

## $\alpha_1$ -Antitrypsin: Molecular Pathology, Leukocytes, and Tissue Damage

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### Introduction: $\alpha_1$ -antitrypsin and the serpins

The maintenance of health requires a balance between the defensive and offensive systems of the body. One such balance that is now just becoming understood at the molecular level is the one between the enzymes that break down protein and the antiproteases that act as their inhibitors. Of particular importance is the group of closely related enzymes, the serine proteases, which are responsible for triggering the inflammatory cascades of the plasma such as coagulation, kinin release, fibrinolysis, and complement activation. The triggering by these enzymes of the plasma cascades is controlled by an appropriately specialized group of plasma protease inhibitors, e.g., antithrombin, antiplasmin, and the first component of complement (C1)<sup>1</sup> inhibitor. These serine protease inhibitors are now known to belong to the same protein family—the serpins—and consequently to share a common molecular structure and mechanism, Table I (1–6).

The archetype of the serpin family is  $\alpha_1$ -antitrypsin, the inhibitor present at highest concentration in human plasma. Although named  $\alpha_1$ -antitrypsin, its physiologic target is leukocyte elastase rather than trypsin (7), and for this reason, it is alternatively called  $\alpha_1$ -proteinase inhibitor; in this review, the historical name will be retained and sometimes abbreviated to antitrypsin.

Interest has focused on  $\alpha_1$ -antitrypsin because of the frequent occurrence of its genetic deficiency in Europeans and the association of its homozygous deficiency with the development of premature emphysema (8) and progressive liver disease (9). Investigation of the molecular basis of this association (10) has stimulated research into the general pathogenesis of emphysema. More recently, the structural studies of  $\alpha_1$ -antitrypsin have provided an insight into the function of the other plasma serpins and their vulnerability in the shock syndromes (11).

### Structure and function of $\alpha_1$ -antitrypsin

There is evidence to suggest that two genes code for  $\alpha_1$ -antitrypsin (12), but the plasma findings are compatible with the expression of just the two alleles at the one locus; i.e., a homozygote for normal (M) antitrypsin has a genotype MM, a heterozygote for the severe Z-deficiency variant has a genotype MZ, and a homozygote ZZ. Although monocytes (13) and perhaps other cells can produce  $\alpha_1$ -antitrypsin, production for the

plasma is virtually totally dependent on the hepatocyte. Hence, a liver transplant from an MM donor to a ZZ recipient results in a complete change to the normal M plasma antitrypsin.

Antitrypsin is a small glycoprotein that readily diffuses throughout the interstitial fluids. It functions as an inhibitor of the serine proteases in general, but the critical targets are the proteases released by stimulated neutrophil leukocytes: cathepsin G and, more particularly, neutrophil leukocyte elastase (7). Elimination of these proteases occurs through the formation of a tight 1:1 complex with  $\alpha_1$ -antitrypsin, the complex being later removed and catabolized within the circulatory system. Antitrypsin is therefore a suicidal protein, with a normal circulatory life of 6 days, and with the ability to respond to stress by increased synthesis to give a fourfold increase in plasma concentration in the acute-phase state (14).

The mechanism of inhibition is best explained in terms of the tertiary (crystallographic) structure of  $\alpha_1$ -antitrypsin (15). This shows it to be a globular, highly ordered molecule, with 30% of its structure in helical form and 40% as  $\beta$ -sheets. There are two regions in the molecule of unordered exposed sequence that are vulnerable to enzymic attack (3): an amino-terminal sequence of 20 residues, and a loop, containing the reactive center situated near the carboxy terminus of the molecule (Fig. 1). Cleavage of the amino-terminal sequence is known to occur and there is a suspicion, but no evidence as yet, that the released pentapeptide has a physiologic role; certainly this is so with some other members of the serpin family, notably angiotensinogen.

