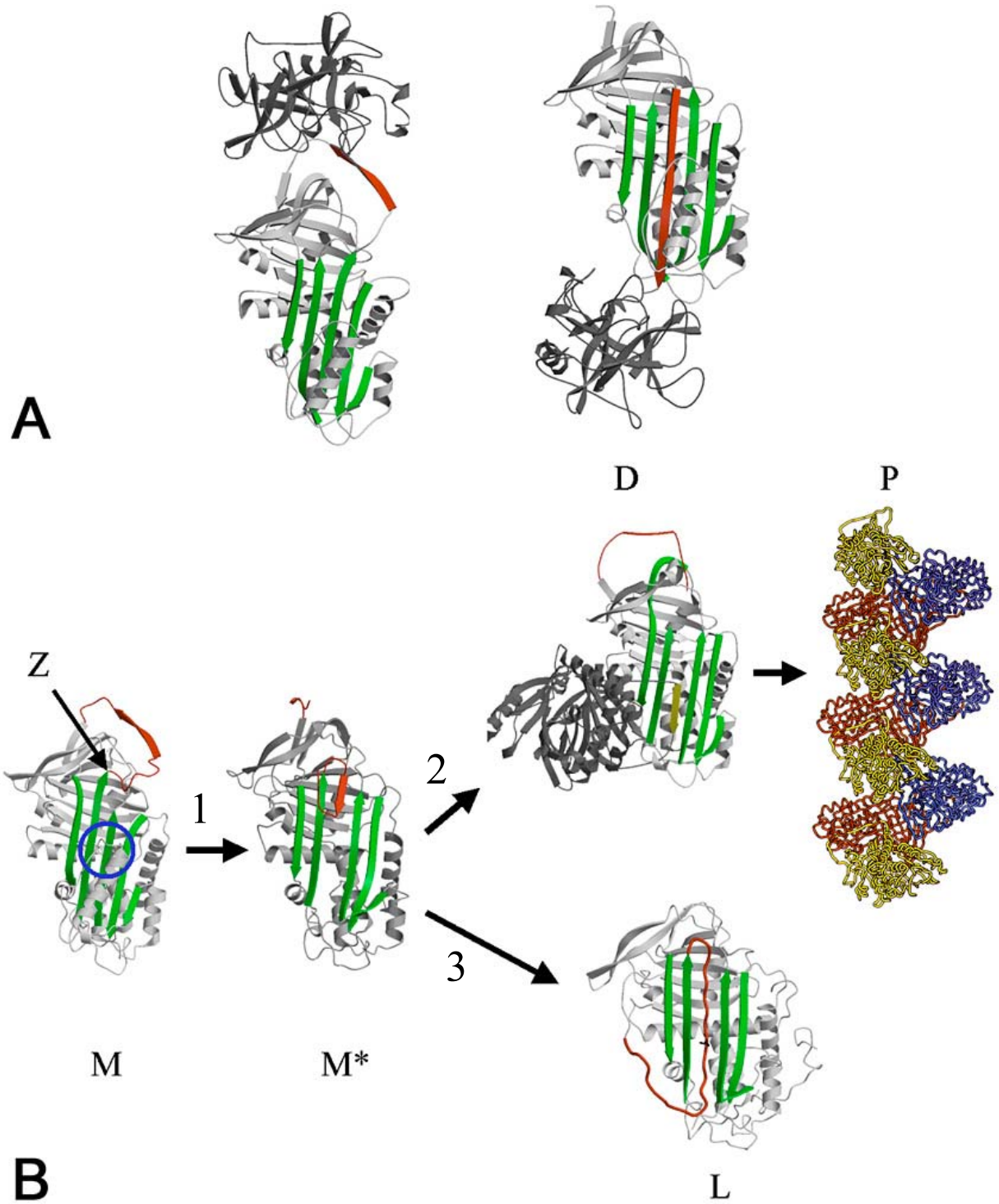


Figure 1. (a) Alpha$_1$-antitrypsin can be considered to act as a mousetrap (14, 25, 180). Following docking (left) the neutrophil elastase (grey) is inactivated by movement from the upper to the lower pole of the protein (right). This is associated with insertion of the reactive loop (red) as an extra strand into β-sheet A (green). Reproduced from (118) with permission. (b) The structure of α_1-antitrypsin is centered on β-sheet A (green) and the mobile reactive centre loop (red). Polymer formation results from the Z variant of α_1-antitrypsin (Glu342Lys at P_{17}; arrowed) or mutations in the shutter domain (blue circle) that open β-sheet A to favour partial loop insertion (step 1) and the formation of an unstable intermediate (M*) (72, 76). The patent β-sheet A can either accept the loop of another molecule (step 2) to form a dimer (D) which then extends into polymers (P) (14, 64, 67) or its own loop (step 3) to form a latent conformation (L) (146, 181). The individual molecules of α_1-antitrypsin within the polymer are coloured red, yellow and blue. Reproduced from reference (72) with permission.

enzyme cleaves the P1-P1' peptide bond of α_1-antitrypsin (20) and the proteinase is inactivated by a mousetrap action (Figure 1a) that swings it from the upper to the lower pole of the protein in association with the insertion of the reactive loop as an extra strand (s4A) in β-sheet A (21-25). This altered conformation of α_1-antitrypsin bound to its target enzyme is then recognised by hepatic receptors and cleared from the circulation (26-28). The remarkable 'mouse trap' action of α_1-antitrypsin is central to its role as an effective inhibitor of serine proteinases. Paradoxically, it is also its 'Achilles heel' as point mutations in these mobile domains make the molecule vulnerable to aberrant conformational transitions such as the one that underlies α_1-antitrypsin deficiency.

2.2. Alpha-1-antitrypsin deficiency

2.2.1. Common mutations

Alpha-1-antitrypsin deficiency was first described as a clinical entity in 1963 by Laurell and Eriksson who noted an absence of the alpha-1 band on serum protein electrophoresis (29). Over 70 naturally occurring variants have been described and characterised by their migration on isoelectric focusing gels - the proteinase inhibitor or PI system (30). The commonest deficiency variants, S and Z, result from point mutations in the α_1-antitrypsin gene (31-33) and are so named as they make the protein migrate more slowly than normal M α_1-antitrypsin on isoelectric focusing gels. Mutations that cause more rapid migration of α_1-antitrypsin are labelled A to L.

