10
Reactive-Centre Variants of α_1-Antitrypsin. A New Range of Anti-inflammatory Agents

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Introduction: engineered evolution

The human plasma protease inhibitor α_1-antitrypsin is the first protein to have been redesigned to acquire a novel activity and to have been produced by genetic engineering (Rosenberg *et al.*, 1984; Courtney *et al.*, 1985). To understand the therapeutic potential of this innovation, it is necessary to look at the general role of the protease inhibitors in the plasma, and the particular role of α_1-antitrypsin in the protection of the elastic tissue of the lungs. The ability to engineer functional changes has also focused attention on the serpins, a group of closely related inhibitors of the serine proteinases (EC 3.4.21). The tens of thousands of different proteins encoded within the human are now known to have originated from a much smaller number of ancestral, primordial proteins. Over a period of less than a billion years, these primordial proteins are believed to have evolved and recombined to give the multiplicity of structures and functions needed in higher organisms. The genetic engineering of α_1-antitrypsin illustrates how the evolutionary process may be reversed or mimicked to convert a protein with one function into that with another (Carrell, 1984).

The primordial member of the serpin family, the group of serine protease inhibitors to which α_1-antitrypsin belongs, is thought to have undergone many modifications during evolution to provide the wide range of specific inhibitors that control the important proteolytic cascades of the blood: coagulation, the clotting of blood; fibrinolysis, the dissolving of clots; complement activation, the triggering of the immune reaction; and kinin release, the production of the small peptides that cause shock. By making a single change to the reactive centre of α_1-antitrypsin, it has been possible to mimic the function of a number of these inhibitors and so produce substances of potential therapeutic value. Furthermore, recombinant inhibitors can be designed to vary the half-lives in the circulation. It should also be possible

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in the future to alter the structures of inhibitors in such a way as to change their distribution between the general circulation and the intercellular and intracellular fluid compartments.

α_1-Antitrypsin and the serpins

The protease inhibitors form 10% of the proteins of human plasma, and together constitute its major component after albumin and the antibodies of the gamma globulin (Travis and Salvesen, 1983). As their name suggests, protease inhibitors inactivate and remove enzymes capable of cleaving tissue proteins. They act as suicidal proteins by forming 1:1 complexes with their target proteases, the resulting complex of enzyme and inhibitor being removed from the circulation for subsequent breakdown.

The large protein, α_2-macroglobulin, which is mostly confined to the vascular compartment, is an inhibitor of a wide range of proteases, and provides a first line of defence against proteolytic enzymes. Specialized inhibitory activity in plasma is, however, provided mainly by the serpins (*Table 1*), the family of serine protease inhibitors of which α_1-antitrypsin is the archetype (Carrell and Travis, 1985; Carrell and Boswell, 1986).

The serpins are each targeted, as relatively specific inhibitors, against members of a family of proteolytic enzymes, the serine proteases, the best-known member of which is trypsin (EC 3.4.21.4). More than 500 million years ago, before the development of the vertebrates, there was presumably a primordial serine protease with a corresponding primordial serine protease inhibitor, i.e. the ancestral serpin. With the passage of time, the primordial protease underwent evolutionary change to give highly specialized derivatives such as thrombin (EC 3.4.21.5), the enzyme that initiates coagulation. This evolutionary divergence of the specialized serine proteases can be deduced from their present-day structure; the fascinating finding is that exactly parallel evolutionary changes were occurring in the serpins. The specialization of the primordial serine protease to thrombin, for instance, was matched over the same time-scale by the evolution of the primordial inhibitor to antithrombin.

Table 1. Serpins: A family of plasma proteinase inhibitors (adapted from Boswell and Carrell, 1986)

Inhibitor	Plasma concentration (μM)	Mass (Daltons)	Reactive centre P_2–P_1–P_1'†	Target
α_1-Antitrypsin	25	51 000	Pro–Met–Ser	N elastase
α_1-Antichymotrypsin	7	69 000	Leu–Met–Ser	Cathepsin G
Antithrombin-III	2	61 000	Gly–Arg–Ser	Thrombin
C_1-Inhibitor	2	104 000	Ala–Arg–Thr	C_1s, Kallikrein
α_2-Antiplasmin	1	70 000	Ser–Arg–Met	Plasmin
Heparin cofactor II	1	66 000	Pro–Leu–Ser	Thrombin
*Angiotensinogen**	*10^{-2}*	*50 000*	?	*Non-functional*

†Conventional numbering based on site of putative cleavage P_1–P_1'

*Angiotensinogen, although a member of the family, is not an inhibitor

In a similar way, the specialization of the serine proteases, plasmin (EC 3.4.21.7) and C_1-esterase (EC 3.4.21.42), has been matched by a parallel specialization of the inhibitors, antiplasmin and C_1-inhibitor. This process has involved more than just inhibitory specificity: for example, in antithrombin the development of a mechanism for its activation by heparin permits a more precise control of blood clotting. Similarly, in antiplasmin a terminal sequence provides an attachment for fibrin and hence allows more effective localized inhibition of plasmin (Holmes *et al.*, 1986).

Structure and function of the serpins

The structure of the serpins is typified by that of α_1-antitrypsin (Carrell *et al.*, 1982). α_1-Antitrypsin is a relatively small glycoprotein which diffuses readily throughout the interstitial fluids where it combines, molecule for molecule, with a particular protease. Although named α_1-antitrypsin, it specifically inhibits elastase (EC 3.4.21.37) which is released by inflammatory white cells, the neutrophil leucocytes. For this reason, α_1-antitrypsin is alternatively called α_1-proteinase inhibitor, but for this review the historical name is retained, and is sometimes abbreviated to antitrypsin.

