UNIVERSITY OF SOUTHAMPTON

FACULTY OF SCIENCE

PHYSIOLOGY AND PHARMACOLOGY

THE MOLECULAR PATHOLOGY OF SERINE PROTEINASE INHIBITORS : SECRETION OF NOVEL GENETICALLY ENGINEERED VARIANTS OF HUMAN α_1-ANTITRYPSIN

by **SANJIV KUMAR SIDHAR**

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It is not speech which we should want to know: we should know the speaker.

It is not things seen which we should want to know: we should know the seer.

It is not sounds which we should want to know: we should know the hearer.

It is not mind which we should want to know: we should know the thinker.

Kaushitaki Upanishad 3.8

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ABSTRACT

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THE MOLECULAR PATHOLOGY OF SERINE PROTEINASE INHIBITORS : SECRETION OF NOVEL GENETICALLY ENGINEERED VARIANTS OF HUMAN α_1-ANTITRYPSIN

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The hepatic secretory protein α_1-antitrypsin, a member of the serpin family of serine proteinase inhibitors, is the most abundant proteinase inhibitor in human plasma. α_1-Antitrypsin plasma deficiency variants include the common Z variant ($Glu^{342} \rightarrow Lys$) and the rarer S_{iiyama} ($Ser^{53} \rightarrow Phe$) and M_{Malton} (Phe^{52} deleted) α_1-antitrypsins. These variants form hepatic inclusion bodies within the endoplasmic reticulum and may predispose individuals to the lung disease emphysema, and to the development of liver cirrhosis. It has been proposed that the accumulation of these abnormal proteins occurs by a common mechanism of loop-sheet polymerization, with the insertion of the reactive centre loop of one molecule into a ß-pleated sheet of another.

Oligonucleotide-directed mutagenesis using the polymerase chain reaction (PCR) was used to reconstruct these mutations. In vitro transcription of the PCR templates produced messenger RNA that was microinjected into Xenopus oocytes to investigate the biosynthesis, glycosylation, and secretion of normal (M) and abnormal variants. All three deficiency variants duplicated the secretory defect seen in hepatocytes. Digestion with Endoglycosidase H localised all three deficiency variants to a pre-Golgi compartment, suggesting a common site for the accumulation of non-secreted inhibitor.

Two complementary approaches were taken to investigate in detail the loop-sheet polymerization process. This involved: (a) mutations in the hydrophobic core underlying the A sheet to prevent loop insertion and (b) mutations in the loop which would restrict loop mobility and impede insertion into the A sheet, a necessary prerequisite to polymerization and protein aggregation.

The non-inhibitory serpin ovalbumin is unable to undergo the conformational change, typical of inhibitory serpins, because of sequence differences in the reactive centre loop. Mutants of ovalbumin were constructed in an attempt to increase loop mobility and induce inhibitory activity, and also to investigate the association between loop mobility and secretory capacity.

α_1-Antitrypsin and ovalbumin mutants were also constructed by expression-PCR with the 5'-primer incorporating a SP6 RNA polymerase promoter, to allow direct transcription without the need for cloning into a transcription vector. These transcripts translated efficiently in vitro but not in oocytes.

A decrease in the intracellular accumulation of α_1-antitrypsin Z and S_{iiyama} variants was achieved with the mutations designed to restrict loop entry into the A sheet, and lends supports to the principle of loop-sheet polymerization.

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Finally, I wish to thank my parents for their love and support throughout my educational studies.

ABBREVIATIONS

A	adenine
α_1-AT	α_1-antitrypsin
AMV	alfalfa mosaic virus
ATP	adenosine triphosphate
BC11	reactive centre loop peptide of eleven residues
BC13	reactive centre loop peptide of thirteen residues
BiP	immunoglobulin heavy-chain-binding protein
bp	base pairs
C	cytosine
CBG	corticosteroid binding globulin
CD	circular dichroism
cDNA	complementary DNA
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphospahte
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
Endo H	endoglycosidase H
ER	endoplasmic reticulum
fMLP	N-formyl-methionine-leucine-proline
FT-IR	Fourier transform-infrared spectroscopy
G	guanine
HPLC	high pressure liquid chromatography
Hsp	heat shock protein

IL	interleukin
K_{ass}	association rate constant
kb	kilobase pairs (1 000 nucleotide bases)
kDa	kilodalton
L state	latent state
LB	Luria broth
met	methionine
MOPS	3-[N-Morpholino]propane-sulfonic acid
mRNA	messenger RNA
NMR	nuclear magnetic resonance
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pi	proteinase inhibitor variant
PMSF	phenylmethylsulphonyl fluoride
rATP	adenosine triphosphate
rCTP	cytidine triphosphate
RER	rough ER
RFLP	restriction fragment length polymorphism
rGTP	guanosine triphosphate
RNA	ribonucleic acid
RNase	ribonuclease
rNTP	riboncleoside triphosphate
r.p.m	revolutions per minute
rUTP	uridine triphosphate
SDS	sodium dodecyl sulphate
SEC	serpin-enzyme complex
SOE	splicing by overlap extension
serpin	member of serine proteinase inhibitor superfamily
S→R	serpin 'stressed' to 'relaxed' conformational change

SR1/SR2	serpin receptor 1/serpin receptor 2 pathway
T	thymine
Taq	*Thermus acquaticus*
TBE	Tris-borate-EDTA buffer
TBG	thyroxine binding globulin
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TNF	tumour necrosis factor
tPA	tissue-type plasminogen activator
Tris	tris(hydroxymethyl)-aminomethane
TUG	transverse urea gradient
U	unit
UNI	universal primer
uPA	urokinase-type plasminogen activator
UTL	untranslated leader sequence
UV	ultraviolet light

AIMS

Mutants of the inhibitory serpin α_1-antitrypsin and the non-inhibitory ovalbumin will be constructed by site-directed PCR mutagenesis to investigate the role of individual residues in the reactive centre loop that contribute to antiproteinase activity and loop-sheet polymerization.

Initially the S_{iiyama} (Ser^{53}→Phe) and M_{Malton} (Phe^{52}) α_1-antitrypsin variants will be constructed and the biosynthesis, and secretion of these variants in the *Xenopus* oocyte secretory system will be compared to Z and normal α_1-antitrypsin. Activity assays will be performed on oocyte secreted and retained material, and intracellular polymers of α_1-antitrypsin will be isolated by gel filtration chromatography.

The link between the secretory defect and the mechanism of loop-sheet polymerization will be examined *in ovo* by preventing entry of the reactive centre loop into the A sheet by three approaches. Firstly, secretion of normal, Z and S_{iiyama} α_1-antitrypsins containing an additional point mutation at position 51, Phe^{51}→Leu, designed to close the A sheet will be examined. Secondly, mutations of M and Z α_1-antitrypsin designed to hinder loop mobility and so prevent insertion into the A sheet will also be generated. Thirdly, oocytes will be co-injected with reactive loop peptide to block loop entry into the A sheet .

The secretory properties of loop hinge mutations of ovalbumin designed to undergo the S→R conformational change will be examined in oocytes, to determine the relationship between loop mobility and secretion. The P_{14}, P_{11} and P_{12} residues of ovalbumin will be replaced with the conserved residues present in inhibitory serpins. The secretory properties of a mutant of ovalbumin designed to contain a P_{17}Glu→Lys as present in Z α_1-antitrypsin will be investigated.

Ovalbumin and α_1-antitrypsin mutants will also be constructed by expression-PCR which involves incorporating a bacteriophage promoter in the 5'-amplimer, to allow direct transcription of PCR amplified reactions without the need for cloning into a transcription vector. Transcripts will be expressed *in ovo* and protein expression compared to expression from vector transcribed RNAs.

1. INTRODUCTION

1.1 Serine Proteinases

Proteinases are ubiquitous enzymes involved in peptide bond cleavage in a range of proteolytic processes such as blood coagulation, fibrinolysis, complement activation, release of signal peptides, virus maturation, inflammation, phagocytosis, and hormone and growth factor processing. Proteinases are classified on the basis of the prominent functional group in the active site into four families: serine, cysteine, aspartic and metalloproteinases. To date, the majority of proteinase known and characterized belong to the serine proteinase family (reviewed by Kraut, 1977). Although most serine proteinases have no absolute substrate specificity, many show a preference for a particular amino acid on the amino terminal side of the scissile bond. For example trypsin preferentially cleaves on the carboxyl terminal of Lys or Arg residues and elastase prefers small uncharged side chains.