A recent review of 70 surveys has provided an assessment of the frequency and distribution of the S and Z α_1-antitrypsin alleles throughout Europe (34). The greatest frequency of the S allele occurs within the Iberian Peninsula and gradually reduces in the direction of south to north and from west to east. S α_1-antitrypsin (Glu264Val) is found in up to 28% of Southern Europeans and although it results in plasma α_1-antitrypsin levels that are 60% of the M allele, it is not associated with any pulmonary sequelae. In contrast, the Z allele is most common in northwest Europe with frequencies declining from west to east and from north to south. The Z variant (Glu342Lys) results in a more severe deficiency that is characterised in the homozygote by plasma α_1-antitrypsin levels of 10% of the normal M allele and 60% in the MZ heterozygote (50% from the M allele and 10% from the Z allele). The Z mutation results in the accumulation of α_1-antitrypsin as inclusions in the rough endoplasmic reticulum of the liver (35). These inclusions predispose the homozygote to juvenile hepatitis, cirrhosis (36, 37) and hepatocellular carcinoma (38). Furthermore, the lack of circulating protein predisposes the homozygote to early onset panlobular emphysema (39-41).

2.2.2. Clinical features

The liver disease associated with the Z allele of α_1-antitrypsin results from the 'overload' of abnormal Z α_1-antitrypsin within the endoplasmic reticulum of hepatocytes. The accumulation of abnormal protein starts *in utero* (42) and is characterised by the accumulation of diastase-resistant, periodic acid-Schiff positive inclusions of α_1-antitrypsin in the periportal cells (35, 43) (Figure 2). Seventy-three percent of Z α_1-antitrypsin homozygote infants have a raised serum alanine aminotransferase in the first year of life but in only 15% of people is it still abnormal by 12 years of age (36, 37, 44, 45). Similarly serum bilirubin is raised in 11% of PI*Z infants in the first 2-4 months but falls to normal by 6 months of age. One in ten infants develops cholestatic jaundice and 6 per cent develop clinical evidence of liver disease without jaundice. These symptoms usually resolve by the second year of life but approximately 15% of patients with cholestatic jaundice progress to juvenile cirrhosis. The overall risk of death from liver disease in PI*Z children during childhood is 2-3% with boys being at more risk than girls.

All adults with the Z allele of α_1-antitrypsin have slowly progressive hepatic damage that is often subclinical and only evident as a minor degree of portal fibrosis. However up to 50% of Z α_1-antitrypsin homozygotes present with clinically evident cirrhosis and occasionally with hepatocellular carcinoma (38). The accumulation of α_1-antitrypsin within hepatocytes in association with severe plasma deficiency is also seen with 2 other rare mutations: the Siiyama variant (Phe53Ser) which is the commonest cause of α_1-antitrypsin deficiency in Japan (46, 47) and Mmalton (48), which is also known as Mnichinan (49) and Mcagliari (50), and results from deletion of 52Phe. The Mmalton mutation is the commonest cause of α_1-antitrypsin deficiency in Sardinia. Both of these alleles probably also cause liver disease but there is currently insufficient evidence to conclusively state that this is the case. The risk of liver disease in individuals who are heterozygous for the Z allele (i.e. PI*MZ) is uncertain.

The respiratory disease associated with α_1-antitrypsin deficiency usually present with increasing dyspnoea with cor pulmonale and polycythaemia occurring late in the course of the disease. Chest radiographs typically show bilateral basal emphysema with paucity and pruning of the basal pulmonary vessels. Upper lobe vascularisation is relatively normal. Ventilation perfusion radioisotope scans and angiography also show abnormalities with a lower zone distribution (51). High resolution CT scans with 1-2 mm collimation are the most accurate method of assessing the distribution of panlobular emphysema and for monitoring progress of the pulmonary disease (52, 53) although this currently has little clinical value. Lung function tests are typical for emphysema with a reduced ratio of forced expiratory volume in 1 second to forced vital capacity (FEV1/FVC), gas trapping (raised ratio of residual volume to total lung capacity) and low gas transfer factor. The onset of respiratory disease can be delayed to the 6th decade in never-smokers with PI*Z α_1-antitrypsin deficiency and these individuals often have a normal life span (54). Lung disease is characteristically seen in PI*Z α_1-antitrypsin homozygotes but PI*MZ individuals also have a slightly greater risk of emphysema if they smoke (55-58) although this view is controversial (59).