The serpins inactivate proteases because their reactive centres function as a relatively specific substrate for each enzyme. The reactive centre of the inhibitor is believed to resemble an ideal substrate for the target enzyme: the substrate enters but is not hydrolysed and so does not leave the active site of the enzyme. Protease and serpin are thus locked together as the intermediate enzyme–substrate complex, through their active site and reactive centres, respectively (Travis and Salvesen, 1983). Of course, the complex involves more than just active-site and reactive-centre interaction, but this is the basis for determining specificity, and is therefore the basis of the direct potential for simple engineered changes in function.

The substrate bait provided by the reactive centre of the serpins is primarily based on a single peptide bond—that between the P_1 and P_1' amino-acid residues (*see Table 1*). This is characteristically X–Ser, where the amino acid X determines specificity. In α_1-antitrypsin the reactive centre is Met–Ser, neutrophil elastase characteristically cleaving at methionine residues, and for antithrombin the reactive centre is Arg–Ser, thrombin characteristically cleaving at arginine residues. Other residues such as the P_2 amino acid also play a part in determining specificity, and it would be more accurate to classify the reactive centre of α_1-antitrypsin as Pro–Met–Ser and of antithrombin as Gly–Arg–Ser. Nevertheless, as the initial results from natural and engineered mutants have demonstrated, it is primarily the single P_1 residue that determines the specificity of each inhibitor (Carrell and Travis, 1985).

Crystallographic techniques have been applied to investigate the structure and mechanism of action of α_1-antitrypsin (Loebermann *et al.*, 1984). Unfortunately, the present crystallographic structure does not show the conformation of the reactive centre because, so far, it has not been possible to crystallize any of the serpins in their native form. They do, however, readily

crystallize in their inactive post-complex form, i.e. subsequent to induced separation of the protease–inhibitor complex, to yield an inactive product cleaved at its reactive centre (*Figure 1c*), and well able to crystallize. This product, on X-ray crystallography, shows a globular, highly ordered molecule, with 30% of its structure in helical form and 40% as β-sheets.

This crystallographic structure of α_1-antitrypsin, cleaved at its reactive centre, is surprising in that the methionine 358 and serine 359 that formed the reactive centre of the active α_1-antitrypsin molecule are now seen to be separated by a distance of 69 Å in the two associated peptides formed by cleavage of the α_1-antitrypsin molecule. To reconstruct the intact molecule, it would be necessary to remove a complete strand from the middle of a six-membered β-sheet, and bend it back to form an exposed 16-residue loop of sequence (*Figure 1b*). That such a loop would be greatly strained is demonstrated by the markedly differing heat stabilities of the two forms: typically, the native serpins precipitate from solution at 60°C, but their cleaved forms are stable in solution at 100°C (Carrell and Owen, 1985).

This reconstruction of the molecular conformation of intact α_1-antitrypsin, with a strained and metastable exposed loop, may explain the comparative instability of the native structure and thus the difficulty in crystallization. The strained loop holding the reactive centre in an exposed position may provide a stressed Met–Ser bond, with a conformation mimicking the transition state in serine proteolysis and hence providing an ideal model substrate.

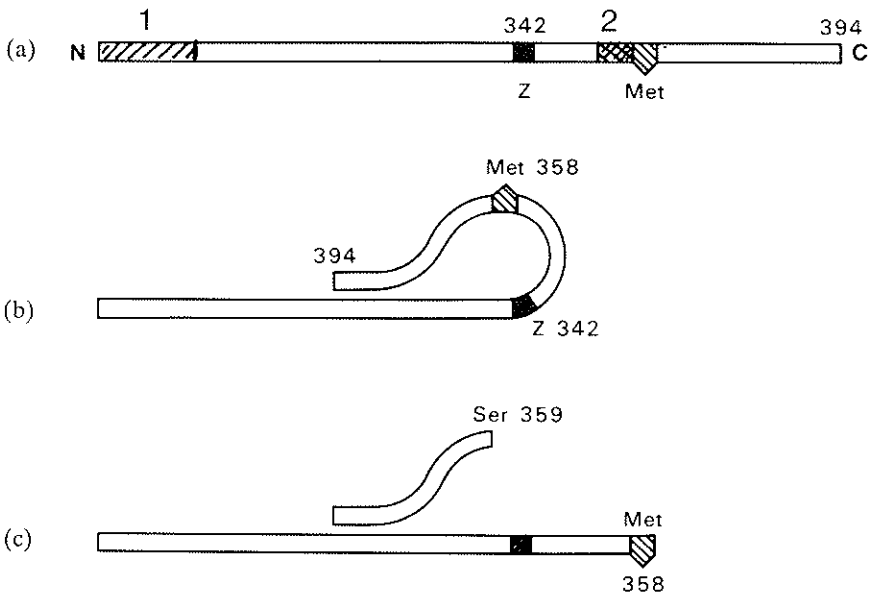

Figure 1. Schematic structure of α_1-antitrypsin. (a) Primary structure showing domains; 1, *N*-terminal tail; 2, loop cleavage segment; Met, reactive centre; Z, hinge regions and site of Z mutation. (b) Native conformation showing exposed loop between the hinge and the reactive centre. (c) Crystallographic, inactive, cleaved structure.

The exposed loop may also make the molecule vulnerable to disruption by cleavage anywhere within this area. If rupture does occur, the ends of the loop spring irreversibly apart to give a much more stable and relaxed, but inactive, molecular structure. Proteases in certain snake venoms and bacterial secretions exert their effects by inactivating serpins in this way. Inactivation by cleavage of the exposed loop also provides a physiological mechanism by which the serpins may be inactivated locally by enzymes secreted by white cells during inflammation (Kress *et al.*, 1979; Carrell and Owen, 1985).