The serine proteinase reaction mechanism has been determined using crystallographic and NMR studies (reviewed by Steitz & Shulman, 1982; Fersht, 1985). Hydrolysis begins with acylation, the formation of a covalent acyl-enzyme intermediate, in which the carboxyl (C_1) atom of the substrate is esterified to the reactive serine hydroxyl (—OH) of the enzyme (Figure 1.1). Acyl-enzyme intermediate production proceeds through a negatively charged tetrahedral-intermediate transition state. A histidine (His) residue in the enzyme acts as a general base by accepting a proton from the hydroxyl group of the serine and so increasing the nucleophilicity of the serine —OH. The resulting positively charged histidine stabilizes the negatively charged transition state and the histidine in turn is stabilized by a electrostatic interaction with a negatively charged aspartate (Asp). The three side chains from Ser, His and Asp are close to each other in the active site to form a catalytic triad that is at the heart of all serine proteinases. Tight binding and further stabilization of the tetrahedral intermediate is achieved by hydrogen bond formation between the main-chain atoms of the proteinase and that of

the substrate. The second step, deacylation, is essentially the reverse of the first with hydrolysis of the acyl-enzyme intermediate by a water molecule in the role of the amine ($-NH_2$) group.

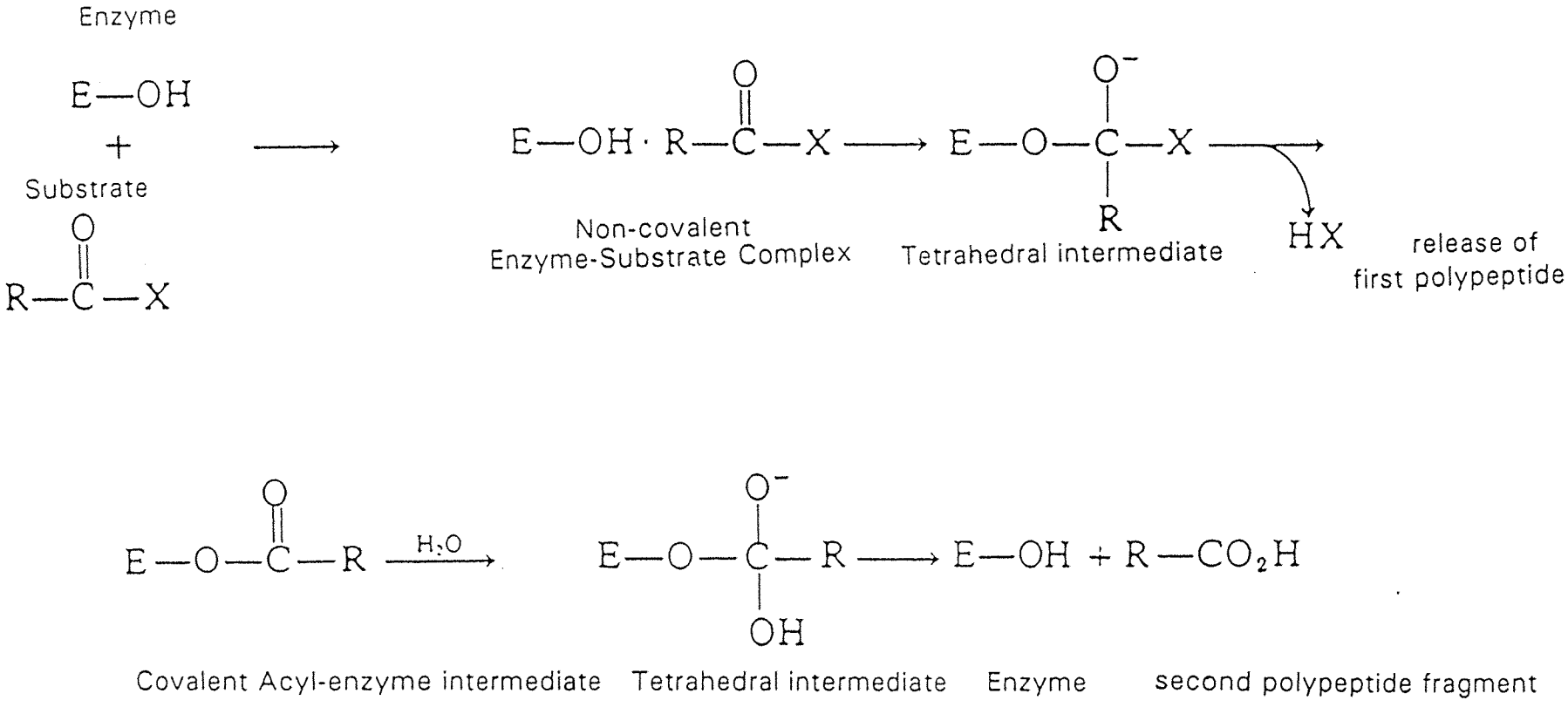


Figure 1.1 Serine proteinase hydrolysis of peptide bonds within a polypeptide chain. The reactive centre residue of the enzyme (E—OH) hydrolyses the peptide bond (C—X, where X is the remaining polypeptide chain) via a tetrahedral intermediate transition state. Hydrolysis of the acyl-enzyme intermediate releases the second product peptide ($R—CO_2H$) and restores the serine hydroxyl group of the enzyme.

1.2 Proteinase Regulation

If uncontrolled, excess proteinase activity in organisms can be self destructive. The two principal methods regulating proteinase activity are proteolytic cleavage or zymogen activation of proteinases, and inhibition of proteinases by host antiproteinases.

1.2.1 Zymogen Activation

Many enzymes are synthesized as inactive precursors, known as a zymogens or proenzymes, that are subsequently activated by cleavage of one or a few peptide bonds to expose the active site. Examples include the pancreatic enzymes that hydrolyze proteins. The proenzymes trypsinogen and chymotrypsinogen are secreted as inactive precursors by the pancreas (Huber & Bode, 1978). Cells lining the duodenum secrete enteropeptidase which hydrolyzes a unique lysine-isoleucine peptide bond in trypsinogen on its entry from the pancreas. Hydrolysis triggers a conformational change in trypsinogen which exposes a binding pocket for the substrate, to form trypsin. Trypsin in turn activates all the other pancreatic proenzymes: chymotrypsinogen, proelastase, and procarboxypeptidase. Thus, the action of proteolytic enzymes in the duodenum is controlled by the activation of trypsin.

Blood clotting occurs by a cascade of zymogen activations (Davie, 1986), the activated form of one clotting factor catalyzing the activation of the next precursor (Figure 1.2). From one tiny signal the cascade of events produces a large amplification, ensuring a rapid response to bleeding. Clotting is triggered by two mechanisms: a) the *extrinsic pathway* is triggered by factors released from tissues as a consequence of trauma or injury; b) the *extrinsic pathway* is activated on injury by contact with abnormal surfaces. Both pathways converge on a single common pathway that results in the conversion of fibrinogen to a soluble thread-like mesh of

fibrin, that traps blood cells to form a blood clot. Many of the activated clotting factors are serine proteinases— kallikrein, factors XII, XI, IX, VII, X, and thrombin. Clotting is confined to the site of injury by the short half-life of the clotting factors due to removal by the liver, degradation by proteinases and specific inhibitors. The single most important inhibitor is antithrombin III, a plasma serine proteinase inhibitor, that inactivates thrombin as well as the other serine proteinases in the clotting cascade, by forming irreversible 1:1 complexes (For a recent review see Perry, 1994). Antithrombin III inhibitory activity is enhanced by heparin, a negatively charged polysaccharide formed by mast cells near the walls of blood vessels and on the surface of endothelial cells (Rosenberg, 1975). Once the structural integrity of an area protected by a clot is restored, further clotting is prevented by plasmin, a serine proteinase that hydrolyses fibrin in a process known as fibrinolysis (reviewed by Doolittle, 1984). Zymogen activation is also important in the regulation of fibrinolysis; plasmin is formed by the proteolytic inactivation of plasminogen by the serine proteinases, urokinase- (uPA) and tissue-type plasminogen activator (tPA) (Saksela & Rifkin, 1988). The major physiological regulator of both uPA and tPA is plasminogen activator inhibitor-1 (PAI-1) (Lijnen *et al.*, 1991; Lawrence *et al.*, 1994). Zymogen activation by cleavage of a single peptide bond cleavage is irreversible and so a different strategy is required to keep the activities of proteinases in check. This mechanism involves specific proteinase inhibitors that mimic the transition states of proteinase substrates and bind to the proteinases very tightly.