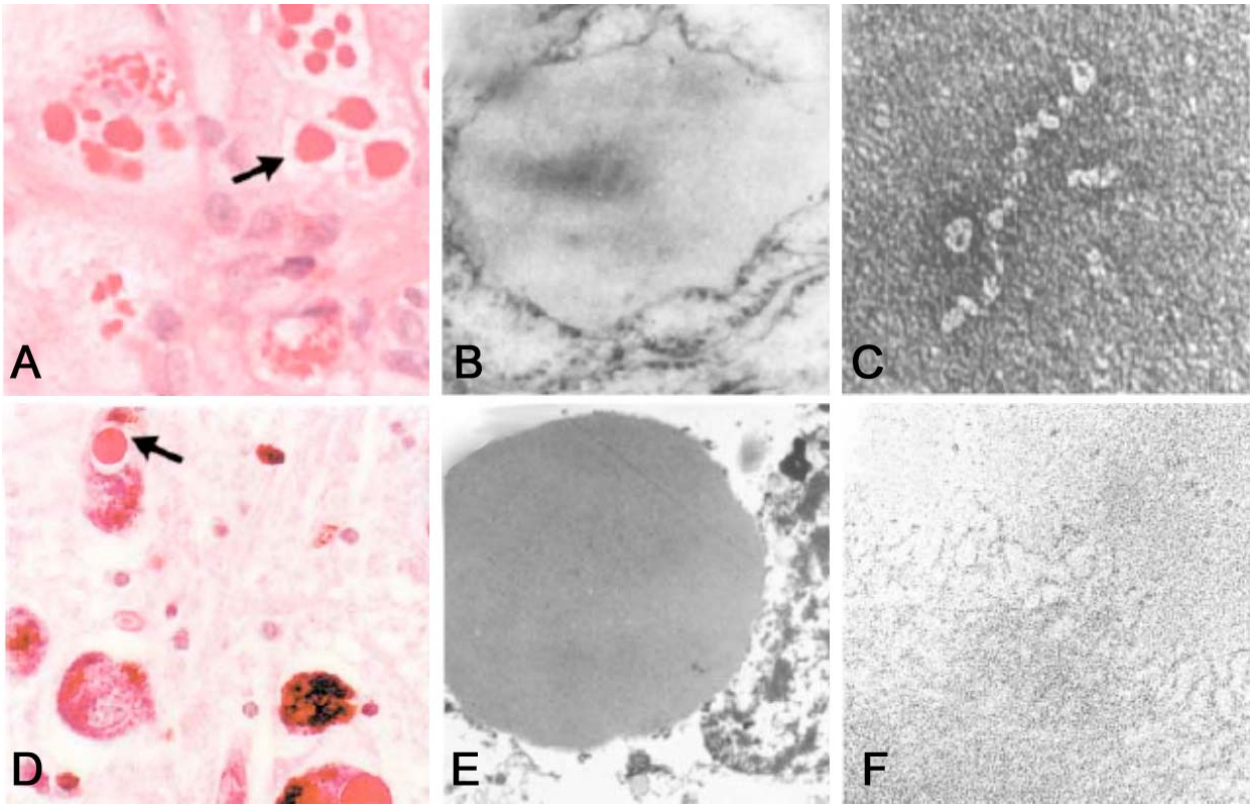


Figure 2. Z α_1-antitrypsin is retained within hepatocytes as intracellular inclusions. These inclusions are PAS positive and diastase resistant (Figure 2a, arrowed) and are associated with neonatal hepatitis and hepatocellular carcinoma. Figure 2b. Electron micrograph of an hepatocyte from the liver of a patient with Z α_1-antitrypsin deficiency shows the accumulation of α_1-antitrypsin within the rough endoplasmic reticulum. These inclusions are composed of chains of α_1-antitrypsin polymers shown here from the plasma of a Siiyama α_1-antitrypsin homozygote (Figure 2c) and from the liver of a Z α_1-antitrypsin homozygote (Figure 2f). Similar mutations in α_1-antitrypsin deficiency and neuroserpin encephalopathy result in similar intracellular inclusions of α_1-antitrypsin and neuroserpin. They are shown here in hepatocytes and neurons with PAS staining (Figures. 2a and d respectively) and as endoplasmic aggregates of the abnormal proteins on electron microscopy (Figures 2b and e respectively). Electron microscopy confirms that the abnormal neuroserpin forms bead-like polymers and entangled polymeric aggregates identical to those shown here with Z α_1-antitrypsin (Figures 2c and f respectively). (Magnification left to right: x200, x20,000, x220,000). Figure reproduced from reference (119) with permission.

2.3. Molecular pathology of the liver disease associated with mutants of α_1-antitrypsin

2.3.1. PI*Z α_1-antitrypsin

There is now overwhelming evidence that the liver disease associated with the Z variant of α_1-antitrypsin is due to a failure of secretion and accumulation of aggregated protein rather than the plasma deficiency of α_1-antitrypsin. Strong support is provided by the recognition that null alleles, which produce no α_1-antitrypsin, are not associated with cirrhosis (30). Moreover, the overexpression of Z α_1-antitrypsin in animal models results in liver damage (60, 61). Our understanding of the molecular basis of α_1-antitrypsin deficiency came from the recognition that the normal, active protein undergoes a profound conformational transition to inhibit its target proteinase, neutrophil elastase (Figure 1a). Moreover heating normal M α_1-antitrypsin at non-physiological temperatures resulted in the formation of high molecular mass polymers (62-65). Polymer formation was blocked by the incorporation of reactive loop peptides that annealed as strand 4 in β-sheet A (62-65).

The Z mutation of α_1-antitrypsin (Glu342Lys) is at residue P17 (17 residues proximal to the P1 reactive centre) at the head of strand 5 of β-sheet A and the base of the mobile reactive loop (Figure 1b). The mutation opens β-sheet A, thereby favouring the insertion of the reactive loop of a second α_1-antitrypsin molecule to form a dimer (14, 64, 66, 67). This can then extend to form polymers that tangle in the endoplasmic reticulum of the hepatocyte to form inclusion bodies (Figure 2). Support for this came from the demonstration that purified plasma Z α_1-antitrypsin formed chains of polymers when incubated under physiological conditions (64). The rate of polymer formation was accelerated by raising the temperature to 41°C and could be blocked by peptides that competed for annealing to β-sheet A (64, 65, 68). The role of polymerisation *in vivo* was confirmed by the finding of α_1-antitrypsin polymers in inclusion bodies from the liver of a Z α_1-antitrypsin homozygote with cirrhosis (64, 69) and in

hepatic cell lines expressing the Z variant (70). Moreover, point mutations that block polymerisation increased the secretion of mutants of α_1-antitrypsin from a *Xenopus* oocyte expression system (71).

The pathway of α_1-antitrypsin polymerisation has been assessed by biochemical, biophysical and crystallographic analysis and is shown in Figure 1b (66, 72). Step 1 represents the conformational change of α_1-antitrypsin to a polymerogenic monomeric form (M*), step 2 represents the formation of polymers (P), and step 3 represents a side pathway which leads to the formation of the stable, monomeric, inactive latent conformation (L) in which the reactive loop is fully incorporated into β-sheet A. The presence of the unstable, polymerising intermediate M* was predicted from the biophysical analysis of polymer formation (66), the demonstration of an unfolding intermediate (73-75), and solving the crystal structure of a polymerogenic mutant of α_1-antichymotrypsin (72). The Z mutation forces α_1-antitrypsin into a conformation that approximates the unstable M* and hence favours polymer formation (76).

The quality control mechanisms within the hepatocyte that handle polymers are now being elucidated (77-79). Elegant studies have demonstrated that it is the trimming of asparagine linked oligosaccharides that target Z α_1-antitrypsin polymers into an efficient non-proteosomal disposal pathway within hepatocytes. However, the proteosome has an important role in metabolising Z α_1-antitrypsin in some hepatic (80) and extrahepatic (81, 82) mammalian cell lines. Moreover, there is increasing evidence that the retained Z α_1-antitrypsin stimulates an autophagic response within the hepatocyte (83, 84). Despite our increased understanding of the disposal pathway, it still remains unclear how the accumulation of Z α_1-antitrypsin causes cell death and liver cirrhosis.

The temperature and concentration dependence of polymerisation (62, 64, 66), along with genetic factors (85, 86), may account for the heterogeneity in liver disease amongst individuals who are homozygous for the Z mutation. The synthesis of α_1-antitrypsin rises during episodes of inflammation as part of the acute phase response. At these times, the formation of polymers is likely to overwhelm the degradative pathway, thereby exacerbating the formation of hepatic inclusions and the associated hepatocellular damage. This hypothesis has been challenged by cell studies which do not show an increase in intracellular Z α_1-antitrypsin in response to raised temperatures (87). However our recent data in a *Drosophila* model of serpin deficiency shows a clear temperature dependence of polymerisation *in vivo* (88). There is also anecdotal clinical evidence to support the role of temperature in exacerbating the liver disease associated with Z α_1-antitrypsin from the prospective study of Sveger and colleagues in Sweden (36, 37). They screened 200 000 newborn babies and identified 120 Z homozygotes whom they have followed into late adolescence. Two of these patients developed progressive jaundice during the course of the study, in one this followed an acute appendicitis and in the other severe pneumonia. Other asymptomatic infants developed marked derangement of liver function tests in association with coryzal illnesses and eczema. Further prospective studies are required to assess whether pyrexial episodes occur more frequently and increase the burden of intra-hepatic polymers in Z α_1-antitrypsin homozygotes who develop liver disease compared to those individuals who remain asymptomatic.