Comparison of the peptide structures of the serpins allows the definition of four homologous functional domains (*Figure 1a*), each providing potential for engineered modifications (Loebermann *et al.*, 1984; Carrell and Boswell, 1986). The domains include the reactive centre, the exposed loop, and the hinge for the loop; the fourth domain is the amino-terminal peptide sequence, which is readily available to proteolytic attack, thus permitting cleavage and release of small peptides from the end of the molecule. The best-known example of this is the release of the peptides angiotensin I and II, which control blood pressure, from the plasma serpin angiotensinogen.

α_1-Antitrypsin deficiency

Interest in α_1-antitrypsin was stimulated by Laurell and Eriksson's observation in 1963 of a frequent genetic deficiency of the inhibitor associated with premature onset of the lung disease, emphysema. Northern European populations carry two common variants of α_1-antitrypsin, both resulting in a plasma deficiency of the inhibitor. About 1 in 25 Britons is a heterozygous carrier of the Z variant which causes severe deficiency, and 1 in 15 is a carrier of the S variant leading to mild deficiency (Editorial, 1985). Consequently, about 1:1000 will carry two genes, ZZ or SZ, that cause a plasma deficiency of α_1-antitrypsin sufficient to predispose them to chronic lung damage. Attention has focused on the Z variant, which has been shown by gene mapping studies to have originated in a single Northern European (the primordial Viking!) some 6000 years ago (Cox, Woo and Mansfield, 1985). Since then, the gene has spread throughout Europe as a balanced polymorphism, i.e. as a mutation of which the disadvantage in the homozygote must be balanced by a survival advantage in the heterozygote. What that advantage was, is speculative. It must presumably have improved the chances of survival against a disease virulent among Europeans. Tuberculosis has been proposed as the disease in question because decreased inhibition of white cell elastase in the deficient individual would encourage recovery by increased liquefaction and thus better isolation of the inflammatory foci that characterize tuberculosis.

The mutation in the Z variant of α_1-antitrypsin is the replacement of the hinge residue 342 glutamic acid by lysine (Jeppsson, 1976). Although the Z polypeptide is synthesized normally, only 15% of the product is secreted, while the rest accumulates in the liver cells. The homozygote for the Z variant gives rise to two problems: slow progressive liver disease (Sharp *et al.*, 1969) and a severe plasma deficiency of α_1-antitrypsin. This deficiency

of plasma inhibitor increases the susceptibility of lung tissue to attack by white cell enzymes. The elastic tissue of the lungs is particularly vulnerable to the elastase released by neutrophil leucocytes during inflammation. Normally, the action of elastase is controlled by plasma α_1-antitrypsin, but genetic deficiency can allow elastase activity to proceed unchecked, with consequent destruction of lung tissue. The cumulative effect over a period of years is a crippling loss of lung elasticity, a disease process known as emphysema (Cohen, 1983).

The study of α_1-antitrypsin deficiency has opened up the wider concept of a protease–antiprotease balance in the protection of the lung (*Figure 2*). This concept is the basis of a current pharmaceutical effort, particularly in genetic engineering, to develop synthetic elastase inhibitors to supplement the deficient α_1-antitrypsin, and also to tip the balance against the other causative factors indicated in *Figure 2*.

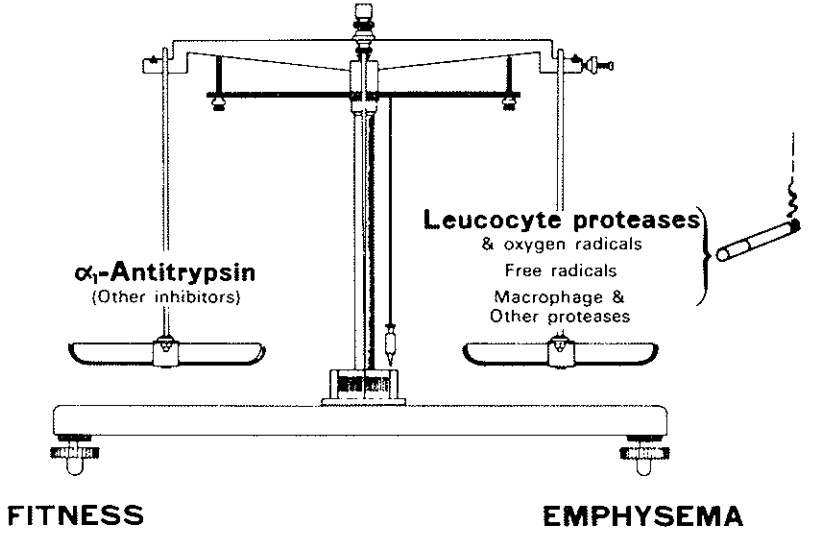

Figure 2. Protease–antiprotease balance and lung damage.

Smoking and emphysema

Genetic deficiency of α_1-antitrypsin by itself causes only 3% of the total number of cases of emphysema, the great majority resulting from habitual cigarette smoking. The study of α_1-antitrypsin deficiency has provided the evidence for this direct causal relationship between cigarette smoking and emphysema, and has provided an understanding of the mechanism involved. When the morbidity of individuals with α_1-antitrypsin deficiency was examined, a striking correlation was found between the onset of emphysema and the smoking history of the individual (Larsson, 1978; Janus, Phillips and Carrell, 1985). In fact, the life-span of a deficient (ZZ) individual who does not smoke is not greatly lessened, if at all, from the norm.