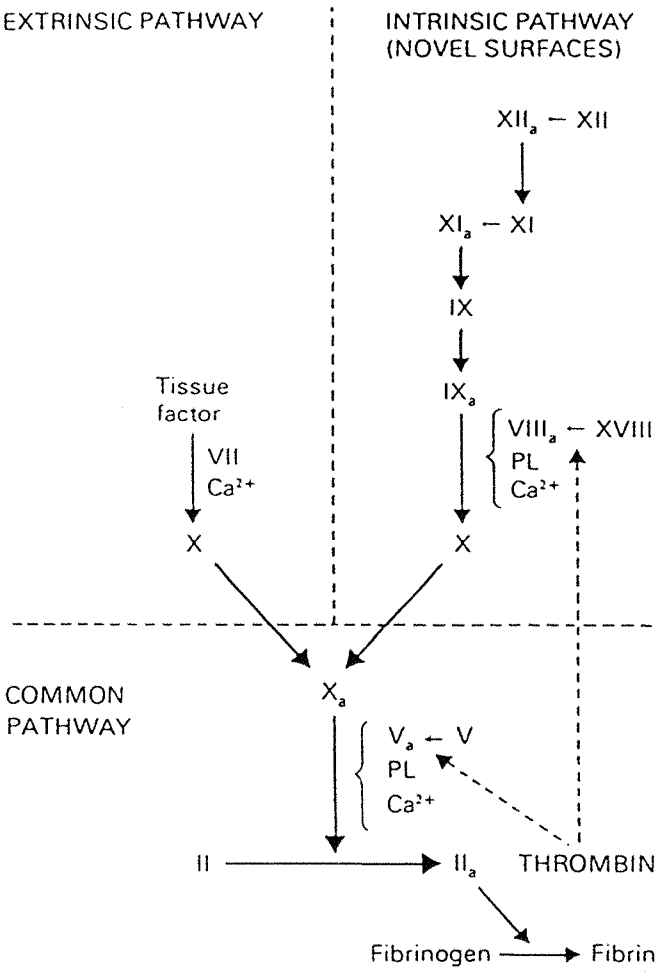


Figure 1.2 Blood Clotting Cascade. Plasma factors of the intrinsic, extrinsic, and final common pathways are activated by zymogen activations to produce a fibrin clot. The activation of one clotting factor catalyses the activation of the next. a = activated form; PL = phospholipid. Factor II = Prothrombin; II_a = Thrombin.

Table 1.1 X-ray crystal structures of serine proteinase inhibitors and their complexes with proteinases. Representative structures from different families are listed; all known structures from the serpin family are recorded.

Structure	Abbreviation	Resolution (Å)	Reference
1. BPTI (Kunitz, Kunin) family			
Bovine Pancreatic Trypsin Inhibitor	BPTI (I)	1.5	Deisenhoefer & Steigemann, 1975
BPTI (crystal form (III)	BPTI (III)	1.7	Eigenbrot *et al.*, 1990
Amyloid ß-protein Precursor Inhibitor	APPI	1.5	Hynes *et al.*, 1990
2. Kazal family			
Japanese Quail Ovomucoid 3rd domain	OMJPQ3	1.9	Papamokos *et al.*, 1982
Porcine Pancreatic Secretory Trypsin Inhibitor	PSTI	1.8	Bolognesi *et al.*, 1982
Turkey Ovomucoid 3rd domain:Bovine α-Chymotrypsin	OMTKY3:CHT	1.8	Fujinaga *et al.*, 1987
3. STI (STI-Kunitz) family			
Soybean Trypsin Inhibitor:Porcine Trypsin	STI:PT	2.6	Sweet *et al.*, 1974
4. SSI family			
Streptomyces Subtilisin Inhibitor	SSI	2.3	Mitsui *et al.*, 1979
5. Potato inhibitor 1 (PI-1) family			
Eglin c: Subtilisin Carlsberg	Eglc:SCAR	1.2	Bode *et al.*, 1987
CI-2	CI-2	2.0	McPhalen & James, 1987

continued.....

Structure	Abbreviation	Resolution (Å)	Reference
6. Potato inhibitor 2 (PI-2) family			
Chymotrypsin Inhibitor-1	CI-1:SGPB	2.1	Greenblatt *et al.*, 1989
7. Chelonianin family			
Mucous Proteinase Inhibitor:Chymotrypsin	MPI:CHT	2.5	Grütter *et al.*, 1988
8. Bowman-Birk family			
Azuki Beans Protease Inhibitor:Bovine Trypsin	AB-I:BT	3.0	Tsunogae *et al.*, 1986
Peanut Inhibitor A-II	A-II	3.3	Suzuki *et al.*, 1987
9. Squash seed inhibitors			
Cucurbita Maxima Trypsin Inhibitor-I:Bovine Trypsin	CMT-I:BT	2.0	Bode *et al.*, 1989
10. Hirudin			
Desulfato Hirudin variant 2K47: Human α-Thrombin	HIRV2:HUTHR	2.3	Rydel *et al.*, 1990
11. *Ascaris* inhibitors			
Ascaris Chymotrypsin/Elastase Inhibitor:Porcine Elastase	C/E-I	2.4	Huang *et al.*, 1994

continued.....

Structure	Abbreviation	Resolution (Å)	Reference
12. Serpins			
Reactive-site cleaved α_1-Proteinase Inhibitor: tetragonal form (I)	α_1-PI*T(I)	3.0	Loebermann *et al.*, 1984; Engh *et al.*, 1989
α_1-PI* hexagonal form	α_1-PIH*	3.1	Engh *et al.*, 1989
α_1-PI* tetrahexagonal form II	α_1-PIT*	3.0	Engh *et al.*, 1989
Reactive-site cleaved α_1-Proteinase Inhibitor S-variant (Glu^{264}→Val)	α_1-PIS	3.1	Engh *et al.*, 1989
Reactive-site cleaved α_1-Antichymotrypsin	α_1-AChy	2.7	Baumann *et al.*, 1991
Reactive-site cleaved Equine Leukocyte Elastase Inhibitor	HLEI	2.0	Baumann *et al.*, 1992
Reactive-site cleaved Chicken Ovalbumin (Plakalbumin)	PLA	2.8	Wright *et al.*, 1990
Chicken Ovalbumin	OVA	2.0	Stein *et al.*, 1990
Latent Plasminogen Activator Inhibitor-1	PAI-1	2.6	Mottonen *et al.*, 1992
Native Recombinant Antichymotrypsin (P_3-P_3' α_1-AT)	rACT.P_3-P_3'	2.5	Wei *et al.*, 1994
Dimeric antithrombin III		3.0 3.2	Carrell *et al.*, 1994 Schreuder *et al.*

1.2.2 Proteinase Inhibitors

Proteinase inhibitors represent the third largest group of functional proteins in mammals by weight after albumin and the immunoglobulins (Travis & Salvesen, 1983). These inhibitors play a key role in the regulation of the proteolytic processes mentioned above. Although endogenous inhibitors are nearly always proteins, small non-proteinaceous inhibitors directed against host proteinases are produced in some micro-organisms (Laskowski & Kato, 1980). An account of the inhibitors of serine proteinases follows.

1.3 Serine Proteinase Inhibitors

In a now classical review article Laskowski and Kato (1980) for the first time grouped the serine proteinase inhibitors into 17 distinct superfamilies on the basis of sequence homology, structural similarity and the mechanism of binding. The X-ray crystal structure of at least one representative is known from 12 of these families (see Table 1.1). Structural analyses of other members and other families are underway.

