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The construction of novel protease inhibitors by modification of the active centre of α_1-antitrypsin

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Several variants of α_1-antitrypsin (α_1-AT) have been produced by site-directed mutagenesis and expression in *E. coli*. The variant α_1-AT(Met-358→Val) replaces the oxidation-sensitive Met residue at the active centre with an inert Val residue, is fully active as an inhibitor of neutrophil elastase and is resistant to oxidative inactivation. This molecule could be particularly useful in the treatment of pulmonary emphysema caused by hereditary or acquired α_1-AT deficiency. It is found that α_1-AT(Met-358→Arg) no longer inhibits elastase but is an efficient, heparin-independent thrombin inhibitor. This shows that alteration of the active centre to incorporate a thrombin-specific sequence targets the inhibitor to thrombin. Preliminary studies with other mutants indicate that with this approach, inhibitors of other serine proteases like cathepsin G and porcine pancreatic elastase can also be designed. Analysis of further variants has allowed the identification of residues at the active centre that are essential for the molecule to function as an inhibitor.

Site-directed mutagenesis (Zoller & Smith 1982) has been used to construct variants of the human serine protease inhibitor α_1-antitrypsin (α_1-AT). In these experiments three questions have been addressed.

(*a*) Can the susceptibility of α_1-AT to oxidative inactivation (Carp & Janoff 1978) be alleviated by substituting inert for sensitive side chains?

(*b*) Is it possible to alter the specificity of inhibition by modification of the active-centre sequence?

(*c*) Can residues be identified at or near the active centre that are essential for function?

The raw data for these studies come from the primary sequences of the serine protease inhibitor family and from a detailed knowledge of the substrate specificities of the proteases themselves. In the course of this analysis several α_1-AT variants of potential therapeutic value have been produced.

Serine proteases

Serine proteases are among the most widely studied and best understood groups of proteins. They function either in degradative proteolysis (for example, the neutrophil proteases elastase and cathepsin G), or as more specific enzymes in zymogen activation. Many important biological processes are controlled by serine protease action. For instance, blood coagulation is regulated by a cascade of serine protease zymogen activation events, culminating in the conversion of prothrombin to thrombin which then cleaves fibrinogen to clot-forming fibrin (Grette 1962). Some examples of serine proteases with very different biological roles are listed

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in table 1. Also shown is the primary specificity of each protease, defined as the preferred amino-acid residue at the cleavage site. Serine proteases are often classified according to their primary specificity, for example, thrombin and kallikrein are termed trypsin-like because they cleave at Arg or Lys residues (Fruton 1975). However, both of these enzymes, unlike trypsin, cleave limited substrate proteins at a very limited number of specific sites, illustrating the importance of as yet ill-defined secondary specificities. The concept of primary and secondary specificity is of central importance in the design of new protease inhibitors.

TABLE 1. SERINE PROTEASES AND THEIR BIOLOGICAL ROLE

serine protease	primary substrate specificity	function
trypsin	Arg (Lys)↓	digestive tract
chymotrypsin	Trp (Phe, Tyr, Leu)↓	proteases
pancreatic elastase	Ala (Ser)↓	
neutrophil elastase	Val↓	inflammation
cathepsin G	Phe, Leu↓	
thrombin	Arg (Lys)↓	coagulation
kallikrein	Arg↓	kinin formation
factors X, IX, XII, etc.	various	coagulation

SERINE PROTEASE INHIBITORS

Because serine proteases are involved in the regulation of diverse biological systems it is of central importance that their own activities be finely controlled. This is generally achieved by control of activation of the proenzyme and by the action of an inhibitor. Inhibitors of most serine proteases have been described. However, understanding their true physiological role has proved difficult because each protease can be blocked by several different inhibitors and these are often capable of inhibiting various proteases. For example, α_1-AT inhibits all known serine proteases but with widely different k_{ass} values, and neutrophil elastase can be inhibited by α_1-AT, cathepsin G, bronchial mucus inhibitor, and α_2-macroglobulin (Bieth 1985). To estimate the *in vivo* relevance of *in vitro* data it is necessary to consider the relative physiological concentration and kinetics of inhibition of the different inhibitors (Bieth 1984). By using these criteria it has been possible to define the major *in vivo* inhibitor of many serine proteases, some of which are shown in table 2.

