[Prion 1:1, 15-20; January/February/March 2007]; ©2007 Landes Bioscience

Mini-Review

Protein Misfolding and the Serpinopathies

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Original manuscript submitted: 01/22/07
Revised manuscript submitted: 02/06/07
Manuscript accepted:02/06/07

Previously published online as a *Prion* E-publication:
http://www.landesbioscience.com/journals/Prion/abstract.php?id=3974

KEY WORDS

serpins, α1-antitrypsin, neuroserpin, polymerization, dementia, conformational disease, serpinopathies

ABBREVIATIONS

RCL	Reactive Centre Loop
PAS	periodic acid Schiff
FENIB	familial encephalopathy with neuroserpin inclusion bodies
ER	endoplasmic reticulum

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (UK), the Wenner Gren Foundations, the Swedish Society for Medical Research and the Isaac Newton Trust Cambridge European Trust.

ABSTRACT

The serpins are the largest superfamily of protease inhibitors. They are found in almost all branches of life including viruses, prokaryotes and eukaryotes. They inhibit their target protease by a unique mechanism that involves a large conformational transition and the translocation of the enzyme from the upper to the lower pole of the protein. This complex mechanism, and the involvement of serpins in important biological regulatory processes, makes them prone to mutation-related diseases. For example the polymerization of mutant α_1-antitrypsin leads to the accumulation of ordered polymers within the endoplasmic reticulum of hepatocytes in association with cirrhosis. An identical process in the neuron specific serpin, neuroserpin, results in the accumulation of polymers in neurons and the dementia FENIB. In both cases there is a clear correlation between the molecular instability, the rate of polymer formation and the severity of disease. A similar process underlies the hepatic retention and plasma deficiency of antithrombin, C1 inhibitor, α_1-antichymotrypsin and heparin co-factor II. The common mechanism of polymerization has allowed us to group these conditions together as a novel class of disease, the serpinopathies.

Serpins (or serine protease inhibitors) are the largest family of protease inhibitors. They have been found in all major branches of life including viruses, prokaryotes and eukaryotes.[1-3] Despite their name there is increasing evidence that serpins can also inhibit other classes of proteases as demonstrated by the viral serpin CrmA and recently by a plant serpin, serpin1.[4,5] They can even play a non-inhibitory role in events as diverse as blood pressure regulation (angiotensinogen), chromatin condensation (MENT), tumor progression (maspin), protein folding (hsp47) and hormone transport (cortisol and thyroxine binding globulin).[6]

One of the most important roles of serpins is the regulation of enzymes involved in proteolytic cascades. Among these serpins are α_1-antitrypsin, α_1-antichymotrypsin, C1 inhibitor, antithrombin and plasminogen activator inhibitor-1, which play an important role in the control of proteases involved in the inflammatory, complement, coagulation and fibrinolytic pathways, respectively.[1,3] The serpin superfamily is characterised by more than 30% homology with the archetypal serpin α_1-antitrypsin and conservation of tertiary structure.[7,8] Serpins adopt a metastable conformation composed in most cases of 9 α-helices, three β-sheet (A to C) and an exposed mobile reactive centre loop (RCL). This flexible RCL typically contains 20 residues that act as a pseudo substrate for the target protease (Fig. 1A).[9-15] After formation of a Michaelis complex[16,17] the enzyme cleaves the P1-P1' bond of the serpin, releasing the P1' residue and forming an ester bond between the protease and the serpin.[18,19] This is then followed by a dramatic conformational transition from a stressed to relaxed conformation with the enzyme being pulled from the upper to the lower pole of the serpin and the insertion of the reactive loop as an extra strand in β-sheet A.[20-25] As a consequence of this conformational change the thermal stability of the serpin is greatly enhanced. Whereas a typical serpin in its native state exhibits a midpoint of thermal denaturation of around 50–60°C, a cleaved serpin with its RCL fully incorporated into β-sheet A denatures at temperatures >120°C.[9,26,27] Another consequence is the inactivation of the enzyme, stabilised at the acyl-intermediate and unable to proceed further to deacylation of the complex.[24,28] This serpin-protease complex then binds to members of the lipoprotein receptor family and is cleared from the circulation.[29-31]