2.3.2. Shutter domain mutants

Although many α_1-antitrypsin deficiency variants have been described, only two other (besides the Z allele) mutants of α_1-antitrypsin have similarly been associated with profound plasma deficiency and hepatic inclusions: α_1-antitrypsin Siiyama (Ser53Phe) and Mmalton (deletion of phenylalanine at position 52). Both of these mutants are in the shutter domain underlying the bifurcation of strands 3 and 5 of β-sheet A (Figure 1b). The mutations disrupt a hydrogen bond network that is based on 334His and bridges strands 3 and 5 of the A sheet (89), causing it to part to allow the formation of folding intermediates (74, 75) and loop-sheet polymers *in vivo* (90, 91). Polymerisation also underlies the mild plasma deficiency of other variants that perturb the shutter domain: S (Glu264Val) and I (Arg39Cys) α_1-antitrypsin (92, 93). These point mutations cause less disruption to β-sheet A than does the Z variant. Thus, the rates of polymer formation are much slower than that of Z α_1-antitrypsin (66) and this results in less retention of protein within hepatocytes, milder plasma deficiency, and the lack of a clinical phenotype. However, if a mild, slowly polymerising I or S variant of α_1-antitrypsin is inherited with a rapidly polymerising Z variant, then the two can interact to form heteropolymers within hepatocytes leading to inclusions and finally cirrhosis (93-95).

2.4. Molecular pathology of the lung disease associated with PI*Z α_1-antitrypsin

Alpha-1-antitrypsin levels are greatly reduced in the lungs of individuals with α_1-antitrypsin deficiency (96). Moreover, the α_1-antitrypsin that is available to protect the lungs is approximately 5-fold less effective at inhibiting neutrophil elastase than normal M α_1-antitrypsin (65, 97-99). The single most important factor in the development of emphysema in patients with α_1-antitrypsin deficiency is smoking (41, 100). The combination of antiproteinase deficiency and cigarette smoke can have a devastating effect on lung function (40, 101), probably by allowing the unopposed action of proteolytic enzymes. The inhibitory activity of Z α_1-antitrypsin can be further reduced as the Z mutation favours the spontaneous formation of α_1-antitrypsin loop-sheet polymers within the lung (102). This conformational transition inactivates α_1-antitrypsin as a proteinase inhibitor, thereby further reducing the already depleted levels of α_1-antitrypsin that are available to protect the alveoli (see (103) for recent review). Moreover the conversion of α_1-antitrypsin from a monomer to a polymer converts it to a chemoattractant for human neutrophils (104). The magnitude of the effect was similar to that of the chemoattractant C5a and was present over a

Table 1. Mutants of the serpins cluster in the shutter domain

Serpin template number	49	50	51	52	53	54	55	56	57	58	Effect of polymer formation
Serpin template amino acid	Asn	Ile	Phe	Phe	Ser	Pro	Val	Ser	Ile	Ala	
α_1-antitrypsin				ΔPhe	Phe						Accumulation: cirrhosis Lack of inhibitor: emphysema
Neuroserpin					Pro			Arg			Accumulation: dementia
C1-inhibitor				Ser		Leu					Lack of inhibitor: angio-oedema
Antithrombin						Thr					Lack of inhibitor: thrombosis
α_1-antichymotrypsin							Pro				Lack of inhibitor: emphysema

The homologous sequences at the commencement of the B-helix in α1-antitrypsin, neuroserpin, antithrombin, C1-inhibitor and α1-antitchymotrypsin have been plotted on the serpin template rather than their own residue number (1). The replacements are shown that result in polymerisation and disease

range of physiological concentrations (EC_{50} 4.5 ± 2 g/ml). Polymers also induced neutrophil shape change and stimulated myeloperoxidase release and neutrophil adhesion (104). The chemoattractant properties of α_1-antitrypsin polymers may explain the excess number of neutrophils in bronchoalveolar lavage (105) and in tissue sections of lung parenchyma (106) from individuals with Z α_1-antitrypsin deficiency. Moreover polymers may contribute to the excess inflammation that is apparent even in individuals with Z α_1-antitrypsin deficiency with very early lung disease (107). Any pro-inflammatory effect of polymers is likely to be exacerbated by inflammatory cytokines, cleaved or complexed α_1-antitrypsin (108), elastin degradation products (109) and cigarette smoke which themselves cause neutrophil recruitment. Thus our understanding of the biological properties of α_1-antitrypsin provides novel pathways for the pathogenesis of emphysema in individuals who are homozygous for the Z mutation (104).

3. DISEASE CAUSED BY THE POLYMERISATION OF OTHER SERPINS: THE SERPINOPATHIES

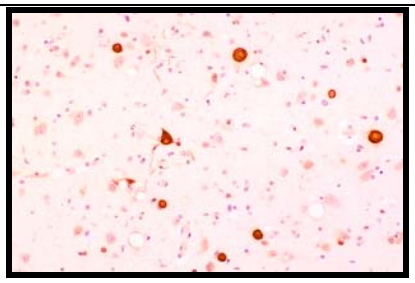
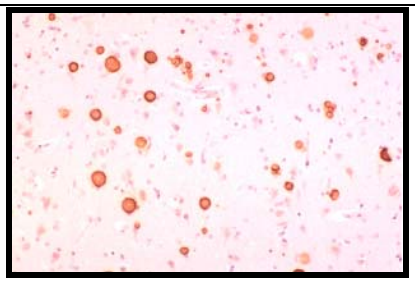
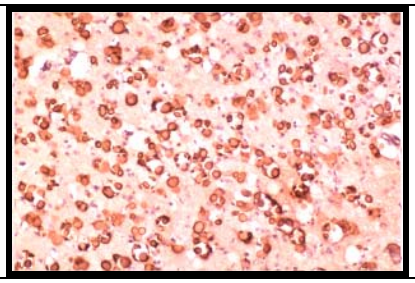
3.1. Antithrombin, C1 inhibitor and α_1 - antichymotrypsin

Alpha-1-antitrypsin is the archetypal member of the serine proteinase inhibitor or serpin superfamily. This family includes members such as α_1 -antichymotrypsin, C1 inhibitor, antithrombin and plasminogen activator inhibitor-1 which play an important role in the control of proteinases involved in the inflammatory, complement, coagulation and fibrinolytic cascades respectively (12, 13). The family is characterised by more than 30% sequence homology with α_1-antitrypsin and conservation of tertiary structure (1, 110). Consequently, physiological and pathological processes that affect one member may be extrapolated to another. The phenomenon of loop-sheet polymerisation is not restricted to α_1-antitrypsin and has now been reported in mutants of other members of the serpin superfamily to cause disease. Naturally occurring mutations have been described in the shutter (Table 1) and other domains of the plasma proteins C1-inhibitor (Phe52Ser, Pro54Leu, Ala349Thr, Val366Met; Phe370Ser, Pro391Ser) (111, 112), antithrombin (Pro54Thr, Asn158Asp, Phe229Leu) (113, 114) and α_1-antichymotrypsin (Leu55Pro, Pro229Ala) (72, 115, 116). In all cases the residue numbers are based on the serpin template to allow comparison between members of the family (117). These mutations destabilise the serpin architecture to allow the formation of inactive reactive loop into β-sheet polymers. The polymers are most likely to form within the endoplasmic reticulum of the liver but are rapidly degraded. They are usually not associated with the formation of inclusions and the liver disease seen in individuals with Z α_1-antitrypsin deficiency, as C1-inhibitor, antithrombin and α_1-antichymotrypsin are synthesised at approximately 10% of the rate of α_1-antitrypsin. However one variant of α_1-antichymotrypsin (Pro229Ala) has been shown to form granular inclusions within hepatocytes analogous to those formed by Z α_1-antitrypsin (115). This mutation also allows the spontaneous formation of polymers *in vitro* (B. Gooptu and D. Lomas, unpublished observations). The individual with the Pro229Ala α_1-antichymotrypsin mutation was infected with the hepatitis C virus and it seems likely that the viral infection drove the inflammatory response which increased the production of α_1-antichymotrypsin polymers to form the inclusions. The lack of circulating protein in individuals with C1-inhibitor, antithrombin and α_1-antichymotrypsin deficiency allows uncontrolled activity of proteolytic cascades and hence angio-oedema, thrombosis and chronic obstructive pulmonary disease respectively (117). In view of the common underlying disease mechanism we have grouped these conditions together as the serpinopathies (106, 118, 119).