The reactive center of  $\alpha_1$ -antitrypsin is formed by the amino acid methionine at position 358, situated on an exposed loop of the molecule where it forms a bait for the target enzymes. This 16 residue, exposed, loop of sequence is under considerable tension, as it holds the molecule in a stressed metastable form. This stressed conformation is thought to strain the reactive center 358–359 Met–Ser bond such that it precisely fits the active site pocket of the serine proteases and, in particular, of leukocyte elastase. In this way the serpins are thought to act as ideal substrates for their target proteases, in each case the enzyme and inhibitor forming such a close fit that they remain locked together until the complex is removed from the circulation.

The precise specificity of inhibition of each of the serpins is substantially, though not completely, dependent on the single amino acid at its reactive center (Fig. 1 B). Thus  $\alpha_1$ -antitrypsin has a methionine that neatly fits into the active center of elastase, whereas antithrombin has an arginine that neatly fits the active center of thrombin. This concept is important as it explains the ability to change the specificity of  $\alpha_1$ -antitrypsin by natural or engineered mutations; e.g., replacement of the 358 methionine by arginine changes antitrypsin from an inhibitor of elastase to an inhibitor of thrombin (16).

The disadvantage of exposure of the reactive center on a stressed loop is that it renders the molecule vulnerable to inac-

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1. Abbreviations used in this paper: C1, first component of complement; FEV<sub>1</sub>, forced expiratory volume.

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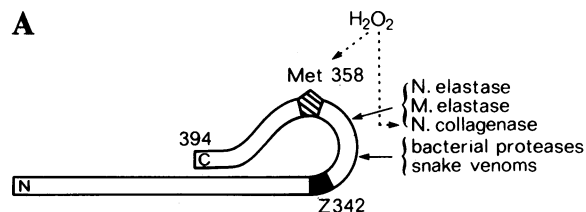
Table I. Serpins: A Family of Plasma Proteinase Inhibitors

Inhibitor	Concentration $\mu\text{M}$	Mass daltons	Target
$\alpha_1$ -Antitrypsin	25	51,000	N elastase
$\alpha_1$ -Antichymotrypsin	7	69,000	Cathepsin G
Antithrombin III	2	61,000	Thrombin
C1 inhibitor	2	104,000	C1s, kallikrein
$\alpha_2$ -Antiplasmin	1	70,000	Plasmin
Heparin cofactor II	1	66,000	Thrombin
PC inhibitor	$10^{-2}$	57,000	Activated protein C
PA inhibitor	$10^{-4}$	50,000	Plasminogen activator
Angiotensinogen	$10^{-2}$	50,000	Nonfunctional

tivation by cleavage of the loop, the ends of which spring irreversibly apart to give a much more stable and relaxed molecular structure. This susceptibility to cleavage is utilized pathologically by proteases in snake venoms and bacterial secretions (e.g., *Pseudomonas aeruginosa*), as a mechanism to overcome inhibitor defenses (17, 18). Inactivation by cleavage of the exposed loop also occurs physiologically, presumably to create an inhibitor-free zone around a center of inflammatory activity (9, 19).

#### Leukocytes, elastase, oxidation, and tissue damage

The association of  $\alpha_1$ -antitrypsin deficiency with the development of premature emphysema led to the wider proposal that



#### B

Serpin	Reactive site	Inhibits	Oxidation
$\alpha_1$ -Antitrypsin	Pro Met Ser Ile	Elastase	+
Antithrombin III	Gly Arg Ser Leu	Thrombin	-
C1 inhibitor	Ala Arg Thr Leu	C1s, kallikrein	-
Pittsburgh mutant	Pro Arg Ser Ile	Thrombin, kallikrein	-
Valine mutant	Pro Val Ser Ile	Elastase	-