1.3.1 Standard Mechanism Inhibitors

Typical serine proteinase inhibitors are relatively small proteins (or protein domains) of between 29 and 190 amino acid residues, with an exposed reactive site binding loop of a characteristic canonical conformation which reacts with its cognate serine proteinases (Laskowski & Kato, 1980). Protein inhibitor—serine proteinase interaction has been studied in detail, particularly for the soybean-trypsin inhibitor (STI)—bovine ß-trypsin system (Findenstadt *et al.*, 1974). The interaction known as the 'standard mechanism' involves equimolar association between the inhibitor and the proteinase in the manner of a good substrate. The residue recognized by the primary binding site of the target proteinase is designated P_1; residues P_2,

P_3, P_4 etc. are amino-terminal to P_1 and residues P_1', P_2', etc. are carboxyl terminal to P_1 (using nomenclature of Schechter & Berger, 1967). The standard mechanism scheme can be represented as (Finkenstadt *et al.*, 1974; Quast *et al.*, 1978) :

$$E + I \rightleftharpoons EI \rightleftharpoons E + I^*$$

where E is the proteinase, and I is the virgin (intact) inhibitor. Inhibition resembles hydrolysis of normal substrates except that the complex EI is rapidly formed and is much more stable than the Michaelis enzyme-substrate complex; the second-order association rates (k_{ass}) are very high (typically 10^5 M^{-1} s^{-1}) (Laskowski & Kato, 1980). The reactive site peptide bond, P_1-P_1', is hydrolysed to yield free enzyme and cleaved inhibitor (I^*) (Ardelt & Laskowski, 1985). Although the specificity constant, k_{cat}/K_m, for hydrolysis of this peptide bond is very high 10^4-10^6 $M^{-1}s^{-1}$ (Finkenstadt *et al.*, 1974; Estell *et al.*, 1980), the individual values of k_{cat} and K_m are several orders of magnitude lower than those for normal substrates (Laskowski & Kato, 1980), resulting in extremely slow hydrolysis of the bond. Furthermore, hydrolysis does not proceed to completion; instead an equilibrium is established between virgin inhibitor and inhibitor with peptide bond cleaved ('modified' inhibitor) (Finkenstadt *et al.*, 1974; Laskowski & Kato, 1980).

Interactions between proteinases and different serine proteinase inhibitor families have been investigated using X-ray crystallography and NMR (reviewed by Bode & Huber, 1992). The reactive-site peptide bond, P_1-P_1', is intact within the complex, with the P_1 carbonyl carbon in close proximity (approximately 2.7 Å) to the nucleophilic O^γ in the catalytic serine residue (Huber *et al.*, 1974; Read & James, 1986; Bode & Huber, 1992). Other interactions also contribute to the stability of the complex. In particular, the P_3-P_3' exposed residues make the strongest interactions with a proteinase and have a characteristic rigid extended canonical conformation amongst different inhibitor families (Mitsui *et al.* 1979; Read & James,

1986; Bode & Huber, 1992). Although the similar dihedral angles ϕ and Ψ (Table 2) show a good conservation of the reactive-site, a certain conformational readjustment of these residues is allowed for optimal enzyme-inhibitor interaction. Inhibitors from these different families have a common reactive-site loop conformation while displaying completely different overall structures. Bode and Huber (1991) distinguished the solvent-exposed proteinase binding loop from the major part of the inhibitor which forms a 'scaffold' for the exposed reactive site loop. The rigidity of the reactive centre loop obstructs nucleophilic attack on the C_α carbonyl atom of the catalytic serine, thereby peptide bond cleavage is very slow, if at all.

1.3.2 The Serpin Superfamily

The serpin (**ser**ine **p**roteinase **in**hibitor) superfamily of inhibitors (Carrell & Boswell, 1986) are much larger (350 amino residues) than the small protein inhibitors of serine proteinases outlined above, and show deviations from the standard mechanism of inhibition with respect to complex stability and reversibility of inhibition. Although serpins seem to interact with their target proteinases like the canonical inhibitors via an exposed binding loop, the resulting complexes are very stable. Ultimately the complexes dissociate to reveal cleaved serpin that is no longer inhibitory, ie. serpin inhibition is irreversible.

Table 1.2 Conformational angles ϕ, ψ (°) of the reactive site loops between sites P_3 and P_2' of different serine proteinase inhibitors.

	P_3		P_2		P_1		P_1'		P_2'	
Inhibitor	ϕ	ψ	ϕ	ψ	ϕ	ψ	ϕ	ψ	ϕ	ψ
BPTI	-84	-7	-86	159	-110	21	-77	171	-129	81
2SSI	-130	147	-72	145	-92	89	-118	167	-116	90
1CSE	-139	168	-62	143	-115	45	-97	168	-117	110
OMTKY	-131	150	-68	160	-107	32	-74	159	-113	107
ATI	-156	143	-85	-154	-74	40	-108	87	-122	121

BPTI, Bovine pancreatic trypsin inhibitor (Huber *et al.*, 1974); 2SSI, *Streptomyces* subtilisin inhibitor (Mitsui *et al.*, 1979); 1CSE, Eglin-c complexed with subtilisin Carlsberg (Bode *et al.*, 1987); OMTKY, Turkey ovomucoid inhibitor (Fujinaga *et al.*, 1987); ATI, *Ascaris* trypsin inhibitor at pH 2.4 (Grasberger *et al.*, 1994).

1.4 The Serpins

The acronym SERPIN was coined by Carrell and Travis (1985) for serine proteinase inhibitors that consist of small monomeric glycoproteins of greater than 350 amino acids in length, possessing close sequence homology in the C-terminal region and a well conserved tertiary structure. On the basis of protein homology, over 60 proteins have been classified as serpins, distributed throughout nature in vertebrates, invertebrates, plants, and viruses (Huber & Carrell, 1989) (Table 1.3). Despite the name serpin, some members lack inhibitory ability against serine proteinases, and others may act as both inhibitors or substrates of the target proteinase, depending on the proteinase and/or the reaction conditions. Typical serpins include most plasma inhibitors (α_1-antitrypsin, antithrombin, and C1 inhibitor) that control enzymes of the major biologically important proteolytic cascades such as coagulation, fibrinolytic, kinin and complement activation systems. Non-inhibitory serpins include egg-white ovalbumin (function unknown), and those serpins which have developed specialized physiological roles as carrier proteins (thyroxine- and cortisol- binding globulins), or peptide hormone precursors (angiotensinogen). The partial primary structures of α_1-antitrypsin and antithrombin III first revealed a relationship between the two plasma serine proteinase inhibitors (Carrell *et al.*, 1979). Subsequent studies on cloned DNA showed a 30% sequence homology between α_1-antitrypsin and antithrombin III (Kurachi *et al.*, 1981). A similar homology (30%) of these two proteins with the non-inhibitory serpin ovalbumin was revealed by Hunt and Dayhoff (1980) using analysis of amino acid sequence identity. This suggests that members of the serpin family have diverged from a common ancestral serpin over a period of 500 million years (Hunt & Dayhoff, 1980). Phylogenetic analysis by sequence and structural alignment (Marshall, 1993), reveals that as much diversity exists between the plant serpin, barley protein Z, and mammalian serpins as exists among the mammalian serpins. This high variation among mammalian serpins reflects the variability in composition of the reactive centre loop (Huber & Carrell, 1989), although

the overall serpin structure is highly conserved.

Apart from serpin classification into inhibitory and non-inhibitory members, Remold-O'Donnell (1993) has proposed a separate protein family within the serpin superfamily: the 'ovalbumin-like' (Ov-serpins) family. Ov-serpins embrace a small number of serpins that can be either intracellular or secreted. The family is not recognized on the basis of amino acid homology alone; features of the Ov-serpins are their lack of classical N-terminal signal sequences and their susceptibility to inactivation by thiol-reactive reagents by oxidation of residues in close proximity to their reactive sites (Remold-O'Donnell *et al.,* 1992; Ray *et al.*, 1992). Members of the Ov-serpins include ovalbumin, plasminogen activator inhibitor 2, placental thrombin inhibitor, human leukocyte elastase inhibitor, and horse leukocyte elastase inhibitor (Remold-O'Donnell *et al.,* 1989; von Heijne *et al.*, 1991; Remold-O'Donnell *et al.,* 1992; Coughlin, *et al.*, 1993). Physiochemical comparisons within the family may provide an insight into the structural features responsible for inhibitory activity in some Ov-serpins (plasminogen activator inhibitor 2 and elastase inhibitor) and the absence of inhibitory function in ovalbumin.

In spite of C-terminal sequence homology amongst the serpins, the N-terminal region shows considerable variation in length, composition and degree of glycosylation (Bock *et al.*, 1986, Huber & Carrell, 1989), conferring additional functional domains to some serpins. Examples of such specialized domains include heparin binding sites in antithrombin III, protease nexin-I and plasminogen activator inhibitor-1 and the binding of dermatan sulphate by heparin cofactor II (Huber & Carrell, 1989; Grootenhuts & van Boeckel, 1991).

Table 1.3 Some members of the serpin family and their general function; based on Potempa *et al.* 1994.