3.2. Familial encephalopathy with neuroserpin inclusion bodies (FENIB)

The process of disease related polymerisation is most strikingly displayed by the inclusion body dementia, familial encephalopathy with neuroserpin inclusion bodies (FENIB) (120-122). This is an autosomal dominant dementia characterised by eosinophilic neuronal inclusions of neuroserpin (Collins' bodies) in the deeper layers of the cerebral cortex and the substantia nigra. The inclusions are PAS positive and diastase resistant and bear a striking resemblance to those of Z α_1-antitrypsin that form within

Table 2. Mutations, quantity of cerebral inclusions of neuroserpin and severity of disease in patients who died of FENIB (124)

Mutation	Histology of inclusions at post-mortem	Predicted instability	Age of onset of symptoms	Clinical Manifestations
Ser49Pro		+	48	Dementia, tremor, seizures in terminal stages
Ser52Arg		++	24	Myoclonus, status epilepticus, dementia
His338Arg	N/A	+++	15	Myoclonic seizures, dementia, tremor, dysarthria
Gly392Glu		++++	13	Myoclonus, status epilepticus, dementia, chorea

The diagnosis in the individual with the His338Arg mutation was determined on brain biopsy and not at post mortem. In all cases the quantity of inclusions correlates with the predicted or measured degree of neuroserpin instability. Note the earlier age of onset of disease with the increasing numbers of inclusion bodies

the liver (Figure 2). The observation that FENIB was associated with a mutation Ser49Pro (Ser53Pro on the α_1-antitrypsin template, Table 1) in the neuroserpin gene that was homologous to one in α_1-antitrypsin that causes cirrhosis (Ser53Phe) (90) strongly indicated a common molecular mechanism. This was confirmed by the finding that the neuronal inclusion bodies of FENIB were formed by entangled polymers of neuroserpin with identical morphology to those present in hepatocytes from a child with α_1-antitrypsin deficiency related cirrhosis (121). Moreover we have recently shown that recombinant Ser49Pro neuroserpin has a greatly accelerated rate of polymerisation when compared to the wild type protein (123).

The direct relationship between the magnitude of the intracellular accumulation of neuroserpin and the severity of disease is clearly shown by the recent identification of other mutations of neuroserpin in families with FENIB (124). In the original family with Ser49Pro neuroserpin (neuroserpin Syracuse) the affected family members had diffuse small intraneuronal inclusions of neuroserpin with an onset of dementia between the ages of 45 and 60 years (120-122). However, in a second family, with a conformationally more severe mutation (neuroserpin Portland; Ser52Arg) and larger inclusions, the onset of dementia was in early adulthood; and in a third family, with yet another mutation (His338Arg) there were more inclusions and the onset of dementia in adolescence. The most striking example was the family with the most 'polymerogenic' mutation of neuroserpin, Gly392Glu (Table 2). This replacement of a consistently conserved residue in the shutter region resulted in large multiple inclusions in every neurone, with affected family members dying by age 20 years (124). Thus FENIB shows a clear genotype-phenotype correlation, with the severity of disease correlating closely with the propensity of the mutated neuroserpin to form polymers.

The cellular handling of neuroserpin has been

assessed by transiently transfecting COS cells with wildtype neuroserpin and mutants of neuroserpin that cause FENIB. The most striking feature of the cell model is the retention of Syracuse (Ser49Pro) and Portland (Ser52Arg) neuroserpin as intracellular aggregates composed of polymers of mutant neuroserpin, similar to the loop-sheet polymers of mutant neuroserpin that can be isolated from the brains of individuals affected by FENIB (125) (Figure 3a). Moreover, N-glycan digestion of metabolically labelled neuroserpin and co-staining with antibodies against neuroserpin and the ER chaperone calreticulin demonstrated that neuroserpin aggregates are contained within the ER (Figure 3b). This is in keeping with the localisation of mutant neuroserpin within the brains of individuals with FENIB.

These findings, added to the prior evidence from α_1-antitrypsin-cirrhosis (64), strongly indicate that intracellular protein aggregation is by itself sufficient to cause neurodegeneration (124). A possible mechanism by which the polymers cause cell death is apparent from recent studies showing that the accumulation of protein aggregates can overwhelm and inhibit the ubiquitin-proteosome pathway (126) and directly trigger cell death (127, 128).

4. SEEDING OF POLYMERISATION

The polymerisation of α_1-antichymotrypsin is unusual in that the addition of pre–formed α_1-antichymotrypsin polymers to monomeric α_1-antichymotrypsin progressively abolished the initial lag phase in the light scatter profile during polymer formation (129, 130). Moreover the shape of the curve was transformed from sigmoidal to hyperbolic. This behaviour is characteristic of a nucleation–dependent process and has never been previously described in polymerisation of other members of the serpin superfamily (64, 66, 113, 131, 132). It resembles the mechanism of polymerisation of β-amyloid and α-synuclein in Alzheimer's and Parkinson's disease respectively (133, 134). In order to explain the mechanism by which α_1-antichymotrypsin polymers accelerate the polymerisation of the monomeric protein it is important to consider the structure of the polymer (Figure 1b). The final molecule in any polymer chain must undergo a conformational change that makes it a more efficient acceptor or donor of reactive loops in the loop–sheet polymerisation reaction. The binary complex of α_1-antichymotrypsin with an exogenous reactive loop peptide bound as s4A was used to investigate whether the effect could be due to more efficient loop donation (129). The binary complex has a loop that is available for donation but has a complete six–stranded β-sheet A and so cannot accept a reactive loop from another molecule of α_1-antichymotrypsin. The light scatter assay demonstrated a concentration dependent acceleration in α_1-antichymotrypsin polymerisation upon addition of binary complex. Cleaved α_1-antichymotrypsin did not accelerate the polymerisation reaction, demonstrating that completion of β-sheet A with a sixth strand was not in itself the cause of seeding.