A striking feature of most serine protease inhibitors is the homology of their reactive-centre sequences with the preferred cleavage site of the target protease, at least in terms of primary

TABLE 2. SERINE PROTEASE INHIBITORS AND THEIR TARGETS

inhibitor	P2–P1	target enzyme
α_1-antitrypsin	–Pro–Met–	elastase
antithrombin III	–Gly–Arg–	thrombin
α_1-antichymotrypsin	–Leu–Leu–	chymase
C_1-inhibitor	–Ala–Arg–	kallikrein
mouse contrapsin	–Arg–Lys–	'trypsin'

The proteases listed are the principal *in vivo* targets of the corresponding inhibitors. In some cases other important roles are not excluded, for example α_1-antichymotrypsin also inhibits cathepsin G *in vivo*. The second column shows the amino-acid residues at the P1 and P2 positions of the active centre of each inhibitor. P1 is the position at the NH_2-terminal side of the scissile peptide bond.

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specificity (Chandra *et al.* 1983). For example, thrombin cleaves preferentially at Arg residues and the active-centre sequence of antithrombin III is Ala–Gly–ARG–Ser. Similarly, cathepsin G is chymotrypsin-like with a specificity for Phe or Leu residues. Its *in vivo* inhibitor, α_1-antichymotrypsin, has the sequence thr–Leu–LEU–Ser at the active centre. Antithrombin III, α_1-AT, and α_1-antichymotrypsin belong to a family of proteins that share 30% amino-acid homology, indicating that they have evolved by divergence from an ancestral protease inhibitor (Chandra *et al.* 1983).

α_1-Antitrypsin: general properties

The substance α_1-AT (relative molecular mass 51000) (Carrell *et al.* 1982; Beatty 1982) is produced primarily in the liver and is secreted into the plasma where it circulates at a concentration of *ca.* 1.3 mg $(dl)^{-1}$. It is a single-chain glycoprotein of 394 amino acids with three N-linked carbohydrate side chains (Mega *et al.* 1980). The amino-acid sequence has been determined and confirmed at the nucleotide level (Long *et al.* 1984). Neutrophil elastase is inhibited by α_1-AT with very fast kinetics ($k_{ass} = 6 \times 10^7$ M^{-1} s^{-1}) (Beatty *et al.* 1980), and is probably its only *in vivo* target.

Inhibition by α_1-AT involves the formation of a very stable bimolecular complex which renders the protease inactive even to small oligopeptide substrates. Complex formation is irreversible, but the complex can break down, albeit very slowly, to give active protease and inactive cleaved inhibitor. However, the complex is probably cleared *in vivo* before any significant breakdown occurs. Studies with model-protein and oligopeptide substrates have determined the secondary or extended substrate specificity for neutrophil elastase. The α_1-AT active centre displays strong homology with neutrophil elastase substrates and thus, like the other inhibitors mentioned above, acts as bait for the protease.

Attempts have been made to solve the X-ray structure of α_1-AT. Löbermann *et al.* (1984) could not crystallize native α_1-AT but did determine the structure of a derivative cleaved at the active centre (the fragments remain associated after cleavage). The data showed that in the cleaved protein the two halves of the active centre were located 67 Å† apart at either end of the molecule. This observation suggests that in native α_1-AT the active centre is in a strained loop at the surface of the molecule which, upon proteolytic attack, springs apart accompanied by a large conformational change.

Pathology of α_1-antitrypsin

X-ray crystallography data show that α_1-AT probably adopts a very compact structure with almost the entire polypeptide chain involved in well defined secondary structure elements. This allows the molecule to diffuse freely into the interstitium and tissues, where it acts as an inhibitor of neutrophil elastase. The high incidence of severe pulmonary emphysema associated with hereditary deficiency of α_1-AT indicates that its most important site of action is the lung (Eriksson 1964). Decreased levels lead to an excess of elastolytic activity in the lower lung and progressive destruction of the connective tissue of the alveolar structures (Gadek & Crystal 1981).

† 1 Å = 10^{-1} nm = 10^{-10} m.