Despite the evolutionary advantage conferred upon serpins by the remarkable mobility of the native state, their complexity is also their weak point.[19,32] Mutations affecting the serpins can lead to a variety of diseases, resulting from either a gain or loss of function.[6,19] For example mutations can cause aberrant conformational transitions that result in the

retention of the serpin within the cell of synthesis. This will lead to either protein overload and death of the cell in which the serpin is synthesised, or disease as a consequence of the resulting plasma deficiency. Such a mechanism underlies diseases as diverse as cirrhosis, thrombosis, angio-oedema, emphysema and dementia. We review here the common mechanism underlying these diseases that we have grouped together as the serpinopathies.[33-35] The aggregation and accumulation of conformationally destabilized proteins is an important feature of many neurodegenerative diseases, including Alzheimer's and Parkinson's disease and the spongiform encephalopathies. Indeed we have used the serpinopathies as a paradigm for these other 'conformational diseases'.[36]

POLYMERIZATION OF α_1-ANTITRYPSIN IN THE PATHOGENESIS OF CIRRHOSIS AND EMPHYSEMA

α_1-antitrypsin is an acute phase glycoprotein that is synthesised by, and secreted from, the liver. It inhibits neutrophil elastase and therefore plays an important role in the control of the inflammatory response. More than 100 allelic variants have been described with the most clinically relevant being the S (Glu264Val) and Z (Glu342Lys) alleles.[37-40] The S allele is found in up to 28% of southern Europeans[39] and reduces plasma levels of α_1-antitrypsin to 60% of the normal M protein. The Z allele is present in 4% of northern Europeans[39] and reduces the plasma level to 10–15% of normal. The decrease in plasma level is not associated with any clinical phenotype in the S homozygote but it has dramatic effects in those who are homozygous for the Z allele. The Z mutation causes the retention of α_1-antitrypsin in hepatocytes as diastase resistant, periodic acid-Schiff (PAS) positive inclusions that cause both neonatal and adult liver disease (Fig. 2).[41-44]

Following synthesis, misfolded monomeric Z α_1-antitrypsin is degraded by the proteosome but 10–15% folds normally and traffics through the secretory pathway to be released into the circulation. Disease results from the proportion of Z α_1-antitrypsin that folds to form polymers. These accumulate within the endoplasmic reticulum (ER) of hepatocytes to form inclusions and hence cause disease.[44,45] Biochemical, biophysical and crystallographic studies have elucidated the molecular basis of the polymerization of Z α_1-antitrypsin. The mutation associated with the Z allele is located at the head of strand 5 of β-sheet A and the base of the mobile reactive loop (Fig. 1B). This mutation causes a conformational transition and the formation of an unstable intermediate that we have called M*. M* is characterised by partial insertion of the RCL and opening of β-sheet A. The patent β-sheet A can then accept the loop of another molecule to form a loop-sheet dimer, which extends to form longer chains of loop-sheet polymers.[44-49] Polymers activate the ER overload response but their ordered nature allows them to escape the surveillance of the unfolded protein response. Indeed this pathway is only activated in the presence of a secondary insult.[50,51] More recently it has become apparent that polymers can be handled by autophagic pathways within hepatocytes.[52-55]

Further evidence of the importance of α_1-antitrypsin polymers in the pathogenesis of liver disease is provided by two other mutants of α_1-antitrypsin that are similarly associated with plasma deficiency and hepatic inclusions: α_1-antitrypsin Siiyama (Ser53Phe)[56] and Mmalton (ΔPhe52).[57] The Siiyama variant is the commonest cause of α_1-antitrypsin deficiency in Japan whilst the Mmalton variant is the commonest cause of α_1-antitrypsin deficiency in the isolated island of Sardinia. Both of these mutants disrupt a hydrogen bond