	Target Enzyme(s)	Regulatory Function	Reference
Vertebrate			
α_1-Antitrypsin	Neutrophil elastase	ECM remodelling	Beatty *et al.*, 1980
α_1-Antichymotrypsin	Cathepsin G, Chymase	ECM remodelling, Prohormone conversion, Inflammatory response regulation	Travis & Salvesen, 1983 Andreasen *et al.*, 1990
α_2-Antitplasmin	Plasmin	Fibrinolysis	Travis & Salvesen, 1983
Antithrombin III	Thrombin	Blood Coagulation	Pratt & Church, 1993
Heparin cofactor II	Thrombin	Blood coagulation	Pratt & Church, 1993
Active protein C inhibitor	Active protein C	Blood coagulation	Pratt & Church, 1993
C1-inhibitor	C1 esterase	Blood coagulation	Travis & Salvesen, 1983
PAI-1	u-PA, t-PA	Cell migration, Fibrinolysis, Blood Coagulation ECM remodelling	Preissner & Jenne, 1991 Andreasen *et al.*, 1990
PAI-2	u-PA, t-PA	Fibrinolysis, ECM remodelling	Andreasen *et al.*, 1990
Protease nexin-1	Thrombin, u-PA, Plasmin	Cell differentiation, ECM remodelling	Monard, 1988
Leukocyte inhibitors	Neutrophil elastase	Intracellular proteolysis	Dubin *et al.*, 1992
Kallistatin	Kallikrein	?	Chai *et al.*, 1993
Placental thrombin inhibitor	¿	Intracellular proteolysis	Coughlin *et al.*, 1993
Maspin	#	Tumour suppression	Zou *et al.*, 1994
Ep45	#	?	Holland *et al.*, 1992
Cortisol binding globulin	None	Hormone transport	Hammond *et al.*, 1987
Thyroxine binding globulin	None	Hormone transport	Flink *et al.*, 1986
Angiotensinogen	None	Blood Pressure	Doolittle, 1983
Ovalbumin	None	?	Hunt & Dayhoff, 1980
Uterine milk portein	None	?	Ing & Roberts, 1989
PEDF	None	Cell differentiation	Steele *et al.*, 1993
Invertebrate			
Limulus ICI	Factor C	Blood Coagulation	Miura *et al.*, 1994
Plant			
Barley Protein Z	¿	?	Brandt *et al.*, 1990
Viral			
Vaccinia virus	¿	Viral pathogenicity	Buller & Palumbo, 1991
Cowpox virus (CrmA)	IL-1ß converting enzyme	Viral pathogenicity, Inflammatory response regulation	Ray *et al.*, 1992
Myxoma virus SERP-1	t-PA, u-PA, Plasmin	Viral Pathogenicity	Lomas *et al.*, 1993c

Abbreviations used are: ECM, extracellular matrix; u-PA/t-PA, urokinase/tissue plasminogen activator; Ep45, *Xenopus laevis* estrogen-regulated protein; PEDF, pigment epithelium-derived factor; ICI, intracellular coagulation inhibitor
¿, target enzyme unknown but inhibitory activity demonstrated; #, inhibitory activity unconfirmed; ?, function unknown.

The best studied members of the serpins are those present in human plasma (Travis & Salvesen, 1983). The physiological importance of serpins in homeostatic control is demonstrated by the correlation between serpin inactivation or deficiency and the incidence of pathological disorders. Examples of clinical syndromes observed are: lung and liver disease with α_1-antitrypsin deficiency (Eriksson, 1964) and α_1-antichymotrypsin (Lindmark & Eriksson, 1991; Faber *et al.*, 1993), thrombosis with antithrombin III deficiency (Beresford & Owen, 1990), and haemorrhage with α_2-antiplasmin or plasminogen activator inhibitor-1 (PAI-1) deficiency (Aoki *et al.*, 1979; Schleef *et al.*, 1989). Deficiencies may arise from either abnormally low levels of functional serpin, or from normal levels of dysfunctional serpin as a result of mutation or inactivation by non-target proteinases secreted by pathogenic organisms. Extensive reviews of human serpins have been published (Carrell & Boswell, 1986; Huber & Carrell, 1989; Crystal, 1990). A knowledge of the structure and mechanism of serpin function is fundamental to the development of treatments for these diseases.

1.4.1 Serpin-proteinase Interaction

The inhibitory mechanisms of particular serpins have been investigated by various research groups, and the results have often been taken to be representative of a common serpin inhibitory mechanism. This assumption is based on the close sequence homology between serpins and the overlapping specificities seen in the family. The specificity of serpins for target proteinases is determined, in part, by the P_1 residue at the reactive site that resembles the substrate-cleavage site of the proteinase (Travis & Salvesen, 1983, Carrell *et al.*, 1987). α_1-Antitrypsin Pittsburg, a naturally occurring mutant of α_1-antitrypsin with the P_1 methionine substituted for arginine (P_1 in wild-type antithrombin III) results in a change in α_1-antitrypsin inhibitory specificity from elastase to thrombin (Lewis *et al.*, 1978). Similar specificity of P_1Arg for thrombin have been demonstrated with mutagenic studies on α_1-antichymotrypsin where P_1Leu→Arg changes the specificity

from chymotrypsin and cathepsin G to thrombin and trypsin (Rubin *et al.*, 1990), and P_1Leu→Arg in heparin cofactor II increases the rate of thrombin inhibition (Derechin *et al.*, 1990). A number of P_1 recombinant variants of α_1-antitrypsin have been synthesized and the inhibitory specificity correlated with the reactive site residue (Courtney *et al.*, 1985). However, several studies on engineered mutants of α_1-antitrypsin (George *et al.*, 1985; Matheson *et al.*, 1989; Avron *et al.*, 1991), antithrombin III (Austin, 1990; Theunissen *et al.* , 1993), t-PAI-1 (Madison *et al.*, 1990) and C1-inhibitor (Eldering *et al.*, 1993) have revealed that in addition to the P_1 residue, P_1', P_2', P_3', P_3, and P_5 sites are also important determinants in serpin-proteinase interactions.

Serpin-serine proteinase reaction mechanisms investigated include α_1-antitrypsin with elastases and trypsin (Oda *et al.* 1977; Beatty *et al.* 1982), α_2-antiplasmin with plasmin, chymotrypsin with trypsin (Potempa *et al.* 1988; Shieh *et al.* 1989; Longstaff & Gaffney, 1991), and antithrombin with thrombin (Björk *et al.*, 1982; Olson, 1985). Initial studies on α_2-antiplasmin binding to plasmin resulted in the mechanism of binding shown in Scheme 1, where E is the serine proteinase and I is the inhibitor

SCHEME 1 $$E + I \rightleftarrows EI \rightarrow [EI'] \rightarrow E + I^*$$

This reaction mechanism has also been reported as a general serpin-proteinase binding model by Travis & Salvesen (1983). Initial formation of a reversible non-covalent Michaelis complex, EI, is followed by the irreversible formation of an intermediate [EI'] which involves some interaction at the reactive centre peptide bond. Such interaction may involve formation of a tetrahedral intermediate, involving an ester linkage between the carbonyl group of the serpin active centre (P_1) and the $^{\gamma}$-hydroxyl of the proteinase active site serine, or the reaction may proceed further by

cleavage of the scissile bond (P_1-P_1 ') yielding an acyl-enzyme intermediate (Longas *et al.* 1980). The nature of the stable serpin-proteinase complex has not been proven.

The EI' complex is not dissociable upon boiling with SDS or urea but is sensitive to nucleophiles, a property unique to the serpin class of proteinase inhibitors (Moroi & Yamasaki, 1974; Owen, 1975; Cohen *et al.* 1978). The high stability of the complex can be accounted for if a covalent bond were to exist between the enzyme and inhibitor (Cohen *et al.*, 1978). The most likely structure of EI' is an acyl-intermediate, as seen in the acylation step of serine proteinase substrate hydrolysis (as discussed above). However, the denaturing conditions may invoke covalent intermediates and reactive centre cleavage. Studies on α_2-antiplasmin and trypsin demonstrated that the acyl-intermediate was unlikely to be present during serpin-proteinase interaction because the complex EI' could dissociate reversibly to the active inhibitor and enzyme (Shieh *et al.*, 1989). Furthermore, ^{13}C-NMR studies by Matheson and Travis (1991) have indicated that the covalent bond in the α_1-antitrypsin-pancreatic elastase complex is in a tetrahedral conformation with the P_1-P_1 ' bond intact. Currently there is no crystal structure of an intact serpin or a serpin complex to define the nature of the contacts which lock the serpin-proteinase into a stable complex.