The acceleration of polymerisation is not a non-specific effect of serpin polymers as neither short nor long chain polymers of α_1-antitrypsin nor polymers of antithrombin were able to accelerate the polymerisation of native α_1-antichymotrypsin. Sonication of α_1-antichymotrypsin polymers further increased the rate of native α_1-antichymotrypsin polymerisation supporting the hypothesis that it is the polymer end, probably the free-reactive loop end, that seeds the polymerisation of the monomeric protein (129). This result was particularly striking as the sonicated α_1-antichymotrypsin was able to accelerate polymerisation of native α_1-antichymotrypsin under physiological conditions, that is at body temperature. Thus the polymerisation of at least one serpin, α_1-antichymotrypsin, can be accelerated by seeding. This observation may have implications for the deposition of α_1-antichymotrypsin in the β-amyloid plaques of Alzheimer's disease (135).

5. POLYMERISATION OF PLASMINOGEN ACTIVATOR INHIBITOR 1 (PAI-1)

Plasminogen activator inhibitor type 1 (PAI-1) is a member of the serine proteinase inhibitor or serpin superfamily (12, 136). It inhibits urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (137) and as such is an important modulator of events of extracellular proteolysis, fibrinolysis and turnover of extracellular matrix (138). The striking feature of PAI-1 is that it can spontaneously form an inactive latent conformation (see Figure 1b) in which the intact reactive centre loop is stably inserted into β-sheet A (139-142). This controls its ability to act as a proteinase inhibitor *in vivo.* Alpha$_1$-antichymotrypsin (143) and antithrombin (144) also form the latent conformation *in vivo* and antithrombin and α_1-antitrypsin can be induced to adopt this conformation by heating in stabilising concentrations of sodium citrate (145-148). It has long been believed that PAI-1 is unique amongst active serpins in that it does not form polymers. However the crystal structure of recombinant active PAI-1 revealed PAI-1 as a polymer, in which the reactive loop anneals as strand 7A of β-sheet A (149). This is in contrast to other models of serpin polymers in which the loop anneals as either strand A4 or strand 1C sheet of another molecule (Figure 4). A more detailed examination has shown that recombinant native and latent PAI-1 spontaneously form polymers *in vitro* at low pH, though with distinctly different electrophoretic patterns of polymerisation (132). The polymers of both the native and latent species differ from the typical loop-A-sheet polymers of other serpins in that they readily dissociate back to their original monomeric form. The findings with PAI-1 suggest different mechanisms of linkage, each involving β-strand addition of the reactive loop to s7A in native PAI-1 and to s1C in latent PAI-1. Glycosylated native and latent PAI-1 also formed polymers under similar conditions, which may be of *in vivo* importance in the low pH environment of the platelet (132). A similar acid-dependent polymerisation of α_1-antitrypsin has also been reported (150) indicating that α_1-antitrypsin can also be induced to form non β-sheet A polymers under denaturing conditions. More recently classical reactive loop:β-sheet A sheet polymers of PAI-1