The effect of cigarette smoke is manifold (Janoff, 1983), but two measurable consequences are (1) an increase in the number of leucocytes and hence of the potential source of elastase, and (2) a decrease in the activity of α_1-antitrypsin as an elastase inhibitor. This loss of α_1-antitrypsin activity arises from direct oxidation of the reactive centre methionine by radicals in cigarette smoke and also by oxygen radicals released by leucocytes (Janoff, 1983). On oxidation of α_1-antitrypsin, methionine 358 is converted into the much larger methionine sulphoxide which is too bulky to fit the active centre readily and so to inhibit neutrophil elastase. The leucocyte is thus supplied with two mechanisms which allow it to switch off the activity of α_1-antitrypsin: the labile reactive centre methionine can be switched reversibly by the bleaching action of leucocyte oxygen radicals, and the exposed loop can be switched irreversibly by leucocyte enzyme cleavage (George *et al.*, 1984, 1986).

The first of these mechanisms, oxidation of the reactive centre, appears to be the most important. It provides an elegant means by which the leucocyte can create an inhibitor-free environment in its immediate vicinity, allowing it to break down connective tissue around a focus of inflammation (Carrell *et al.*, 1982). In the cigarette smoker, and especially in the presence of a deficiency of inhibitor, excessive neutrophil activity may overcome local defences to destroy normal tissue and, eventually, to cause emphysema (*Figure 3*).

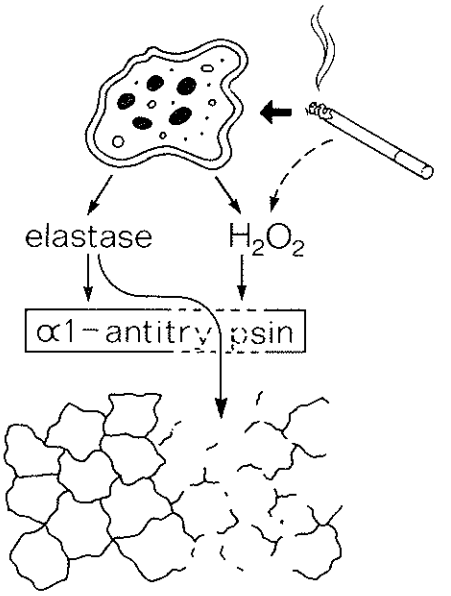

Figure 3. Neutrophil leucocytes when stimulated release oxygen radicals which can oxidize the reactive centre of α_1-antitrypsin allowing local tissue (lung) damage. The process is exacerbated by cigarette smoke.

Mutation and the Pittsburgh variant

The role of the reactive centre of α_1-antitrypsin and the possibility of the genetic engineering of the protein was suggested by a unique natural experiment. In 1978, Dr Jessica Lewis and her colleagues in Pittsburgh published their findings in the case of a 10-year-old boy who had a life-long history of bleeding episodes. They noted, in particular, a greatly increased plasma antithrombin activity, associated with an antitrypsin fraction of changed electrophoretic mobility (Lewis *et al.*, 1978). This finding was of great interest to the group in Christchurch, New Zealand, who had located the position of the reactive centres of α_1-antitrypsin and antithrombin-III on the basis of homologous alignments (Carrell *et al.*, 1980). These homologies (*Figure 4*), together with those to the reactive centres of the unrelated plant protease-inhibitors, suggested that the observed changes could have arisen through a

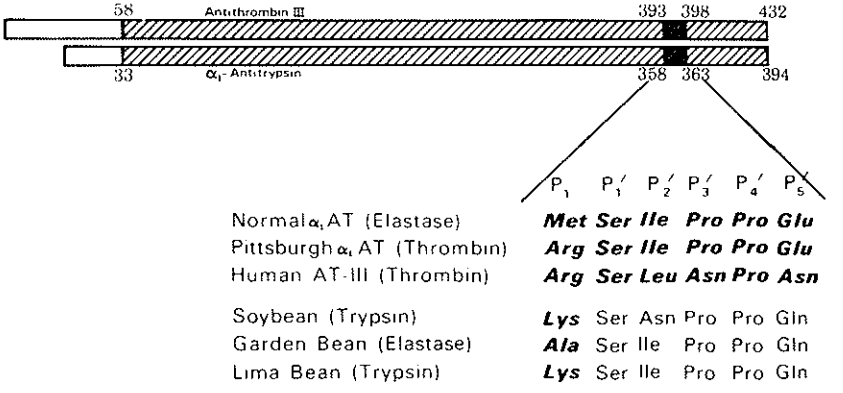

Figure 4. Amino acid sequences of α_1-antitrypsin aligned with antithrombin-III. Their homologous reactive centres also share a homology with the unrelated plant-protease inhibitors. The P_1 residue determines the specificity of inhibition as shown. The change of the P_1 methionine of α_1-antitrypsin to arginine in the Pittsburgh variant changes it from an elastase inhibitor to an antithrombin. (Reproduced with permission from the *New England Journal of Medicine*, Owen *et al.*, 1983.)

mutation of the reactive centre methionine of α_1-antitrypsin to arginine. This prediction was confirmed by isolation and sequence studies of the abnormal electrophoretic component from the Pittsburgh patient, which showed it to be a variant α_1-antitrypsin with residue 358 methionine replaced by arginine (Owen *et al.*, 1983). Functional studies showed that the mutation had, in effect, converted the antitrypsin to an antithrombin (*Table 2*). There was a complete loss of inhibitory activity to pancreatic elastase and a 10^4-fold decrease in the inhibition of neutrophil elastase. This was accompanied by a 10^5-fold increase in activity as a thrombin inhibitor, a figure which matches that of heparin-activated antithrombin. Later studies have shown α_1-antitrypsin Pittsburgh also to be a highly effective inhibitor of plasmin and the