Scheme 1 does not account fully for the serpin inhibitory mechanism. Half-lives for the breakdown of the stable EI' complexes upon prolonged incubation may vary from minutes or hours to several days (Danielsson & Björk, 1983). Moreover, the amount of active enzyme liberated can vary from several percent to complete liberation. Some serpins may act as substrates for certain proteinases under certain conditions and be catalytically inactivated by cleavage at the reactive centre bond. Partial substrate and partial inhibition reactions may also be observed with some serpin-proteinase interactions. Studies on C1-inhibitor with the proteinase kallikrein (Patston *et al.*, 1991), α_1-antichymotrypsin (Rubin *et al.* 1990), and

the heparin-catalyzed inhibition of thrombin by antithrombin (Olson, 1985; Björk *et al.* 1992a) lead to the formulation of a branched reaction scheme, with the serpins functioning as suicide substrates (reviewed by Gettins *et al.* 1993):

SCHEME 2

$$E + I \rightleftarrows EI \rightarrow [EI'] \xrightarrow{k_4} E\text{-}I\dagger \rightarrow E + I^*$$
$$\qquad\qquad\qquad\qquad\quad \downarrow k_3$$
$$\qquad\qquad\qquad\qquad\quad E + I^*$$

The intermediate EI' can either be cleaved at the serpin reactive centre bond as in a normal substrate reaction to produce a cleaved serpin (I^*) and regenerated enzyme, or form a stable bimolecular complex, E-I†, which reacts very slowly (hours to days) to give cleaved serpin and free enzyme. Partitioning of EI' is determined by the partition ratio r, where $r = [k_3/k_4]$, which represents the number of catalytic turnovers per inactivation cycle. When $r = 1$, the rate of formation of the stable enzyme-serpin complex is identical to the rate of serpin cleavage without concomitant proteinase inhibition. When $r = 0$, the reaction leads exclusively to the formation of a stable proteinase-serpin complex. Accordingly when r is greater than 1, substrate turnover predominates over proteinase inhibition (Fersht, 1985).

The partition ratio for the inhibition of serine proteinases by C1-inhibitor is dependent on temperature (Patston *et al.*, 1991); C1-inhibitor acts as a substrate at low temperatures and as an inhibitor above 25°C. This observation that temperature can influence product distribution of the reaction between C1-inhibitor and its target serine proteases indicates a novel mechanism for regulating the activity of this serpin (Patston *et al.*, 1991). Ligands such as the glycoaminoglycan can also potentially alter the product distribution as seen in the activation of antithrombin by heparin (Olson, 1985).

1.5 α_1-Antitrypsin

This current study is concerned mainly with human α_1-antitrypsin (α_1-AT), the archetypal member of the serpins, and the most abundant inhibitor of serine proteinases in human serum. The proteolytic activity of human blood was first recognized a century ago by Fermi & Pernossi (1894; see review of Jacobsson, 1955), and a few years later proteolytic enzymes within blood leukocytes were observed (Opie, 1905). By electrophoresis of human serum, Jacobsson (1955) identified two proteins that inhibited pancreatic trypsin, one which co-migrated with the α_1-globulins and the other with the α_2-globulins. The inhibitor migrating in the α_1-globulins band was purified in 1955 (Schultze *et al.*, 1955), and named 3.5 S-α_1-antitrypsin. Later, this inhibitor was called α_1-antitrypsin (Schultze *et al.*, 1962) and now also called α_1-proteinase inhibitor to reflect its physiological role as an inhibitor of a broad range of serine proteinases (Beatty *et al.*, 1980). Interest in the biochemical and clinical role of α_1-antitrypsin was aroused by Laurell & Eriksson (1963) who observed an association of the chronic lung disease, emphysema, with hereditary α_1-antitrypsin deficiency.

1.5.1 α_1-Antitrypsin Synthesis and Function

α_1-Antitrypsin is a 394 amino acid, 52 kDa monomeric glycoprotein with 3 complex carbohydrate sidechains linked to asparagines 46, 83 and 247 (reviewed by Carrell *et al.*, 1982). There are two major isoforms in the serum, depending on the presence of a bi- or tri-antennary configuration of the carbohydrate side chains (Vaughan *et al.*, 1982). The synthesis and export of α_1-antitrypsin follows the secretory pathway of mammalian cells (reviewed by Kornfeld & Kornfeld, 1985). The α_1-antitrypsin mRNA translation product contains a 24 residue amino-terminal signal peptide (Kurachi, *et al.*, 1981; Carlson & Stenflo, 1982) which is cleaved during translocation across the rough endoplasmic reticulum (RER) membrane. In the ER the protein acquires a block of 14 sugar residues attached to the side

chain nitrogen (N-linked) of asparagines 46, 83 and 247. Core N-linked glycosylation is followed by removal of one mannose and three glucose residues. Transfer vesicles transport protein from the ER to the Golgi Complex (GC). Traversal of the GC involves further trimming of sugar residues and modification of the high-mannose sugars to complex forms. Such modifications involve addition of N-acetylglucosamine, fructose, galactose and sialic acid. Modifications are dependent on the protein conformation presented to the processing enzyme. Fully mature protein is exported from the cell via vesicles.

The predominant role of α_1-antitrypsin is to inhibit neutrophil elastase (EC 3.4.21.37), a single chain 29 kDa glycosylated protease secreted by neutrophils during the host defence mechanism (Heimburger *et al.*, 1971), although α_1-antitrypsin also has the capacity to inhibit a broad spectrum of serine proteases (trypsin and chymotrypsin) to a lesser degree. Human α_1-antitrypsin interacts most rapidly with human neutrophil elastase and in decreasing order with chymotrypsin, pancreatic elastase and the trypsins including plasmin and thrombin (Beatty *et al.*, 1980). The association rate constant (K_{ass}) of α_1-antitrypsin with human neutrophil elastase (-6.5×10^7 $M^{-1}s^{-1}$) is a thousand fold higher than the interaction with human anionic trypsin (-6.3×10^4 $M^{-1}s^{-1}$). α_1-Antitrypsin diffuses into the alveoli of lungs, a site of destruction of neutrophils from the circulation and the first line of defence against entry of foreign particles, and protects digestion of elastin fibres by inhibition of neutrophil elastase. During the host defence reaction, inflammatory cells are recruited to the lungs to combat microbes and other irritants such as smoke or noxious inhalants in the lungs. In α_1-antitrypsin deficiency reduced levels of α_1-antitrypsin in the serum, and hence the lung, do not provide adequate protection against elastase, resulting in progressive lung damage. α_1-Antitrypsin represents > 90% of the anti-neutrophil elastase activity in pulmonary alveolar lavage fluid (Gadek *et al.*, 1981) and so plays a pivotal role in maintaining lung elasticity and preventing excessive proteolytic activity. In normal individuals (PiM) serum

levels range from 150 to 350 mg/dl (Gadek *et al.*, 1981). However, individuals with serum levels below 35% of normal values (70 to 80 mg/dl) may develop chronic destructive lung disease in the form of emphysema - or liver disease if individuals are secretion defective. The plasma half-life of α_1-antitrypsin is 4-5 days, with a daily production rate of about 34 mg/kg body weight (Laurell *et al.*, 1977; Perlmutter & Pierce, 1989). α_1-Antitrypsin diffuses into most organs and is present in most body fluids. Lavage fluid from the lower respiratory tract contains α_1-antitrypsin at a level similar to that in serum (Gadek *et al.*, 1981).