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α_1-AT is a highly polymorphous protein with over 50 different variant forms described. The major pathological variant is the Z-protein, which is present in homozygotes at 15% of normal serum levels (Laurell & Jeppsson 1975). This variant contains a single amino acid substitution (Glu-342→Lys), which disrupts a salt bridge in the structure. The Z-protein appears to be synthesized normally but there is a block in its secretion. Incompletely processed mutant protein accumulates and is deposited as insoluble granules in the endoplasmic reticulum of the hepatocyte, possibly as a result of altered folding. The other principal pathological variant is the S-protein (Glu-264→Val). This variant has serum levels at around 60% of the normal, which can be explained by its decreased stability. Together, the Z- and S-alleles display a carrier frequency of around 1 in 10 in European populations, making α_1-AT deficiency one of the most common genetic disorders described (Laurell & Sveger 1975).

Hereditary deficiency of α_1-AT accounts for only around 1% of emphysema cases, the rest being almost entirely the result of cigarette smoking. Several studies have suggested that acquired emphysema is also caused by a protease–antiprotease imbalance (Gadek *et al.* 1979; Carp *et al.* 1982). Analysis of broncho-alveolar lavage fluids from heavy smokers indicates that a large fraction of the α_1-AT is in an inactive oxidized form that contains four moles of sulphoxy methionine per mole of protein. Because the P1 residue at the active centre is a methionine it was proposed that inactivation is probably a result of oxidation of this residue. Oxidative conditions arise as a result of the release of reactive oxygen species from the large number of neutrophils recruited to smokers' lungs.

Because pulmonary emphysema seems to result from a deficiency, hereditary or acquired, of α_1-AT it should be amenable to replacement therapy. Pilot clinical trials have been performed, using α_1-AT purified from plasma (Gadek *et al.* 1981). These studies indicated that around 4 g per week of intravenously administered α_1-AT was required to attain protective levels of alveolar antielastase activity. At these levels, 10^3 kg of α_1-AT would be necessary to treat the *ca.* 50000 ZZ-homozygotes in the U.S.A. Recombinant α_1-AT will therefore be required to provide adequate supplies for clinical use.

Production of recombinant α_1-AT

An α_1-AT cDNA (complementary) clone was isolated from a human liver cDNA bank prepared in pBR322. For production in *E. coli* the coding region for the mature α_1-AT polypeptide was transferred to an expression vector that uses the major leftward promoter of bacteriophage λ and a synthetic ribosome binding site (Courtney *et al.* 1984). After modification of the sequences around the translation-initiation codon to optimize expression levels, this strain produces around 15% total-cell protein of α_1-AT free from vector-derived sequences except the initiator methionine.

The *E. coli*-produced α_1-AT has been purified and its properties studied. Recombinant α_1-AT inhibits neutrophil elastase with kinetics identical to natural α_1-AT ($k_{ass} = 6 \times 10^7$ M^{-1} s^{-1}) and it can be used to augment anti-elastase activity in deficient serum (Wewers *et al.* 1985).

The availability of an efficient gene expression system also allows the production of mutant proteins from cDNA clones modified by site-directed mutagenesis. This was performed by using the primer mismatch method with synthetic oligonucleotides carrying the desired mutation (Zoller & Smith 1982). The target sequence was cloned into an M13 vector to provide a single-stranded template for hybridization of the oligonucleotide.

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An oxidation-resistant α_1-antitrypsin variant

Studies with synthetic oligopeptide substrates showed that the specificity of neutrophil elastase is preferentially Val and the substrate with the highest K_{cat}/K_m value has the sequence Ala–Ala–Pro–Val, with Val at the P1 position (McRae *et al.* 1980). Substrates with Met at P1 were cleaved, but with lower efficiency. A possible explanation for the presence of Met rather than Val at the P1 position of the α_1-AT active centre is to allow oxidative inactivation of the inhibitor at the focus of inflammation. This would permit unrestrained elastase activity for efficient localized liquefaction. In pathological states where this process has run out of control (for example in emphysema and also in adult and neonatal respiratory distress syndrome), direct inactivation of elastase by an oxidant resistant inhibitor should be extremely beneficial. We have synthesized an oxidation-resistant derivative of α_1-AT by substituting Val for the active centre Met-358 residue (Courtney *et al.* 1985).