Figure 1. (A) Schematic structure of serpins represented here as  $\alpha_1$ -antitrypsin. A major feature is an exposed loop containing the reactive center and hinged near the Z-mutation position 342. The exposed loop is susceptible to proteolytic cleavage and this provides a physiologic and pathologic switch enabling the irreversible inactivation of the serpins.  $\alpha_1$ -Antitrypsin has a second, reversible switch inasmuch as its reactive-site methionine (Met) can be readily oxidized to give a loss of elastase inhibitory activity. The stimulated neutrophil utilizes both switches to nullify inhibitory activity within its immediate environment. Abbreviations: N, neutrophil; M, macrophage. (B) Inhibitory specificity of serpins is primarily dependent on a single reactive center amino acid. The natural Pittsburgh mutation of the methionine of  $\alpha_1$ -antitrypsin to arginine (Arg) converts it to an inhibitor of thrombin and the contact proteases. The engineered mutation to a valine gives an elastase inhibitor that is not inactivated by oxidants.

protease-antiprotease imbalance is a general contributor to the diverse conditions that result in chronic pulmonary obstructive disease (20). The realization that  $\alpha_1$ -antitrypsin is an inhibitor of neutrophil elastase strengthened the case, because elastase is an endogenous enzyme capable of degrading elastin and collagen. Damage to lung tissue can consequently result from, on the one hand, prolonged exposure to leukocyte proteases, as in chronic inflammation and, on the other, from an insufficiency of appropriate inhibitors, as is the case in antitrypsin deficiency.

However, this axis of neutrophil elastase and  $\alpha_1$ -antitrypsin brought with it a problem: what is the point of the leukocyte secreting an elastase if the surrounding tissue normally contains an excess of an effective inhibitor? A clue came from workers investigating the most common cause of emphysema, that of habitual cigarette smoking. They proposed (21) that a contributing factor could be the inactivation of  $\alpha_1$ -antitrypsin owing to oxidation by radicals contained in tobacco smoke. Their *in vitro* experimental support for this was backed by the concurrent identification of methionine as the reactive center amino acid of antitrypsin (20). Methionine contains a sulfur atom and is readily oxidized to methionine sulfoxide—a derivative which is too large to readily fit the reactive center of elastase. Hence oxidation of  $\alpha_1$ -antitrypsin effectively inactivates it as an inhibitor of elastase.

The demonstration that antitrypsin could also be oxidatively inactivated by stimulated neutrophils (24, 25) was of great interest, in that this provided the explanation (10, 25) for the secretion of elastase by the neutrophil even in the presence of normal concentrations of  $\alpha_1$ -antitrypsin. Stimulated neutrophils release oxygen radicals which create a zone of oxidation around them, in effect forming a microenvironment within which  $\alpha_1$ -antitrypsin is inactivated. This facilitates the localized breakdown of connective tissue necessary for the liquefaction and discrete isolation of an inflammatory locus.

The oxidative proposal for the inactivation of  $\alpha_1$ -antitrypsin and, in particular, the contribution of oxidation to the development of emphysema is now a controversial topic (26). One problem is that attention has focused on the direct oxidation of antitrypsin by tobacco smoke, with the expectation that oxidation will be of a magnitude sufficient to be readily detected in bronchial washings or even in the plasma. This, in retrospect, is an unrealistic expectation and it is not surprising that discrepant results have been obtained. Our conclusion, based on experiments that mimic *in vivo* conditions (25, 27), is that oxidative inactivation of  $\alpha_1$ -antitrypsin does occur and does contribute to chronic lung damage. However, it is largely a localized event, confined to the immediate proximity of the neutrophil leukocyte, with significant damage resulting from the summation of focal damage over a period of years. The progress of this destruction will obviously be accelerated by the presence of a genetic deficiency of  $\alpha_1$ -antitrypsin.

These conclusions concerning the role of the leukocyte do not exclude the possibility that a major contribution to the development of emphysema may be made directly by the as yet little-understood alveolar macrophage, or by the direct oxidative effect of tobacco smoke.