1.5.2 Gene Structure

Human α_1-antitrypsin is encoded by two independent alleles in an autosomal codominant fashion. The gene is ~12.2 kb in length and is located on human chromosome 14 at position q31-32.3 (Lai *et al.*, 1983; Schroeder *et al.*, 1985; Rabin *et al.*, 1986). Mapping to the 14q32.1 locus was achieved by using human-rodent hybrid cell lines (Cox *et al.*, 1987). Initial studies on hepatocyte derived DNA showed the gene to consist of five exons and four introns (Leicht *et al.*, 1982). Subsequent studies on both hepatocytes and macrophages organized the gene into seven exons (I_A, I_B, I_C, II, III, IV and V) and six introns (Figure 1.3) (Long *et al.*, 1984; Perlino *et al.*, 1987). Perlino *et al.* (1987) demonstrated the existence of two mutually exclusive transcription initiation sites. In the hepatocyte transcription begins in the middle of exon I_C (Long *et al.*, 1984) to produce a 1.4 kb RNA with a 49 base 5′ untranslated region from exon II). Most of exon II and all the remaining exons encode the protein sequence of α_1-antitrypsin. Macrophage transcription begins nearly 2000 bp upstream of the hepatocyte promoter and the transcript includes exons I_A, I_B, and all of exon I_C (Perlino *et al.*, 1987). Two distinct mRNA species of 1.4 and 1.6 kb in length are produced in macrophages by alternative postranscriptional splicing involving the excision of exon I_B. The first two exons both contain non α_1-antitrypsin coding sequences and have initiation and termination codes (Perlino *et al.*,

1987) which may be implicated in the regulation of α_1-antitrypsin expression. A few other eukaryotic genes also contain such sequences, termed multiple short upstream open-reading frames. These sequences have been implicated in the transcriptional control of the yeast regulatory protein GCN4 (Mueller & Hinnesbusch, 1984) and a cytomegalovirus ß-gene (Geballe & Mocarski, 1988) and may well be involved in expression of α_1-antitrypsin in macrophages. Currently, no α_1-antitrypsin deficiency phenotype resulting from hindered transcription has been characterized.

Recently, four serpin genes (α_1-antitrypsin, α_1-antichymotrypsin, corticosteroid-binding globulin, and protein C-inhibitor) with similar primary sequence and gene organization were shown to be clustered within 280 kb on the distal region of the long arm of chromosome 14 (14q32) suggesting evolution from a common ancestor (Billingsley *et al.*, 1993). Recombination events occur five times more frequently in this region than in other regions of similar size (Nakamura *et al.*, 1989). Evolution is considered to be by intron-exon shuffling (Carrell & Travis, 1985) because the positions of introns are not conserved among the serpins.

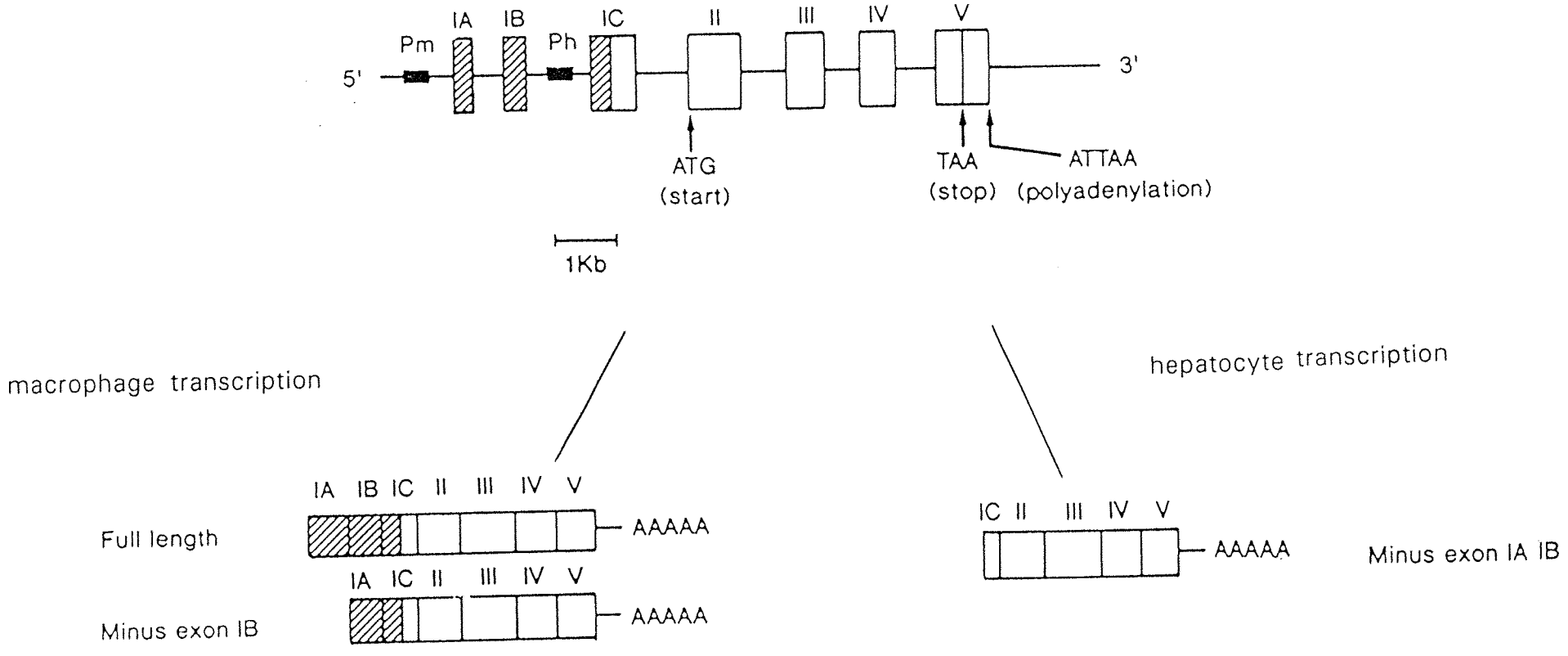


Figure 1.3 Schematic diagram of the human α_1-antitrypsin gene and tissue specific transcription. Boxes represent exons, and lines represent introns. Pm and Ph denote macrophage and hepatocyte specific promoters. 1A, 1B and 1C are the non-coding exons and II-V the coding exons. Shaded areas are transcribed only in macrophages. Alternative splicing in the macrophage can result in excision of exon 1B from the 5′ non-coding region. (Modified from Kalsheker & Morgan, 1990).

1.5.3 Gene Expression and Regulation

The major site of α_1-antitrypsin gene expression is the liver parenchymal cells (hepatocytes) as shown explicitly by the dependence of plasma α_1-antitrypsin on the donor phenotype in patients who have undergone liver transplantation (Hood *et al.*, 1980). α_1-Antitrypsin synthesis has also been detected in mononuclear phagocytes and neutrophils (Perlmutter *et al.*, 1985; Mornex *et al.*, 1986), and α_1-antitrypsin transcripts have also been demonstrated in a variety of human tissues such as gastrointestinal tract, pancreas, kidney, skin, and neural tissue using Northern blotting and immunocytochemical techniques (Ray *et al.*, 1977; Kittas *et al.*, 1982; Carlson *et al.* 1988; Koopman *et al.*, 1989; Molmenti *et al.*, 1993). Moreover, these sites of expression correlate with various pathological disorders such as gastric ulcer, pancreatitis, glomerulonephritis, arthritis and cerebral haemorrhage, observed in some patients with α_1-antitrypsin deficiency. Decreases in the local production or local inactivation of α_1-antitrypsin in different organs may therefore be pathologically related with these disorders.

α_1-Antitrypsin plays a principal role in the acute phase response, serum concentration increasing 3- to 4-fold during acute inflammation, trauma, tissue injury and pregnancy (Brantly *et al.*, 1988, Crystal *et al.*, 1989). The major source of this additional α_1-antitrypsin is the acceleration of α_1-antitrypsin synthesis in hepatocytes (Hood *et al.*, 1980). However in contrast to other acute phase reactants, α_1-antitrypsin synthesis by hepatoma cells (HepG2 and Hep3B) is not modulated by the acute phase mediators interleukin-1 or tumour necrosis factor (Perlmutter *et al.*, 1986a, 1986b). The major regulator of the acute phase response in human hepatocytes is the monokine, interferon ß2/interleukin 6 (IL-6) (Castell *et al.*, 1989). Indeed, IL-6 was shown to increase the levels of α_1-antitrypsin transcription and synthesis by $\approx$2.5- to 3.5-fold in HepG2 and Hep3B cells (Perlmutter *et al.*, 1989).

Both human peripheral monocytes and alveolar macrophages increase their levels of α_1-antitrypsin mRNA in response to IL-6 (Perlmutter *et al.*, 1989) and tumour necrosis factor (Perlmutter *et al.*, 1986). Furthermore, products generated during the inflammatory episode also influence α_1-antitrypsin expression in monocytes and macrophages. In particular, the prototype macrophage inflammatory activator bacterial lipopolysaccharide (LPS) mediates a 5- to 10-fold increase in synthesis of α_1-antitrypsin by both cell types, by increasing the translational efficiency of α_1-antitrypsin mRNA rather than increasing the levels of α_1-antitrypsin mRNA (1.5- to 2.5-fold increase in mRNA transcripts) (Barbey-Morel *et al.*, 1987; Perlmutter & Punsal, 1988). During an inflammatory response activated macrophages and newly recruited macrophages accumulate at the site of injury, releasing reactive oxygen-free radicals (to kill any bacteria) and secrete proteinases including elastase to cause tissue liquefaction (Carrell & Travis, 1985). The oxidants released by stimulated neutrophils oxidize the reactive centre methionine to methionine sulphoxide, with consequent loss of α_1-antitrypsin anti-elastase activity (Johnson & Travis, 1979; Matheson *et al.*, 1979). The oxygen free-radicals are short-lived and have a short radius of activity and so the area of tissue degradation is restricted to the inflammatory locus.