The α_1-AT(Met-358→Val) is an effective inhibitor of human neutrophil elastase, consistent with the idea that inhibitor target specificity is dependent upon homology between the active-centre sequence and the protease substrate. The association rate constant of elastase inhibition was comparable with unmutated α_1-AT. Treatment with 10 mM *N*-chlorosuccinimide inactivates completely plasma α_1-AT and recombinant unmutated α_1-AT whereas α_1-AT(Met-358→Val) retains virtually complete anti-elastase activity (figure 1*a*, *b*). This

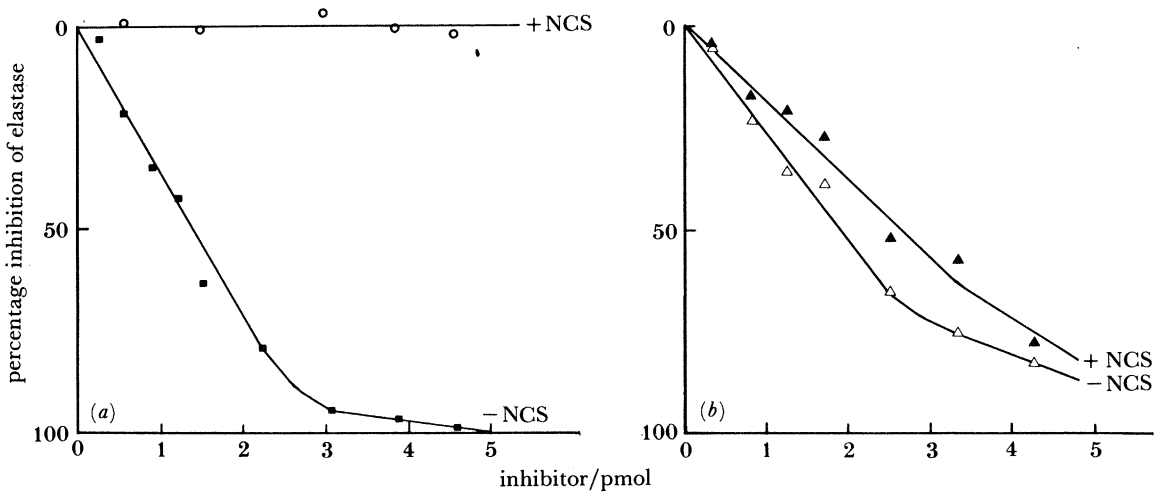

FIGURE 1. The effect of oxidation on unmutated α_1-AT and α_1-AT(Met-358→Arg). Samples either with or without treatment with 10 mM *N*-chlorosuccinimide (NCS) were assayed for neutrophil elastase inhibitory capacity. (*a*) Unmutated α_1-AT treated (○) or untreated (■); (*b*) α_1-AT(Met-358→Val) treated (▲) or untreated (△). Inhibitor concentration was measured by radial immune diffusion. Neutrophil elastase (50 ng per assay) was from Elastin Products (St Louis, Missouri). The substrate (2 mM) was *N*-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Calbiochem). Details of the assay have already been described (Courtney *et al.* 1985).

result shows that inactivation of α_1-AT is due only to modification of the active centre Met. We have preliminary data that other active-site variants (Met-358→Leu, Ile, Ala) are also oxidation-resistant elastase inhibitors. The relative efficiency of these inhibitors is under study.

These properties suggest that α_1-AT(Met-358→Val) could be particularly effective in the treatment of lungs under elastolytic and oxidative stress. We have also found that this analogue is more stable during preparation and storage. It is possible that α_1-AT(Met-358→Val) could

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be effective at lower doses than the unmutated form, an important advantage in view of the large intravenous doses necessary for adequate levels in the lung. A more effective mode of administration at substantially lower doses would be direct application to the lung surface in an aerosol. Experiments are in progress to determine the ideal conditions for particle formation and administration.