Table 2. Protease-inhibitor association constants. Mutants compared with normal serpins

Protease	Serpin (P_2–P_1–P_1')				
	Plasma antitrypsin (Pro–MET–Ser)	Pittsburg antitrypsin (Pro–Arg–Ser)	Valine recombinant (Pro–Val–Ser)	Antithrombin (Gly–ARG–Ser)	C_1–inhibitor (Ala–ARG–Thr)
Human neutrophil elastase	7×10^7	2×10^3	2×10^7	Nil	—
Porcine pancreatic elastase	1×10^5	Nil	1×10^6	Nil	—
Porcine pancreatic trypsin	4×10^4	7×10^6	Nil	2×10^6	—
Human thrombin	5×10^1	3×10^5	Nil	*1×10^6	—
Human kallikrein	7×10^1	1×10^5	—	3×10^2	2×10^3
Human Xa	2×10^2	2×10^4	Nil	—	—
Human XIa	7×10^1	1×10^5	—	2×10^2	2×10^2
Human XIIf	Nil	8×10^2	—	5×10^1	3×10^4
Human Plasmin	2×10^2	2×10^5	Nil	1×10^4	—
Human cathepsin G	4×10^5	2×10^4	7×10^2	Nil	—

*With heparin
(Unpublished results from author's laboratory, and from Scott *et al.*, 1986.)

contact proteases, and the most effective known natural inhibitor of plasma kallikrein (EC 3.4.21.34; Scott *et al.*, 1986).

These investigations of the Pittsburgh mutation provide a full molecular explanation of the patient's bleeding disorder, and also biochemical information on the function of both inhibitors. Most importantly, it was demonstrated that a change of a single amino acid could result in a complete change of inhibitory activity. The authors concluded that the results '. . .will allow the more confident design of specifically targeted inhibitors; in particular, the findings provide an obvious model for the synthesis of an analogue thrombin inhibitor'. It brought closer to reality the conclusion of their earlier study (Carrell *et al.*, 1982) of the molecular pathology of α_1-antitrypsin which suggested that a stable effective inhibitor could be created by the insertion of valine or alanine in α_1-antitrypsin by '. . . the use of recombinant DNA techniques to give a substitution of the reactive centre methionine to decrease lability and increase specificity for elastase'.

The engineered variants

In 1982, when (as mentioned above) the possibility of genetically engineered modifications of α_1-antitrypsin was first being suggested, it seemed almost a proposal from science fiction, yet two years later the production of site-directed mutants had actually been accomplished by three independent groups, to provide oxidation-resistant elastase inhibitors, i.e. 358 valine or alanine in place of 358 methionine, and the 'Pittsburgh' variant with antithrombin action and arginine instead of 358 methionine. The results were presented informally, at a private meeting on proteases and their inhibitors in New Hampshire, in June of 1984, the three groups being Rosenberg and colleagues (Rosenberg *et al.*, 1984) from the Chiron Corporation, Courtney and colleagues (Courtney *et al.*, 1985) from Transgene SA, and D. Schindler and colleagues (unpublished work) from the Biogen Research Corporation.

The interest shown by commercial laboratories was spurred on by the encouragement given to the development of plasma replacement therapy for α_1-antitrypsin by the National Institutes of Health of the USA, and by the likelihood that the material required for this programme would be subsidized under the US orphan-drug legislation, which sponsors the production of drugs for the treatment of uncommon diseases. It is estimated that there are some 60 000 ZZ homozygotes in the USA, each of whom would require 4 g per week of intravenous α_1-antitrypsin. This could, hypothetically, require 12 tonnes of α_1-antitrypsin a year, a demand which could be met only by use of recombinant techniques. In practice, a good deal less than this would be required, as it is neither medically indicated nor is it practical to administer material on this scale. However, even if a selected group only is treated, for example mature individuals with the onset of signs of lung damage, the demand would eventually exceed standard plasma fractionation capacity.

Both companies currently producing trial quantities of mutant α_1-antitrypsin use production techniques based on human cDNA clones of α_1-antitrypsin expressed outside the endoplasmic pathway to give the non-

glycosylated protein. The production of mutant proteins is developed from synthetic oligonucleotides and primer mismatch base insertion. Courtney *et al.* (1985) use *E. coli*, in which the α_1-antitrypsin clone has been transferred to an expression vector which uses the major leftward promoter of bacteriophage λ and a synthetic binding site. The *E. coli* produces α_1-antitrypsin as 15% of total cell protein free from vector-derived sequences, except for the initiator methionine. Rosenberg *et al.* (1984) used yeast in which a human α_1-antitrypsin cDNA clone, minus leader sequence, was inserted, the clone being flanked by glyceraldehyde-3-phosphate dehydrogenase promotion and transcriptional terminators. A similar yield of product was obtained to that produced by *E. coli*, i.e. 10–15% of the total cytosolic protein.

Using these techniques, it has been possible to produce good yields of a range of α_1-antitrypsin variants (*Table 3*). Production so far, in yeast and *E. coli*, has been limited to the non-glycosylated (naked) product, and this has enabled a comparison to be made between the two native proteins, normal and Pittsburgh plasma α_1-antitrypsin, and the naked 358 Met and 358 Arg recombinant products (Travis *et al.*, 1985). This comparison shows that the oligosaccharide side chains are not necessary for the inhibitory function of the protein. There is no significant difference between the inhibitory kinetics of the plasma glycosylated proteins and the naked recombinant products. There is, however, a marked difference in stability.