#### Deficiency and the S and Z variants

Two variants of  $\alpha_1$ -antitrypsin commonly occur in people of European descent (10). Both differ from the normal in only a single amino acid and both result in a plasma deficiency: the S variant giving a production 60% that of the normal M allele,

the Z variant giving a much more severe plasma deficiency of 15% that of the normal. Recent gene-mapping studies (28) have shown that the Z mutation probably arose in a single individual in Northern Europe some 6,000 years ago. Since then the variant has spread through Europe with a frequency gradient extending from north to south (29): 5% of Scandinavians are MZ heterozygotes; 4% of Britons, 1–2% of Southern Europeans, and in the United States, with a heterogeneous white population, 3% are MZ heterozygotes. Curiously, there is a reciprocal distribution of the S variant from 10% in Southern Europe to 5% in the North. As a general rule then, 1 in 10 people of European origin will be a heterozygote for either the S or Z variants, i.e., MZ or MS.

Protection of the lungs seems to be satisfactorily maintained by plasma antitrypsin concentrations down to 40% that of normal so neither the MZ heterozygote (plasma concentration 58% of normal) nor the MS (concentration 80%) has significantly increased risk of lung disease. The association with emphysema is primarily confined to the 1:1,000 Europeans who inherit two deficiency genes, i.e., ZZ (concentration 15% of normal concentration) and SZ (concentration 38%)—but not SS (concentration 60%).

The distribution of the S and Z variants in Europeans must represent a balanced polymorphism in which the fatal disadvantages to the homozygote have been balanced in the past by a survival advantage to the heterozygote that ensured the spread of the genes. Whatever this advantage was, it was likely to be related to a disease that particularly threatened Europeans. One suggestion is that heterozygous deficiency may have provided a survival advantage against tuberculosis (36).

#### *Molecular pathology of the S and Z variants*

The mutations in the S and Z variants, S 264 Glu → Val and Z 342 Glu → Lys, result in the loss of conserved glutamic acids which in each case form important ionic bonds that stabilize the molecule (15). The consequent loss of stability is most marked with the S variant and probably contributes to its low level because of proteolysis of the nascent protein (10). The point mutation causing the S variant also introduces a false splice site in the gene and may lead to some loss of messenger RNA (mRNA) owing to incorrect splicing (31).

The pathogenesis of the Z deficiency is more subtle. There appears to be normal initial production of the Z polypeptide, 15% of which is secreted into the plasma as the fully glycosylated and active inhibitor. However, 85% of the newly synthesized polypeptide appears to be blocked in the endoplasmic production pathway of the hepatocyte, at a stage just prior to final processing of its carbohydrate sidechains, and to its entry into the Golgi secretion system.

Studies using mRNA isolated from normal (MM) and deficient (ZZ) livers showed both messages were equivalent in stability and in their translation and subsequent glycosylation in cell-free systems (32–34). Injection of the normal M and variant Z RNA into a surrogate cell, the toad oocyte, confirmed that there was equivalent initial synthesis of the two polypeptides but showed that, whereas all the M protein was secreted, only a fraction of the Z antitrypsin was secreted, most of the Z product being blocked within the oocyte at the final (high mannose) stage of processing (35–38). This accumulation of the abnormal antitrypsin was accompanied by a burst of lysosomal activity in the oocyte, indicating that the blockage of the Z antitrypsin was accompanied by increased proteolytic degradation (36).

The conclusion from these and other studies (39) is that the defect lies in the transport of the Z antitrypsin at the final stages of its passage through the endoplasmic pathway. The reason for this failure in transport is not known; the amino acid replacement in the Z polypeptide may affect recognition required for the final transport step, or more likely, the mutation may cause a minor perturbation of structure that affects the solubility of the incompletely processed material.

The failure in transport results in the aggregation of Z antitrypsin to give microscopically visible inclusions, most apparent where there is active synthesis, in the periportal hepatocytes (40). Most of the blocked Z polypeptide will be removed by intracellular proteolysis, as indicated by the lysosomal activity observed in the oocyte, and by the consistent microscopic evidence of increased lysosomal activity seen in the hepatocytes of both ZZ and MZ individuals (41).