Altered specificity by mutation of the α_1-AT active centre

Formation of the α_1-AT–elastase inactivation complex is initiated by proteolytic attack at the α_1-AT active centre by the protease. Therefore a mutated α_1-AT with the recognition sequence of a different serine protease at the active centre may alter the specificity of inhibition. For instance, substitution of Leu or Phe for the P1 Met residue should produce an efficient cathepsin G inhibitor, whereas Met-358→Arg should inhibit thrombin or kallikrein. The description of a natural variant with the latter Arg substitution (α_1-AT Pittsburgh) in a 14-year-old boy who died of a severe bleeding disorder supported this proposition (Owen *et al.* 1983).

In *E. coli* α_1-AT(Met-358→Arg) was produced as described for the Val mutant. The new inhibitor efficiently inactivates thrombin and no longer inhibits neutrophil elastase (figure 2*a*, *b*). Thus, as predicted, the single amino acid difference changed completely the specificity of the inhibitor but did not alter its functional capacity. Alteration of the critical P1 residue rendered the active centre sensitive to proteolytic attack by thrombin rather than by elastase. We are currently testing whether α_1-AT(Met-358→Arg) also inhibits other 'trypsin-like' proteases, including plasma kallikrein and factor XIIf.

The natural inhibitor of thrombin is antithrombin III. This inhibitor requires the allosteric interaction of heparin-like cofactors for maximal activity (see figure 2*c*). This property is exploited in the therapeutic use of heparin as an anticoagulant. The heparin-binding domain of antithrombin III is located near the NH_2 terminal, in a region which has no counterpart in α_1-AT. For this reason, the inhibitory activity of α_1-AT(Met-358→Arg) is heparin independent (figure 2*d*).

By inhibiting thrombin efficiently in the absence of cofactors, α_1-AT(Met-358→Arg) is potentially useful as an anticoagulant in the treatment or prevention of venous thrombosis: for example, after surgery, trauma or myocardial infarction. New drugs are needed in this area because there is a considerable risk of haemorrhage with the use of heparin, especially at high doses (Mant *et al.* 1977). Another possible mode of application is after treatment with a thrombolytic agent, when there is a risk of recurrent thrombosis. In disseminated intravascular coagulation (d.i.c.) there is a generalized activation of coagulation leading to the consumption of factors and their inhibitors, including antithrombin III. Because the anticoagulant activity of heparin requires antithrombin III it is not effective in the treatment of d.i.c. α_1-AT(Met-358→Arg) should be advantageous in these cases because it inhibits thrombin directly.

To provide further evidence that alteration of the α_1-AT active-centre P1 residue can change the specificity of inhibition we have created several additional mutants, α_1-AT(Met-358→Leu and Phe) for preferential cathepsin G inhibition and α_1-AT(Met-358→Ala) for preferential porcine pancreatic elastase inhibition. Preliminary results indicate that these mutants have the anticipated activities. This remains to be confirmed by kinetic analysis.