The naked recombinant α_1-antitrypsin is precipitated readily from solution by mild heating whereas plasma α_1-antitrypsin is stable in aqueous solution up to 58°C. Thus the oligosaccharide side chains of the molecule are not required for its activity, but do contribute to the stability of the molecule, presumably because of the hydrophilic shell they form on the exterior of the molecule. As well as the decreased heat stability of the recombinant α_1-antitrypsin, there is also a decreased stability apparent on isolation and storage. In part, this reflects inherent conformational lability due to lack of carbohydrate and can be compensated for by the presence in solutions of stabilizing anions such as citrate. There seems to be a greater susceptibility to oxidation, however, as shown by the better maintenance of inhibitory activity by the 358 valine mutant than by the 358 methionine recombinant product. The naked protein also seems to be more susceptible to proteolytic degradation, and in the handling and storage of the recombinant product, it is desirable to add inhibitors of the non-serine proteases such as EDTA.

A more serious problem is that the absence of oligosaccharide side chains drastically lessens the half-life of the protein in the circulation. This is not surprising because, as well as being less stable, the naked protein is also much smaller and has a greatly decreased negative charge (Travis *et al.*, 1985). These last changes will both favour loss through the glomerular filter of the kidney. Experiments with rabbits show a decrease in circulating half-life from 50 hours for the human plasma α_1-antitrypsin to 8 hours for the naked recombinant product. A similar reduction to a half-life of 8 hours has also been found in the baboon (N. Roosdorp, personal communication).

The other concern is whether the recombinant product will prove to be antigenic in man. Single amino-acid changes are not expected to give rise

Table 3. Reactive centre: engineered and natural variants

Serpin	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	Inhibits	Oxidation
α_1-Antitrypsin	Ala	Ile	Pro	Met	Ser	Ile	Pro	Pro	Glu	elastase	+
Pittsburgh variant	Ala	Ile	Pro	[Arg]	Ser	Ile	Pro	Pro	Glu	thrombin	–
Val-recombinant	Ala	Ile	Pro	[Val]	Ser	Ile	Pro	Pro	Glu	elastase	–
Leu-recombinant	Ala	Ile	Pro	[Leu]	Ser	Ile	Pro	Pro	Glu	Cat G. elastase	–
P_2Cys-recombinant	Ala	Ile	[Cys]	Met	Ser	Ile	Pro	Pro	Glu	non-functional	
Ala-recombinant	Ala	Ile	Pro	[Ala]	Ser	Ile	Pro	Pro	Glu	elastase	–
Christchurch variant	Ala	Ile	Pro	Met	Ser	Ile	Pro	Pro	[Lys]	elastase	+
P_3-P_3'-recombinant	Ala	[Ala	Gly	Arg	Ser	Leu	Asn]	Pro	Glu	non-functional	
Antithrombin	Ile	Ala	Gly	Arg	Ser	Leu	Asn	Pro	Asn	thrombin	–
Denver variant*	Ile	Ala	Gly	Arg	Leu	[Leu]	Asn	Pro	Asn	non-functional	–

Stephens, Thalley and Hirs (1985).

to significant antigenicity, but it is possible that the complete absence of oligosaccharide could reveal new antigenic sites. At present, there is conflicting evidence (Editorial, 1985): results in rabbits indicate detectable antigenicity unique to the recombinant α_1-antitrypsin; however, administration of the recombinant product to the baboon has not produced an antigenic response (N. Roosdorp, personal communication). At this stage, it seems unlikely that antigenicity of the recombinant α_1-antitrypsin will be a problem; the possible presence of contaminating yeast or *E. coli* proteins will be a greater cause for concern.

The rewarding feature of this research is that the recombinant molecules exhibit the predicted inhibitory properties. The 358 arginine yeast product has the same inhibitory spectrum (*Table 2*) as the natural 358 arginine Pittsburgh mutant (P.M. George, R.A. Hallewell and colleagues, unpublished work). The 358 valine α_1-antitrypsin, as predicted, has an inhibitory activity equivalent to that of the native 358 methionine protein but, unlike the native protein, it is not susceptible to oxidative inactivation (Rosenberg *et al.*, 1984; Courtney *et al.*, 1985). The resistance to oxidative inactivation is shown in the gelatine plate experiments illustrated in *Figure 5*.

The physiological advantage of resistance to oxidation is demonstrated by the addition of the valine-recombinant mutant to a system containing connective tissue and stimulated leucocytes (George *et al.*, 1984). The leucocyte enzymes cause rapid destruction of the connective tissue but this is inhibited immediately by addition of the valine-recombinant product. Normally, α_1-antitrypsin is inactivated in the system by oxidation of its 358 methionine, and inhibition of connective tissue breakdown does not occur until the concentration of α_1-antitrypsin reaches a point ten times greater than that