#### *Liver disease*

The mechanism responsible for the Z deficiency is of importance in understanding the cause of the liver disease that is associated with severe  $\alpha_1$ -antitrypsin deficiency. About 10% of ZZ infants develop a neonatal cholestasis; most recover but some (2–3% overall) progress to juvenile cirrhosis (42, 43). As well as the childhood damage, there is also slowly progressive liver damage in the adult. Almost all adult homozygotes show histologic evidence of liver damage and about 20% eventually develop cirrhosis (44). There is also good evidence of a slight, but significant, predisposition of the MZ heterozygote to the development of chronic liver disease (45–47).

There is still debate as to the cause of this associated liver disease. One view (48) is that it is a consequence of the plasma deficiency of inhibitor that renders the hepatocyte more susceptible to proteolytic damage. The second view (10), supported here, is that the liver damage is a direct consequence of the intracellular accumulation of the Z variant. The strongest evidence for this is the recent experimental confirmation of the prediction that the Z abnormality is primarily one of processing rather than synthesis. This prediction is central to the “accumulation” proposal; i.e., that there is normal synthesis of the Z antitrypsin but a massive blockage in its final transport prior to secretion from the hepatocyte. When the blockage of unprocessed material occurs within a cell, there is a massive switch in pathways from metabolism to proteolysis, in a process akin to that of heat shock (49), with a consequent risk to cell vitality. The build-up of Z antitrypsin within the cell represents the overloading of the proteolytic system and, microscopically, necrosis and cell death are observed in the periportal hepatocytes with the greatest accumulation of material (41).

Supporting evidence for the “accumulation” mechanism of damage, as opposed to that of “plasma deficiency,” is the observation of liver changes in MZ heterozygotes who have adequate plasma antitrypsin concentrations (47), and the absence of liver involvement in the rare null homozygotes who have zero plasma concentration (50). The question of which mechanism is responsible is of vital clinical relevance, because if the liver disease does arise from a deficiency of inhibitor, it could be treated by plasma replacement therapy or by stimulants of antitrypsin synthesis such as danazol. However, if, as appears to be the case, the liver damage is a consequence of the intracellular accumulation of antitrypsin, the use of replacement therapy is at best unnecessary, and the use of stimulants such as danazol is potentially dangerous.

The conclusion then (10, 47) is that the liver damage is primarily associated with the Z variant, with significant damage being largely, but not wholly, confined to the ZZ homozygote. Other genetic and hormonal factors also play a part in determining the severity of the damage inasmuch as progression of the neonatal jaundice to a fulminant cirrhosis is much more likely to occur in males, and similarly if there is a history of other affected siblings (42, 51).

#### *Lung disease and treatment*

A loss of lung elasticity is a normal accompaniment of aging but this loss can be accelerated to give the development of premature emphysema in either the smoker or the individual with homozygous  $\alpha_1$ -antitrypsin deficiency. This is illustrated by changes in forced expiratory volume (FEV<sub>1</sub>) observed in a study of 69 ZZ homozygotes (52). The ZZ nonsmoker had an annual FEV<sub>1</sub> decrease of 80 ml as compared with that of 36 ml in the nonsmoking control. The first signs of dyspnea did not appear till after the age of 50 years and severe emphysema did not usually occur until after the age of 60–65. A number of ZZ individuals had a normal life span (up to 87 years) without major respiratory problems. However, the ZZ smoker had a catastrophic progression (44), with an average FEV<sub>1</sub> loss of 300 ml/year and with the onset of emphysema after 15 cigarette pack-years, often by the age of 30 years, with death being likely by the age of 50.

It follows that the first approach to treatment is the detection of the deficiency and the subsequent avoidance of smoking. The outlook for the young ZZ nonsmoker is not necessarily gloomy. There is reason to be confident that a good life span, with respiratory health, is possible if pollutants are avoided and if conservative measures are taken to minimize respiratory inflammation.

Plasma replacement therapy with  $\alpha_1$ -antitrypsin concentrates is now becoming practicable and initial trials are underway (53). However, it will be a daunting procedure on a long-term basis, although it is probably justified in the severely deficient individual with signs or symptoms of the onset of respiratory decline. It will be more difficult to justify in the young ZZ homozygote as a life-long procedure, but intermittent replacement therapy should be useful to provide cover for times of elastase stress, particularly during respiratory infections.