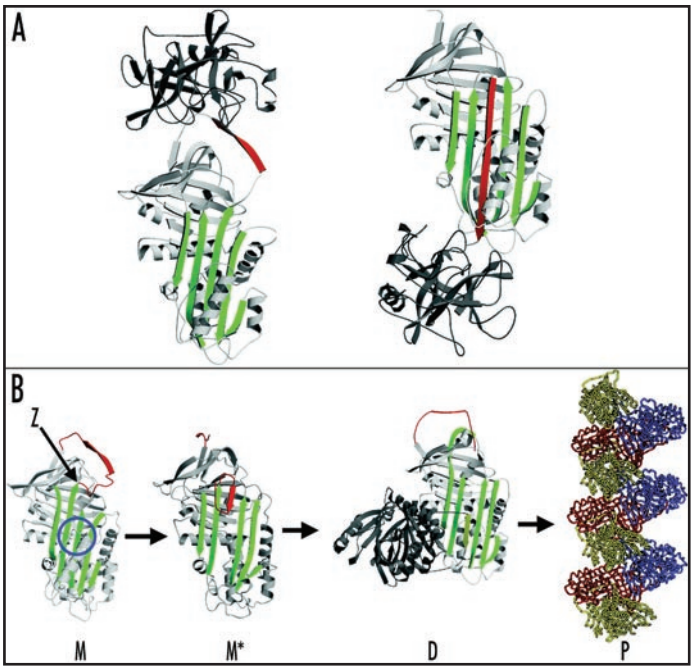

Figure 1. Inhibition of neutrophil elastase by α_1-antitrypsin and the structural basis of polymerization. (A) After docking (left) the neutrophil elastase (grey) is inactivated by movement from the upper to the lower pole of the protein (right). This is associated with the insertion of the RCL (red) as an extra strand into β-sheet A (green). (B) The structure of α_1-antitrypsin is centred 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 P17; indicated by arrow) or mutations in the shutter domain (blue circle) that open β-sheet A to favour partial loop insertion and the formation of an unstable intermediate (M*). The patent β-sheet A then accepts the loop of another molecule to form a dimer (D), which then extends into polymers (P). The individual molecules of α_1-antitrypsin within the polymer, although identical, are coloured red, yellow and blue for clarity. Figure reproduced with permission from Lomas et al.[97]

network based on His334 that bridges strands 3 and 5 of β-sheet A (the shutter domain; Figs. 1A and 2),[58] causing it to open and allow the formation of folding intermediates[59] and loop-sheet polymers in vivo.[60,61] The mild S (Glu264Val) and I (Arg39Cys) variants of α_1-antitrypsin also lie in the shutter domain and can also form polymers in vivo. However they do so at a slower rate and this polymer formation[47] is associated with a mild plasma deficiency and no clinical phenotype.[62,63] However, if a slowly polymerizing S (Glu264Val) or I (Arg39Cys) variant is inherited with a fast polymerizing Z variant then they will form heteropolymers that accumulate within hepatocytes and lead to cirrhosis.[63-65] Thus there is a striking genotype-phenotype correlation between the rate of polymerization, the retention of α_1-antitrypsin within the liver, and the severity of the plasma deficiency.

The reduction in the circulating level of α_1-antitrypsin predisposes the Z homozygote to early onset, panlobular, basal emphysema.[66-68] This predisposition is particularly apparent in Z α_1-antitrypsin homozygotes who smoke as the combination of low levels of α_1-antitrypsin within the lung and the inflammation caused by smoking have a dramatic effect on lung function.[69,70] The intrapulmonary deficiency of α_1-antitrypsin is exacerbated by the effect of the point mutation which reduces the association kinetics with neutrophil elastase by 5-fold and thus the ability of the protein to protect against proteolytic damage. Z α_1-antitrypsin enters the