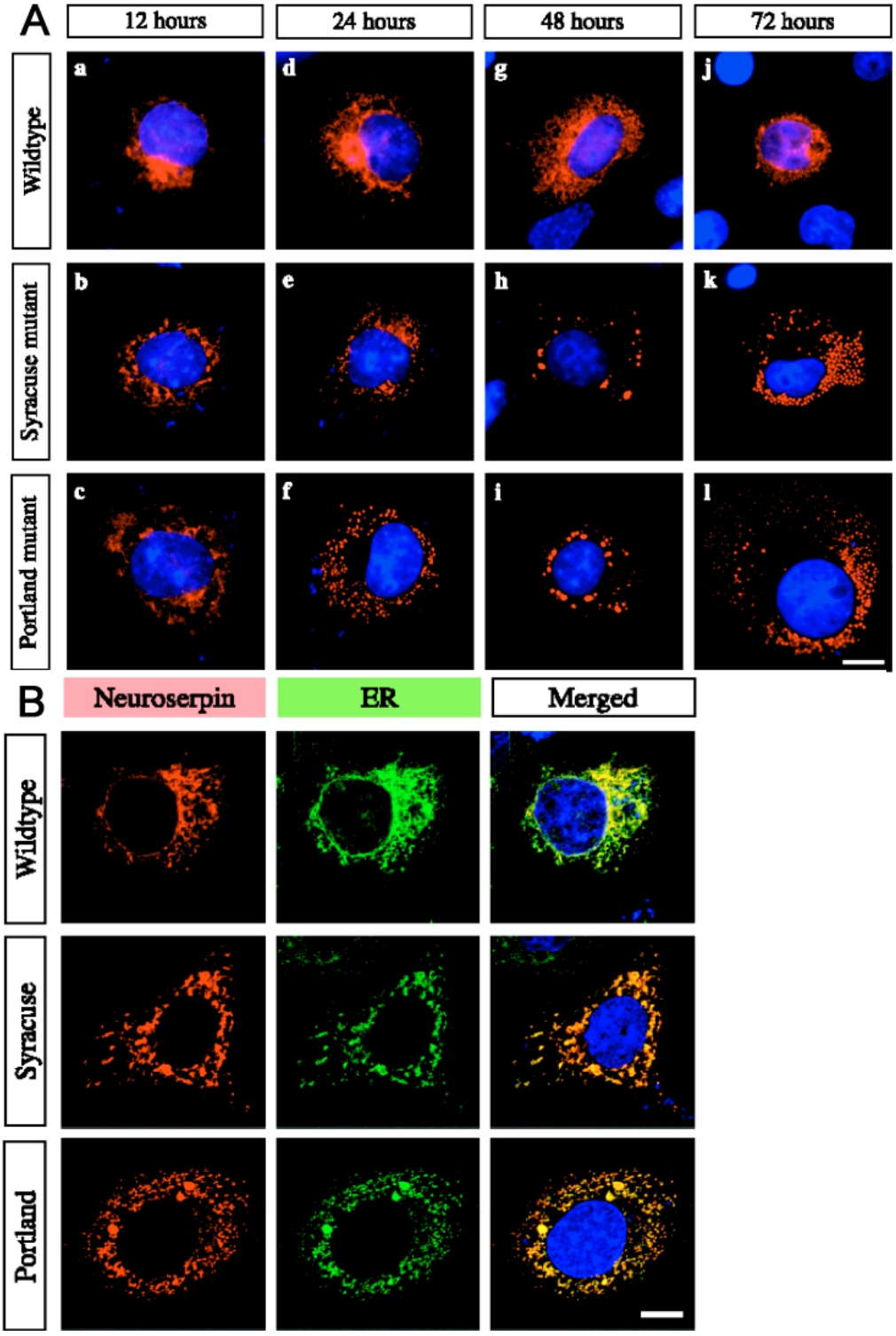


Figure 3. (a) Mutant Syracuse and Portland neuroserpin aggregate within COS-7 transfected cells. a-l. Immunocytochemistry with an anti-neuroserpin antibody showing the distribution of wildtype (a, d, g, j), Syracuse (b, e, h, k) and Portland (c, f, i, l) neuroserpin in COS-7 transfected cells. The nucleus appears blue due to DNA staining with DAPI. Scale bar:10 μm. Over a 3 day period, wildtype neuroserpin shows a normal endoplasmic reticulum staining pattern whereas the neuroserpin mutants form distinct protein aggregates after 24 hours of expression that persist for the 3 days of the experiment. (b) Intracellular localization of wildtype, Syracuse and Portland neuroserpin in COS-7 transfected cells. Confocal microscopy of cells cultured for 24 h after transfection and stained for neuroserpin (labelled with Texas red) and an ER-resident protein, calreticulin (labelled with fluorescein). The merged image shows that the mutant protein is retained within the endoplasmic reticulum. The nucleus appears blue due to DNA staining with DAPI. Scale bar:10 μm. Figure 3a and 3b reproduced from (125) with permission.

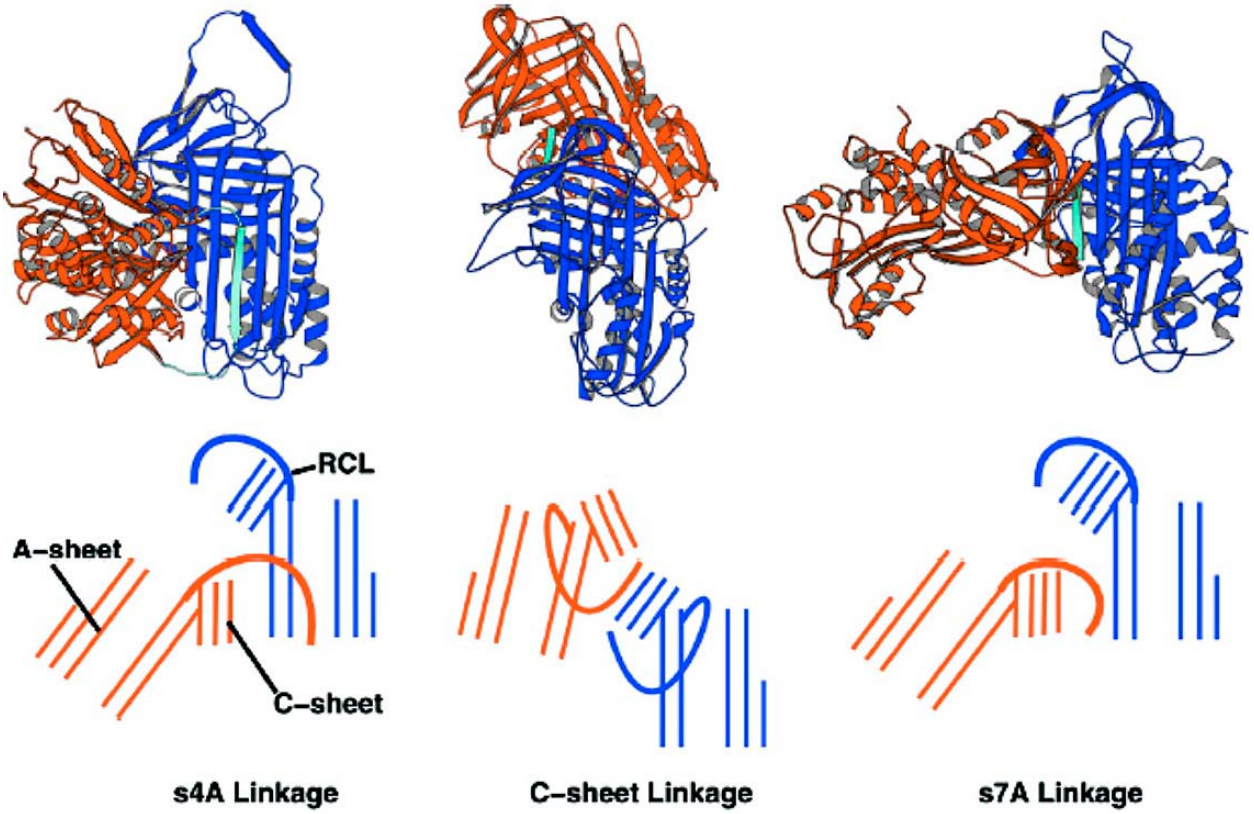


Figure 4. Schematic representation of serpin polymers. The loop-A sheet interaction (left) is believed to underlie the long chain polymers which result from naturally occurring mutants of α_1-antitrypsin (67) but the C-sheet (middle) and s7A linkages (right) have been described in crystal structures of antithrombin and PAI-1 respectively (145, 149). In each of these models, the acceptor molecule is shown in blue, the donor molecule in red and the reactive loop centre loop in cyan. Figure reproduced from reference (132) with permission.

have been formed by incubating the protein with negatively charged organochemical ligands (151, 152). The polymers ranged from dimers to multimers of more than 20 units, had increased thermal stability and were inactive as proteinase inhibitors. Further studies showed that the ligands could also induce the polymerisation of heparin co-factor II but not other serpins such as α_1-antitrypsin, α_1-antichymotrypsin, PAI-1, α_2-antiplasmin, antithrombin, C1-inhibitor, protease nexin 1 or ovalbumin (152)

6. POLYMERISATION OF PLASMINOGEN ACTIVATOR INHIBITOR 2 (PAI-2)

Plasminogen activator inhibitor-2 is a predominantly intracellular serpin. No intracellular target has been identified to date but PAI-2 has been implicated in protecting against apoptosis (153, 154), perhaps by enhancing the tumour suppressor activity of the retinoblastoma protein (155). This effect is mediated, not by the reactive centre loop, but by the loop linking helices C and D (155, 156). PAI-2 is unique in that it is the only native serpin that spontaneously forms polymers under physiological conditions (157, 158). In keeping with the observations for α_1-antitrypsin, the polymerisation of PAI-2 is concentration and temperature dependent and can be inhibited by annealing peptides that are homologous to the reactive centre loop of PAI-2 (158). The X-ray structure of PAI-2 showed a unique configuration of amino acids at the top of β-sheet A (159) but mutational analysis demonstrated that this was not the reason for the propensity to form polymers (160). Instead PAI-2 is held in a 'polymerogenic' configuration with a patent β-sheet A by a disulphide bond between Cys79 (in the flexible CD loop) and Cys161 (at the base of the F-helix) (161). Reduction of this bond 'closes' β-sheet A and abrogates polymerisation. Thus it has been proposed that the Cys79-Cys161 disulphide bond acts as a redox-sensitive switch that converts PAI-2 between an active stable monomeric and a polymerogenic conformation. The role of this switch within the cell, the biological function of intracellular polymers *in vivo*, and the way in which polymers of PAI-2 are handled by cells remains to be elucidated.