#### *Prospects: genetic engineering and the shock syndromes*

The realization that emphysema results from the unhindered activity of elastase has led to a search for inhibitors suitable for therapeutic use. Among those being examined is Eglin c, an elastase inhibitor extracted, and now cloned, from the medicinal leech (54). This provides a highly effective, oxidation-resistant inhibitor of neutrophil proteases. Like a number of other peptide and synthetic inhibitors that are also being assessed for therapeutic use, it has a relatively low molecular weight and hence a short half-life in the plasma. Administration of these low molecular weight compounds will probably have to be by aerosol if any attempt is to be made to offer them as long-term replacements for a deficiency of plasma  $\alpha_1$ -antitrypsin (53).

However, an interesting development is that the most promising use of the elastase inhibitors now appears to be not so much in the prevention of emphysema but in the potential treatment of the acute shock syndromes (55). The shock syndromes can be regarded as a fulminant form of neutrophil-induced tissue damage. They occur when there is widespread neutrophil activation as in septicaemia, endotoxaemia (56, 57), and

when there is surface sequestration of neutrophils as in cardiopulmonary bypass procedures (58). These conditions can give rise to severe shock with gross derangements of the plasma proteolytic cascades: with disseminated intravascular coagulation, kinin release, and complement activation, an endpoint being the development of shock lung (59, 60).

Some of the changes observed in the shock syndromes can now be explained (11) on the basis of our knowledge of the molecular structure of the plasma serpins (Fig. 1). Widespread neutrophil activation results in the massive release of elastase, which in turn can catalytically inactivate the plasma serpins (61, 62) by cleavage of their exposed loops (4, 11). Thus the inbuilt switch provided by the exposed loop, which is of advantage in localized inflammation, becomes grossly disadvantageous when there is a generalized release of elastase. This ability of elastase to catalytically switch off the serpins explains the inactivation of antithrombin, C1 inhibitor, and  $\alpha_2$ -antiplasmin that occurs in the shock syndromes and contributes to the associated derangement of the plasma proteolytic cascades (57, 59).

A rewarding prospect is that the research on  $\alpha_1$ -antitrypsin that has revealed this molecular mechanism in the shock syndromes has also opened up prospects for treatment based on the use of genetically engineered variants of antitrypsin. It was shown earlier (16) that mutation of the reactive center methionine of  $\alpha_1$ -antitrypsin to arginine effectively converted the molecule from an inhibitor of elastase to an inhibitor of thrombin (Fig. 1 B). Based on this principle, it has been possible to design (10, 63) modifications of human  $\alpha_1$ -antitrypsin to target it specifically for therapeutic use. The genetically engineered replacement of the active site methionine, by valine or leucine (64–66), gives a highly effective inhibitor of elastase that is resistant to oxidation and hence to neutrophil inactivation. Mutants such as these should be useful prophylactically to prevent the development of shock, by complexing elastase under conditions in which normal  $\alpha_1$ -antitrypsin is oxidatively inactivated.

An exciting finding (67) is that the arginine mutant of  $\alpha_1$ -antitrypsin, as well as being an inhibitor of thrombin, is also an effective inhibitor of the proteases of the contact system; it is the most efficient known natural inhibitor of kallikrein. Thus, the arginine mutant should potentially be useful in the treatment of established shock, both to replace antithrombin and to prevent the activation of the contact proteases.

The ability to target specifically the engineered variants of  $\alpha_1$ -antitrypsin opens a whole range of therapeutic possibilities. As indicated in Fig. 1 B, it should be possible to mimic selectively the specificity of individual members of the serpin family. Although the serpins have been described here as plasma proteins, some of them, such as the inhibitor of plasminogen activator (5, 68) are primarily membrane bound. If the specificity of these membrane-bound inhibitors can be reproduced, then it may be possible to influence cell proliferation, as has already been demonstrated *in vitro* using the arginine mutant of  $\alpha_1$ -antitrypsin (68).

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