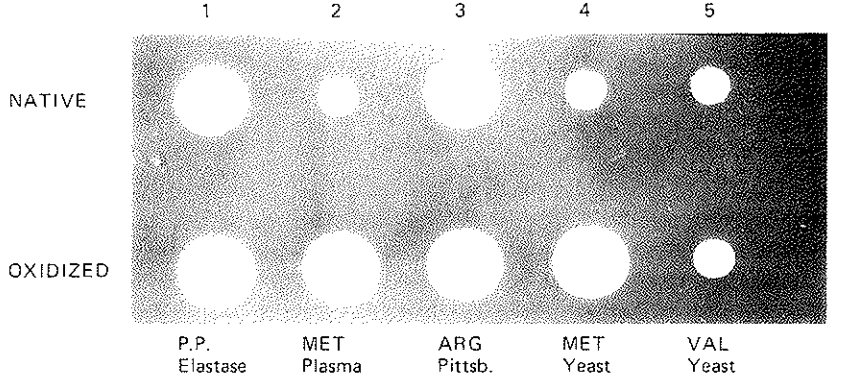

Figure 5. Gelatine plate incorporating an inhibitor to give end-point measurements of porcine pancreatic elastase activity before and after oxidation with chloramine T. The control activity, without inhibitor, is shown in (1). Addition of both plasma (2) and yeast (4) α_1-antitrypsin inhibits elastase but only the valine mutant of yeast (5) retains inhibitory activity after oxidation. The Pittsburgh mutant is an ineffective elastase inhibitor (3) but an effective thrombin inhibitor ($K_{assoc} = 1 \times 10^6$) fully equivalent to heparin-activated antithrombin-III. Plate prepared by P.M. George. (Reproduced with permission from *Trends in Biochemical Science*, Carrell and Travis, 1985.)

required for inhibition by the valine 358 mutant. Recent experiments have shown the valine-recombinant mutant to be similarly resistant to oxidation by cigarette smoke (Janoff, George-Nascimento and Rosenberg, 1986). Other work indicates that the 358 leucine-recombinant mutant of α_1-antitrypsin may be an even more effective inhibitor of neutrophil leucocyte damage because, as well as being an oxidation-resistant inhibitor of elastase, it is also a highly effective inhibitor of the other major leucocyte enzyme, cathepsin G (EC 3.4.21.20) (Courtney *et al.*, 1985; Jallat *et al.*, 1986).

Therapy of emphysema

Initially, it was thought that intravenous α_1-antitrypsin replacement would be of therapeutic value for genetically deficient individuals. However, the epidemiology of the lung disease in α_1-antitrypsin deficiency shows that this deficiency has only a minor effect on health, except in smokers. The first line of treatment, therefore, is to recommend that the individual stop smoking! However, if an individual is unable to give up the smoking habit, he is unlikely to put up with a lifetime of weekly intravenous injections. In addition, because emphysema develops over many years, it will be extremely difficult to assess the efficacy of prophylactic treatment. Although it may become possible to offer replacement therapy with concentrates of plasma α_1-antitrypsin, it is unreasonable to suggest years of therapy with a product of unproven efficacy and unknown long-term side-effects. The routine use of a recombinant product for the prophylactic treatment of emphysema is unlikely until the use of plasma concentrates has been fully assessed by trials, which may take a considerable time.

Although prophylactic use in deficiency states may be unattractive, the use of oxidation-resistant variants of α_1-antitrypsin could be of therapeutic value at times of leucocyte or oxidative stress, such as that occurring in patients with advanced lung damage who are on oxygen therapy, or who have an acute inflammatory episode such as a lung infection. However, this use may be diminished by the forthcoming availability of other elastase inhibitors suitable for short-term use (Editorial, 1985).

Therapy of antithrombin deficiency and depletion

Because the development of modified α_1-antitrypsins has been associated with research on the pathogenesis of emphysema, this has obscured the most obvious use of such compounds as a replacement for plasma antithrombin and the other serpins that control the inflammatory cascades.

Currently, there is a clinical need for a substitute for plasma antithrombin, for the treatment of the genetic deficiency of antithrombin (Editorial, 1983) and for the treatment of the antithrombin depletion which occurs in disseminated intravascular coagulation (Hanada, Abe and Takita, 1985) and in the shock syndromes (Blauhut *et al.*, 1985). The efficacy of the arginine 358 variant of α_1-antitrypsin as a thrombin inhibitor has already been demonstrated *in vivo* in the patient from Pittsburgh. In his case, the excessive

levels eventually proved fatal, but for 14 years he lived with up to 30 g of the antithrombin analogue in his circulation. This concentration is many times greater than the physiological level and the patient's history indicates that the lesser level of arginine 358 α_1-antitrypsin which would provide antithrombotic activity should be both effective and free from major side-effects.

There are other advantages which could favour the introduction of the arginine-recombinant product for therapeutic use. Its effect is immediately assayable and can be monitored constantly by measurement of thrombin-inhibitory activity. Similarly, therapeutic results would be assessable within a matter of days rather than years. The short half-life of the recombinant product, which is a great disadvantage in replacement therapy for antitrypsin deficiency, would be much less so in anticoagulant therapy, and would sometimes be an advantage.

A potential market certainly exists. The genetic deficiency of antithrombin, although not as common as that of α_1-antitrypsin, has much more drastic consequences, and produces significant thromboembolic disease in the heterozygote (Editorial, 1985). Consequently, antithrombin deficiency has an occurrence of symptomatic disease near that of α_1-antitrypsin deficiency—1:3000. Some of these individuals require constant therapy, and most will require cover with antithrombin concentrates during times of risk, such as surgery, at a current cost of US$5000–$15 000 for a standard operative and post-operative period.

Treatment of the common antithrombin depletion syndrome—disseminated intravascular coagulation—forms an even larger potential requirement. The availability of supplies of an effective antithrombin replacement will facilitate the treatment of the acquired antithrombin deficiency that underlies this syndrome although, as with all new agents, it will need to be shown, by animal and clinical tests, that they meet all present standards for efficacy and safety before commercial production can be permitted.