7. ANIMAL MODELS OF THE SERPINOPATHIES

There are 'knockout' mouse models for serpins such as antithrombin, heparin co-factor II, α_2-antiplasmin, hsp47, PAI-1, PAI-2, protease nexin 1, protein C inhibitor, maspin and angiotensinogen that show a range of structural and physiological abnormalities (see (13) for review). Mice

have also been generated that over express the M and Z alleles of α_1-antitrypsin (60, 61, 162). They have a variable severity of liver disease in keeping with the accumulation of polymers of Z α_1-antitrypsin within hepatocytes. More recently we have reported a *Drosophila* model of serpin polymerisation (88). The necrotic (nec) gene in *Drosophila melanogaster* is one of a cluster of serpin transcripts mapping near the base of the second right chromosome arm (163, 164). Lack of function nec mutants hatch as weak adults that develop black melanised spots on the body and leg joints and die within a few days of eclosion (hatching). In addition to the visible phenotype, the TOLL-mediated immune response to fungal infections is constitutively activated in nec mutants (165). The Nec protein consists of a serpin core, that has sequence homology with α_1-antitrypsin, and a polyglutamine-rich N-terminal extension of 79 amino-acids that is not found in other serpins (163, 164). Random nec mutants were prepared by treating the flies with chemical mutagens and X-rays (166). Fourteen 'lack of function' nec mutants were obtained and sequencing of the nec gene identified a range of mutations including both stop codons and single amino acid substitutions. Of particular interest were the Glu421Lys and Gly466Ser mutations that were found on 2 occasions. These mutants are analogous to the naturally occurring Z allele of α_1-antitrypsin (Glu342Lys) and a mutant of antithrombin (Gly424Arg) respectively that both spontaneously form polymers in association with loss of inhibitory function and human disease. Moreover we were able to demonstrate the formation of Nec polymers within the fly. Thus the fly may be useful as a model of serpin polymerisation that causes a 'loss of function' phenotype (167).

8. PREVENTION OF POLYMER FORMATION

There is now strong evidence that polymers of α_1-antitrypsin, and indeed of mutants of all other serpins that are associated with disease, form by an aberrant linkage between the reactive centre loop of one molecule and β-sheet A of another (14, 62-64, 67, 168, 169). This has allowed the development of new strategies to attenuate polymerisation and so treat the associated disease. Three strategies have been pursued to date: (i) chemical chaperones to stabilise the unstable mutant serpin, (ii) filling a surface cavity to block the conformational transition underlying polymer formation and (iii) the use of reactive loop peptides that compete for binding to β-sheet A.

8.1. Chemical chaperones to stabilise serpins and block polymerisation

Chemical chaperones can stabilise intermediates on the folding pathway. Osmolytes such as betaine, trimethyamine oxide and sarcosine all stabilise α_1-antitrypsin against polymer formation (170). However the chaperone trimethyamine oxide had no effect on the secretion of Z α_1-antitrypsin in cell culture (87) as it favoured the conversion of unfolded Z α_1-antitrypsin to polymers (171). In contrast, glycerol increased the secretion of Z α_1-antitrypsin from cell lines (87) most likely as it binds to, and stabilises, β-sheet A (89). 4-phenylbutyrate (4-PBA) also increased the secretion of Z α_1-antitrypsin from cell lines and transgenic mice (87). This agent has been used for several years to treat children with urea cycle disorders and more recently 4-PBA has been shown to increase the expression of mutant (□F508) cystic fibrosis transmembrane regulator protein *in vitro* (172) and *in vivo* (173). These encouraging findings have led to a pilot study that is currently ongoing to evaluate the potential of 4-PBA to promote the secretion of α_1-antitrypsin in patients with α_1-antitrypsin deficiency. Finally a recent study has shown that the chaperone α-crystallin can block the polymerisation of α_1-antichymotrypsin, which is nucleation dependent, but not that of α_1-antitrypsin which is not dependent on nucleation (130). Thus strategies to reduce nucleation may be effective in preventing polymerisation of some serpins.

8.2. Filling a surface hydrophobic pocket to block polymerisation

A second strategy comes from the identification of a hydrophobic pocket in α_1-antitrypsin that is bounded by strand 2A and helices D and E (17, 174). The cavity is patent in the native protein but is filled as β-sheet A accepts an exogenous reactive loop peptide during polymerisation (17). The introduction of either Thr114Phe or Gly117Phe on strand 2 of β-sheet A within this cavity significantly raised the melting temperature of α_1-antitrypsin and retarded polymer formation. Conversely, Leu100Phe on helix D accelerated polymer formation but this effect was abrogated by the addition of Thr114Phe. None of these mutations affected the inhibitory activity of α_1-antitrypsin. The importance of these observations was underscored by the finding that the Thr114Phe mutation reduced polymer formation and increased the secretion of Z α_1-antitrypsin from a *Xenopus* oocyte expression system. Moreover cysteine mutants within the hydrophobic pocket were able to bind a range of fluorophores illustrating the accessibility of the cavity to external agents. These data demonstrate the importance of this cavity as a site for rational drug design to ameliorate polymerisation and treat the associated conformational disease (175). However this approach may not be applicable to all serpins as amphipathic organoligands can bind to this region of PAI-1 to induce polymer formation (151, 152, 176).

8.3. Peptides with homology to the reactive centre loop can compete for binding to β-sheet A and block polymerisation

We have shown previously that the polymerisation of Z α_1-antitrypsin can be blocked by annealing of reactive loop peptides to β-sheet A (64, 68). Such peptides were 11-13 residues in length and could bind to other members of the serpin superfamily (68, 177). This was most clearly demonstrated by the finding that reactive loop peptide of antithrombin inserted more readily to β-sheet A of α_1-antitrypsin and *vice versa* (178). These peptides, although useful in establishing the mechanism of polymerisation, are too long and too promiscuous to be suitable for rational drug design. More

recently, the recognition that the Z mutation forces α_1-antitrypsin into a conformation similar to M* (see Figure 1b) has allowed the design of a 6-mer peptide that specifically anneals to the lower part of β-sheet A and blocks polymerisation (76, 179). This peptide was specific to Z α_1-antitrypsin and did not anneal significantly to other serpins (such as antithrombin, α_1-antichymotrypsin and PAI-1) with a similar tertiary structure. Indeed, smaller trimer peptides have been developed that will also anneal to a patent β-sheet A of antithrombin *in vitro* (89). The aim now is to convert these peptides into small molecule inhibitors that can be used to block aberrant polymerisation *in vivo*.

9. CONCLUSION

The molecular basis of the serpinopathies has now been elucidated with biochemical, cellular and structural studies. The current goals are to determine the cellular response to polymeric serpins and to develop therapeutic strategies to block polymerisation *in vivo*.

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