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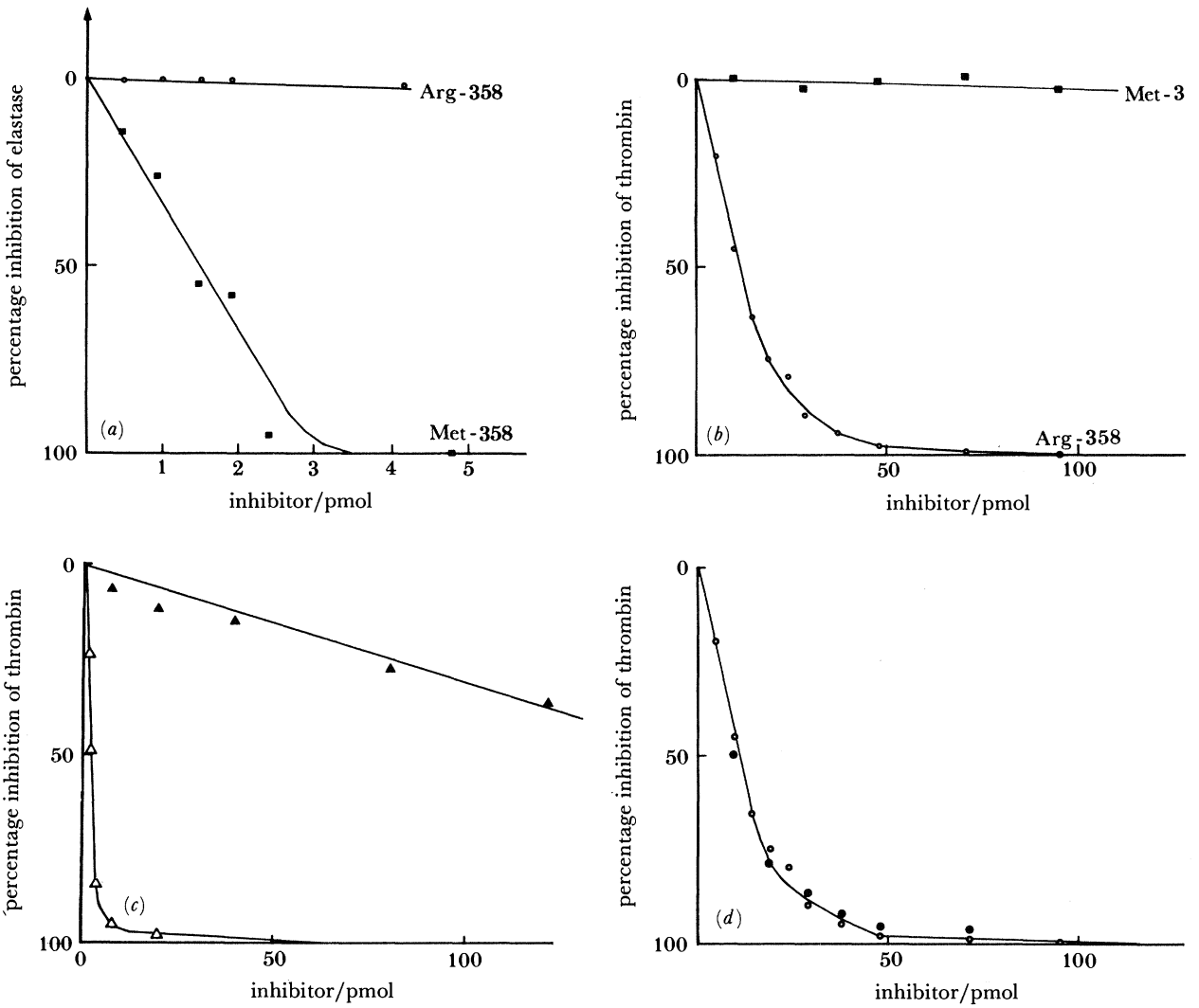

FIGURE 2. Inhibition properties of α_1-AT(Met-358→Arg). (*a*) Elastase inhibition by unmutated α_1-AT (■) and α_1-AT(Met-358→Arg) (○); (*b*) thrombin inhibition by α_1-AT (■) and α_1-AT(Met-358→Arg) (○); (*c*) thrombin inhibition by antithrombin III without (▲) and in the presence of (△) heparin; (*d*) thrombin inhibition by α_1-AT(Met-358→Arg) without (●) and in the presence of (○) heparin. Human thrombin (0.24 units, Sigma) was pre-incubated for 20 min at 23 °C with the inhibitor samples. After addition of 0.1 mM substrate (Chromozym TH, Boehringer Mannheim), initial reaction rates were obtained by measuring the change in absorbance at 410 mM. Where indicated, pre-incubation contained 0.15 units heparin (Sigma).

SEQUENCE REQUIREMENTS FOR FUNCTION

The experiments described above show that modification of the α_1-AT active-centre P1 residue can alter its target specificity. Although the new inhibitors function efficiently they do not carry the preferred extended cleavage sites for their target proteases, as determined from studies with synthetic oligopeptide substrates. For instance, the most effective substrate for neutrophil elastase, Ala–Ala–Pro–Val, differs from the α_1-AT active centre (Ala–Ile–Pro–Met) by two residues and from α_1-AT(Met-358→Val) by one. We have constructed a modified α_1-AT

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with the preferred sequence at the active centre, but found that it was a poor neutrophil elastase inhibitor (table 3). This indicates that minor modifications at the active centre that should not affect its susceptibility to attack by the target protease can lead to loss of function. In this case a single amino-acid substitution (Ile-356 → Ala) results in decreased activity, possibly reflecting the importance of Ile-356 in maintaining the structural integrity of the active centre.