Therapy: The acute shock syndromes

The most promising potential use of the recombinant α_1-antitrypsins is in the acute shock syndromes, not because of the frequency of these conditions, but because of the human suffering that they cause. The shock syndromes cover a wide group of stresses such as septicaemia, endotoxaemia, cardiopulmonary by-pass procedures, and major trauma: all carry the risk of the sudden onset of severe shock, collapse, respiratory failure, and death. Many factors contribute to this, but the most significant is widespread neutrophil leucocyte activation. The activation of neutrophils on this scale causes a derangement of the protective cascades of the blood—coagulation, fibrinolysis, complement activation and kinin release. There is good evidence that this derangement, at least in part, is due to the accompanying massive release of neutrophil elastase (Duswald *et al.*, 1985). As previously described, the release of elastase on this scale places all of the plasma serpins at risk, because elastase can catalytically inactivate them by cleavage of their exposed

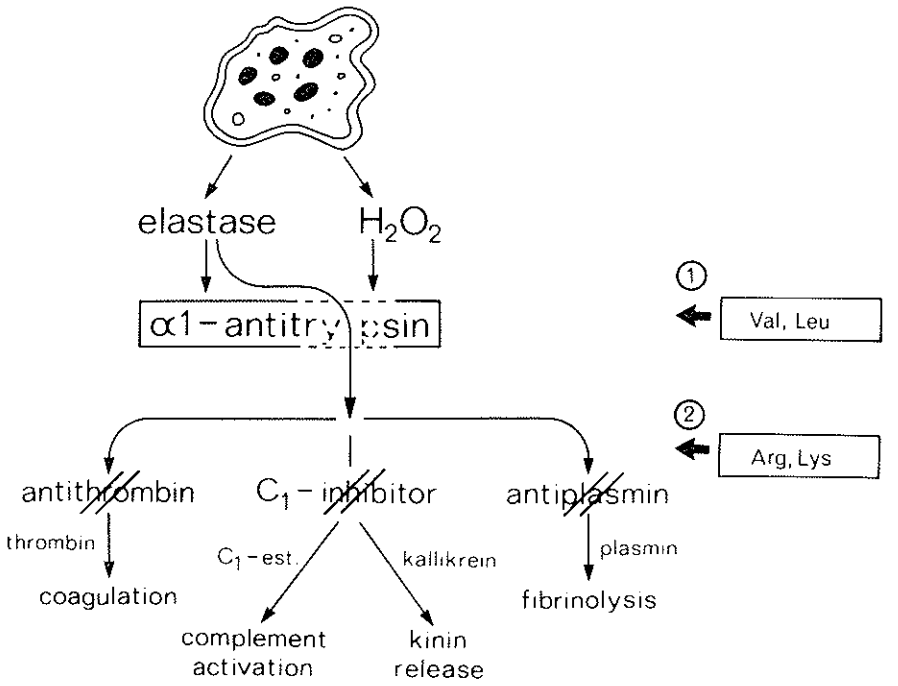

Figure 6. Neutrophil leucocytes and the shock syndromes. Scheme showing α_1-antitrypsin as prime inhibitor of neutrophil elastase but subject to inactivation by oxidation. This allows elastase to cleave and inactivate other tissue serpins with, on a massive scale, the triggering of the inflammatory cascades as seen in the acute shock syndromes. Intervention with recombinant mutants could take place as oxidation-resistant analogues of α_1-antitrypsin (valine or leucine mutants) and as cleavage-resistant analogues of the other serpins (arginine or lysine mutants).

loops. The scheme shown in *Figure 6* illustrates the changes that occur on a massive scale, as opposed to the local damage illustrated in *Figure 3*.

Intervention in acute shock syndromes could take either of two forms: prophylactic inactivation of the released elastase or therapeutic replacement of depleted plasma inhibitors. It is of interest that this could involve both groups of the recombinant antitrypsins. The valine or leucine mutants should be useful in the prevention of the shock syndromes. Both are effective inhibitors of elastase, even under the highly oxidative conditions which accompany massive neutrophil activation. Those patients 'at risk' are readily identifiable, and the recombinant α_1-antitrypsin derivatives have the advantage over other synthetic elastase inhibitors, of long half-life and good tissue distribution.

Once elastase shock has developed, the prime requirement is for replacement of depleted blood factors. In this case, the arginine derivative seems to be ideally suited as a replacement, not only for antithrombin, but also for the inhibitors of the contact proteases, for antiplasmin, and as an inhibitor of kallikrein. The arginine variant has the fortunate incidental advantage in

that, as it is derived from α_1-antitrypsin, its exposed loop is not susceptible to catalytic cleavage by elastase, and hence it is resistant to inactivation by stimulated neutrophils.

Overall, there is good reason to be confident that the engineered mutants will be beneficial in the prevention and treatment of the shock syndromes. In essence (*Figure 6*), the engineered changes stabilize the inhibitors either by providing an oxidation-resistant replacement for α_1-antitrypsin (358 Val or Leu mutants) or by preservation of the exposed loop, as in the cleavage-resistant replacements for the other serpins (358 Arg or Lys derivatives).

Prospects and problems

Those working with the recombinant serpins have no doubts about the eventual value of these products in clinical medicine, and that current problems can be overcome. Eventually it will be possible to produce the fully glycosylated proteins if needed; it is feasible now to modify half-lives and tissue distribution, and it is anticipated that their inhibitory activity can be targeted even more precisely.

The greatest problem is posed by the diverse human organizations that are involved. Prompt development requires the collaboration of three groups: the genetic engineering companies, academia and the pharmaceutical industry. Each has a different time-scale and motivation. The genetic engineering firms have a tempo, excitement, and capital structure which favour projects producing direct returns; if no-one is interested, then on to the next project! The thorough development of the recombinant antitrypsins described here will require resources available only to major pharmaceutical firms. But is it reasonable to expect that these firms can keep pace with all the implications of developments in molecular medicine? Perhaps it is the role of the academic to draw the attention of industry to these implications, and to try to convey the excitement of the potential benefits. This review will be worth while if it achieves just that.

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