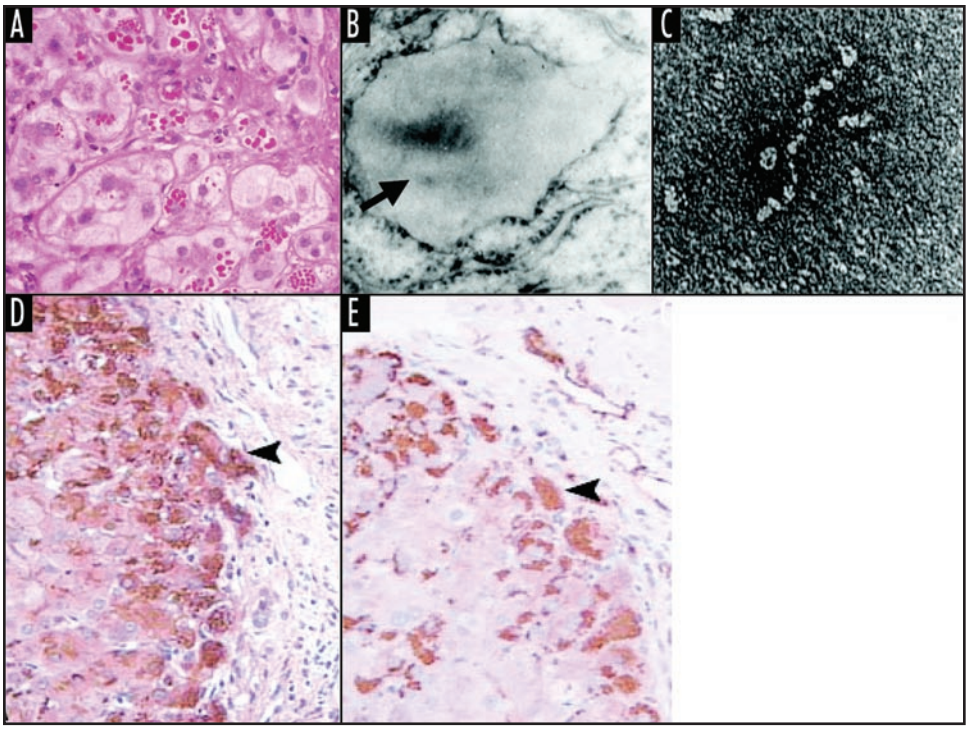

Figure 2. Z α_1-antitrypsin is retained within hepatocytes as intracellular inclusions. These inclusions are PAS-positive and diastase resistant (A) and are associated with neonatal hepatitis and hepatocellular carcinoma. (B) Electron microscopy of a hepatocyte from the liver of a patient with Z α_1-antitrypsin deficiency shows the accumulation of α_1-antitrypsin within the rough ER (arrow). These inclusions are composed of chains of α_1-antitrypsin polymers shown here from the plasma of a Siiyama α_1-antitrypsin homozygote (C). More recently, polymers have been identified within PAS-positive inclusions with a monoclonal anti-polymer α_1-antitrypsin antibody. (D and E) Immunochemistry of the liver from an individual with Z α_1-antitrypsin deficiency, showing staining with an anti-α_1-antitrypsin polyclonal antibody (D, arrow) and a monoclonal anti-polymer α_1-antitrypsin antibody (E, arrow). It is these intracellular inclusions of polymers that are associated with neonatal hepatitis and hepatocellular carcinoma. Figure reproduced with permission from Lomas et al.[97]

lung by passive diffusion. It is also secreted by macrophages and bronchial epithelial cells. In both cases it contains the Z mutation and hence the propensity to form polymers. Indeed polymers of α_1-antitrypsin have been detected in bronchial lavage and tissue sections from Z α_1-antitrypsin homozygotes.[71,72] These polymers are inactive as protease inhibitors and so further deplete the antiprotease screen within the lung. Pulmonary polymers of Z α_1-antitrypsin are chemotactic for neutrophils in vitro and following instillation into the lungs of mice.[72-74] Thus our understanding of the pathways of polymerization has provided new insights into the associated emphysema. However the importance of these polymers in driving the associated inflammation and emphysema remains to be clarified.[75,76]

POLYMERIZATION OF ANTITHROMBIN, C1 INHIBITOR, α_1-ANTICHYMOTRYPSIN AND HEPARIN CO-FACTOR II CAUSES THE RETENTION OF PROTEIN WITHIN HEPATOCYTES AND PLASMA DEFICIENCY

The phenomenon of loop-sheet polymerization 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 and other domains of the plasma proteins C1-inhibitor (Phe52Ser, Pro54Leu, Ala349Thr, Val366Met; Phe370Ser, Pro391Ser), antithrombin (Pro54Thr, Asn158Asp, Phe229Leu) and α_1-antichymotrypsin (Leu55Pro, Pro229Ala). These mutations destabilise the serpin architecture to allow the formation of inactive reactive loop-β-sheet polymers that are also retained within hepatocytes. The associated plasma deficiency results in uncontrolled activation of proteolytic cascades and angio-oedema, thrombosis and chronic obstructive pulmonary disease respectively (reviewed in refs. 6 and 33–35). More recently a mutation in heparin co-factor II (Glu428Lys) has been associated with plasma deficiency but as yet this has not been shown to cause disease.[77] The mutation is of particular interest as it is the same as the Z allele that causes polymerization and deficiency of α_1-antitrypsin. We have shown that this same mutation also causes temperature dependent polymerization and inactivation of mutants of the *Drosophila* serpin Necrotic.[78]

NEUROSERPIN POLYMERS AND THE DEMENTIA FAMILIAL ENCEPHALOPATHY WITH NEUROSERPIN INCLUSION BODIES (FENIB)

Perhaps the most striking disease associated with serpin polymerization is the dementia 'familial encephalopathy with neuroserpin inclusion bodies' or FENIB. This is characterised by the accumulation of mutant neuroserpin as PAS positive diastase-resistant inclusions or Collin's bodies within the deep layers of the cerebral cortex (Fig. 3).[79-81] These inclusions, like those associated with Z α_1-antitrypsin within hepatocytes, are formed of tangles of ordered polymers within the ER. Kindreds with FENIB present with presenile dementia and cognitive deficits unlike those of Alzheimer's or Huntington Diseases.[82]