TABLE 3. α_1-ANTITRYPSIN ACTIVE-CENTRE VARIANTS AND THEIR PROPERTIES

	P4	P3	P2	P1	P1′	P2′	P3′	P4′	inhibition elastase	inhibition thrombin
α_1-AT...	Ala	Ile	Pro	Met ↓	Ser	Ile	Pro	Pro	+	−
	Ala	Ile	Pro	**Val**	Ser	Ile	Pro	Pro	+	−
	Ala	**Ala**	Pro	**Val**	Ser	Ile	Pro	Pro	−	−
	Ala	Ile	**Cys**	Met	Ser	Ile	Pro	Pro	−	−
	Ala	Ile	Pro	**Arg**	Ser	Ile	Pro	Pro	−	+
	Ala	**Ala**	Gly	Arg	Ser	Leu	**Asn**	Pro	−	−

Variants were obtained by site-directed mutagenesis of a cloned α_1-AT cDNA followed by expression in *E. coli*. Neutrophil elastase and thrombin assays were performed by using chromogenic substrates as in the legends to figures 1 and 2. A negative response was scored when a greater than 20-fold excess of inhibitor blocked less than 20% protease activity. Positive inhibition was scored where a twofold excess of inhibitor is sufficient to inhibit the protease 100%.

A similar experiment was performed with respect to thrombin inhibition. Here the α_1-AT active centre was replaced with the active-centre sequence of antithrombin III (Chandra *et al.* 1981). This variant was a very poor thrombin inhibitor, supporting the conclusion that the active centre contains sequences, perhaps Ile-356 in particular, that are essential for function. Modification of Pro-357 also results in inactivation of the inhibitor. α_1-AT(Pro-357 → Cys) is not only unable to inhibit elastase but is no longer recognized by antiserum raised against plasma α_1-AT. Because it was not possible to crystallize native α_1-AT without pre-cleavage there is no structural information available that reveals the conformation of the active centre. We have initiated a series of experiments to crystallize the recombinant molecule and some of the variants described above.

CONCLUSIONS

Despite a lack of structural information describing native α_1-AT it has been possible, based on a knowledge of the inhibitor primary structure and of the cleavage sites of various serine proteases, to make interesting and potentially useful variants by site-directed mutagenesis. By changing the crucial P1 residue at the active centre according to the primary specificity of various proteases, the inhibitor is directed towards different targets. Because serine proteases play a central role in many biological processes, the design of new inhibitors is important for the study of these processes and as possible therapeutic agents for the treatment or prevention of pathological states where control has broken down.

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Discussion

R. W. Carrell (*University of Otago, New Zealand*). I have two comments. The Chairman suggested that the absence of oligosaccharides in the engineered α_1-antitrypsin may make it more readily crystallizable. In fact, the opposite is true. A year ago we examined the first of the genetically engineered variants of α_1-antitrypsin (prepared by Chiron Research Laboratories), in yeast. The lack of oligosaccharide resulted in a marked decrease in thermal stability and the purified protein could not be crystallized in a range of conditions, including those in which the native protein crystallized (personal communication, Dr R. Huber, Munich). Although the presence of carbohydrate will contribute to the heterogeneity of the protein, and hence, presumably, interfere with crystallization, this must be more than counterbalanced by the contribution of the oligosaccharide in maintaining a stable molecular conformation.

A second comment is in response to Professor Hartley's suggestion that the design of the engineered α_1-antitrypsin should be based on models of the reactive centre of the inhibitor. The problem is that we do not know the conformation of the reactive centre. We know its sequence and we know the tertiary structure of the inhibitor after cleavage, but the conformation of the intact reactive centre is, at best, speculative. I agree with Dr Courtney that we do not need to know the conformation to be able to make specific functional changes in the inhibitory

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activity. The primary dependence of this specificity on the single reactive centre amino acid was indicated by the sequence homology of α_1-antitrypsin and antithrombin (Carrell *et al.* 1982) was confirmed by the finding of a mutant converting one to the other (Owen *et al.* 1983) and the predictions that followed have been nicely borne out by the engineered variants reported today and by others (Rosenberg *et al.* 1984).

References

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