The first mutation of neuroserpin that causes FENIB was identified in a large Irish-American family and was termed Syracuse to recognise the origin of the pedigree. The Syracuse mutation (Ser49Pro) is in the shutter domain at an identical location to a previously described mutation in α_1-antitrypsin that forms polymers in association with liver disease (the Siiyama mutation).[80,83] An examination of the brain from affected individuals showed that the inclusions were composed solely of mutant neuroserpin that had formed chains of loop-β-sheet polymers.[80,84] Three other mutations of neuroserpin have since been described, all of which are within the shutter domain: Portland (Ser52Arg), His338Arg and Gly392Glu.[81] There is a direct association between the severity of the mutation (as predicted by molecular modelling) and the number of inclusions and an inverse correlation with the age of onset of dementia. For example, the original family members with the Syracuse mutation showed small diffuse intraneuronal inclusions of neuroserpin and a late onset of dementia between 45 and 60 years old.[79,80,82] A family with the more severe Portland mutation showed larger inclusions and an onset of dementia in their mid-twenties. The family with the His338Arg mutation showed even more inclusions and an onset of

dementia in their mid teens whilst those with the most severe mutation, Gly392Glu, have very large inclusions and an onset of dementia leading to death before the age of 20 years.[81]

The direct correlation between the 'polymerogenicity' of the mutations and the onset of dementia strongly indicates that the intracellular accumulation of neuroserpin is by itself sufficient to cause neurodegeneration. The effect of polymerization on disease was corroborated by in vitro experiments showing a fast rate of polymerization for recombinant Ser49Pro neuroserpin, and an even faster rate for recombinant Ser52Arg neuroserpin which is associated with a more severe clinical phenotype.[85-87] A cell model using transient transfection in COS-7 cells also showed the accumulation of mutant neuroserpin within the endoplasmic reticulum. The intracellular aggregates were composed of polymers similar to the loop-sheet polymers isolated from the brains of individuals affected by FENIB.[88] In keeping with the genotype-phenotype correlation observed in patients, the more severe Portland mutant accumulated more rapidly and its rate of secretion was lower than for the less severe Syracuse mutant.[88]

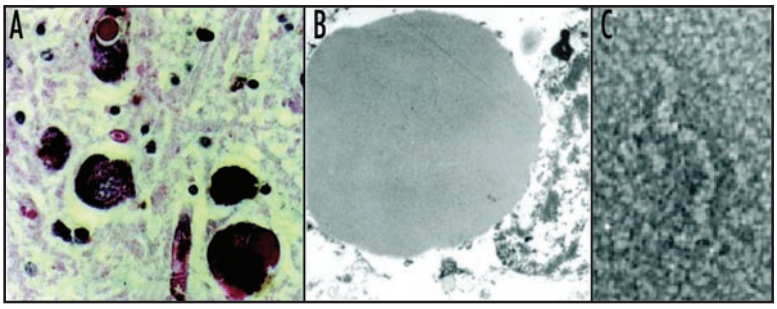

Figure 3. Mutant neuroserpin is retained within neurons as intracellular inclusions. These inclusions stain positive with PAS (A) and can be seen within the ER on electron microscopy (B). Electron microscopy of the isolated inclusions confirms that the mutant neuroserpin forms bead-like polymers identical to those of Z α_1-antitrypsin (C). Figure reproduced with permission from Lomas et al.[97]

STRATEGIES TO PREVENT POLYMERIZATION AND AMELIORATE THE ASSOCIATED DISEASE

Understanding the pathway of polymerization has allowed the development of novel therapeutic strategies to block polymer formation and so ameliorate the associated disease. One strategy is to use peptides to block the aberrant linkage between the RCL of one molecule and β-sheet A of another. Polymerization of Z α_1-antitrypsin can indeed be blocked in vitro by annealing 11-13 amino acid RCL peptides to β-sheet A.[45] The poor specificity of these peptides led to the design of smaller peptides and indeed 4-6-mer peptides can efficiently and specifically block the polymerization of Z α_1-antitrypsin in vitro.[89,90]

Small sugar and alcohol molecules can reduce the rate of polymerization of both α_1-antitrypsin and neuroserpin in vitro, most probably by stabilising β-sheet A.[91] Chemical chaperones stabilise intermediates on the folding pathway[92,93] but 4-phenylbutyric acid, which increased the secretion of Z α_1-antitrypsin in a mouse model of disease,[92] has proved to be ineffective in clinical trials in patients with α_1-antitrypsin deficiency.[94]

Peptides and chaperones are poor therapeutic agents in vivo and so another approach is to use small molecules to block polymerization. A hydrophobic pocket has been identified on the lateral surface of α_1-antitrypsin that is bounded by β-strand 2A and helices D and E but which is distinct from the polymerization interface. The introduction of bulky residues into this pocket retards the polymerization of M α_1-antitrypsin and increases the secretion of Z α_1-antitrypsin from a *Xenopus* oocyte expression system.[14,95,96] Consequently this pocket offers a novel target for rational drug design. The identification of chemical compounds that bind to this cavity is currently underway.

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