α_1-Antitrypsin gene expression in hepatocytes is directed by several nuclear DNA binding proteins (*trans*-factors) interacting with 5′ flanking control signals in the DNA (*cis*-elements). Tissue-specific expression of α_1-antitrypsin in human hepatoma HepG2 cells is predominantly directed by regulatory elements within a 137 nucleotide region upstream of the hepatocyte transcriptional initiation site within exon I_C (DeSimone *et al.*, 1987; Li *et al.*, 1988; Monaci *et al.*, 1988; Frain *et al.*, 1989). The *cis*-acting elements within this region are homologous to the upstream elements of other genes expressed in hepatocytes including haptoglobin, albumin, metallothionein and fibrinogen. Two sequences similar to the proximal portion of the IL-6 response element are located -200 nucleotides upstream of the start site for hepatocyte transcription (Poli & Cortese, 1989). Another

such sequence bearing even greater homology with the IL-6 consensus sequence is located -200 nucleotides upstream of the macrophage transcription initiation site (Perlino *et al.*, 1987).

A number of *trans*-acting factors involved in constitutive expression of α_1-antitrypsin by binding to the upstream *cis*-regulatory elements. One of these protein factor LF-B1, also called hepatocyte nuclear factor-1 (HNF-1), is a 90 kD glycoprotein that is structurally similar to *Drosophila* homeobox-encoding genes and which binds to an element at nucleotides -84 to -70 of the α_1-antitrypsin gene (Frain *et al.*, 1989). The consensus binding site for this factor, GTTAATNATTAAC, located within this domain is also found in the promoter regions of a number of hepatocyte-specific transcription genes, including α-fetoprotein, albumin, transthyretin, pyruvate kinase, fibrinogen and the pre-S1 gene of the hepatitis B virus (Courtois *et al.*, 1988). A second *trans*-acting factor, LF-A1, binds to residues -125 to -100 of the α_1-antitrypsin gene (Monaci *et al.*, 1988). Recently a 68 kD nuclear protein, named HNF-2, has been isolated (Rangan & Das, 1990). Both LF-A1 and HNF-2 are probably the same because they share a common binding site. These proteins presumably act as hepatocyte specific transcriptional activators on account of much limited levels of the factors in nuclear extracts from spleen, brain or HeLa cells. As yet, little is known of the *cis*-acting elements and *trans*-acting factors which direct α_1-antitrypsin expression in extrahepatocytic cell types. A 3′ flanking region of α_1-antitrypsin associated with a nuclear binding factor has been identified recently (Morgan *et al.*, 1993). Individuals who carry a mutation at this site have normal plasma concentrations but in response to inflammation they show a reduction in plasma α_1-antitrypsin levels and a loss of specific binding suggesting other factors also play a role in nuclear factor binding (Morgan *et al.*, 1993).

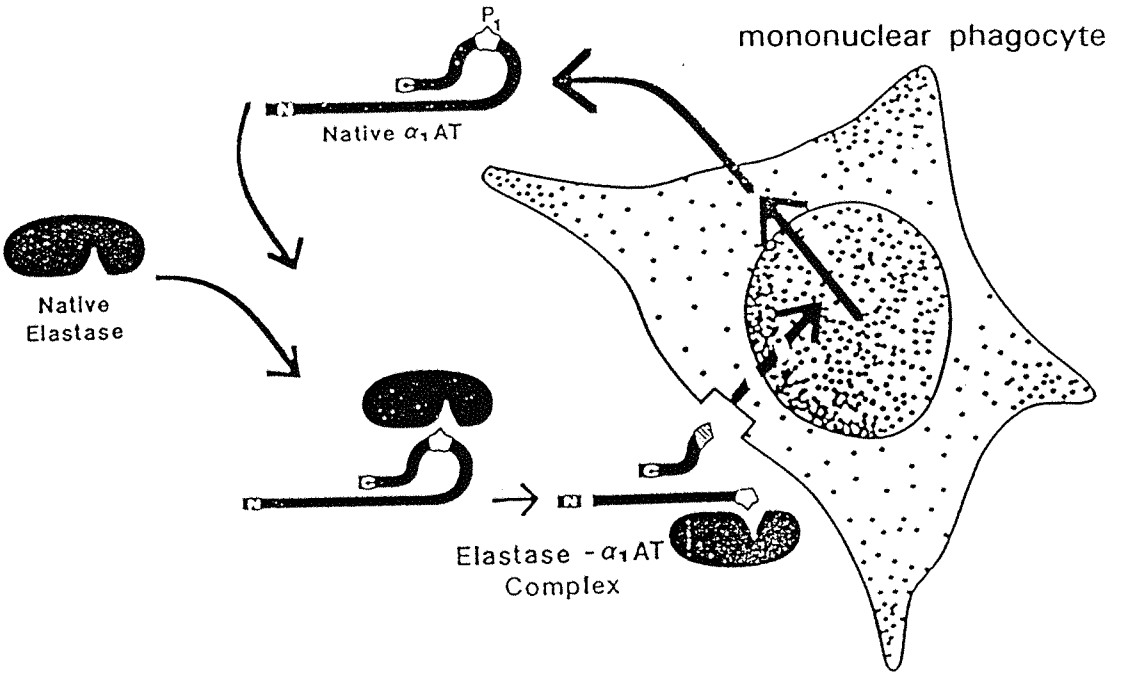


Figure 1.4 Regulation of α_1-antitrypsin synthesis by the serpin-enzyme complex (SEC) receptor. Activation of the SEC receptor on binding of the carboxyl-terminal domain of α_1-antitrypsin in complexes with elastase increases synthesis of α_1-antitrypsin by mononuclear phagocytes. (From Perlmutter & Pierce, 1989)

A novel substrate dependent feedback mechanism for the regulation of α_1-antitrypsin expression in human monocytes by the target enzyme neutrophil elastase has been proposed by Perlmutter *et al.* (1988). Nanomolar concentrations of elastase mediates dose- and time-dependent increases in steady-state levels of α_1-antitrypsin mRNA and the rates of synthesis of α_1-antitrypsin by human monocytes and alveolar macrophages. The mechanism controls the expression of α_1-antitrypsin on the basis of the ratio of elastase to 'anti-elastase' in the local microenvironment. The response is stimulated by the formation of α_1-antitrypsin-elastase complexes (Perlmutter *et al.*, 1988; Perlmutter & Punsal, 1988) and also by synthetic peptides corresponding to a carboxyl-terminal domain α_1-antitrypsin (amino acids 359-374) (Perlmutter *et al.*, 1990a). These peptides bind specifically and saturably to a single class of cell surface receptors present on monocytes and macrophages, known as serpin-enzyme complex (SEC) receptor (Perlmutter *et al.*, 1990a; Joslin *et al.*, 1991) (Figure 1.4). The SEC receptor is so named because it recognize complexes of serpins with proteinases independent of the proteinase in the complex. Two separate pathways for the removal of serpin-proteinase complexes from plasma have been identified. The serpin receptor 1 (SR1) pathway recognizes complexes of proteinases with α_1-antitrypsin, α_1-antichymotrypsin, antithrombin III, and heparin cofactor II (Pizzo *et al.*, 1988; Pizzo, 1989). The second pathway, SR2, recognizes complexes of proteinases with α_2-antiplasmin (Gonias *et al.*, 1982; Pizzo *et al.*, 1988). The SEC receptor mediates the endocytosis and intracellular degradation of α_1-antitrypsin-elastase complexes predominantly catabolized in the liver (Fuchs *et al.*, 1982; Pizzo, 1989) and binding to the receptor increases synthesis of α_1-antitrypsin (Perlmutter *et al.*, 1990a,b; Joslin *et al.*, 1991).

Later studies have shown that the binding of α_1-antitrypsin-elastase complexes to the SEC receptor mediate the directed migration of neutrophils to these α_1-antitrypsin-elastase complexes by chemotaxis (Joslin *et al.*, 1992). A synthetic pentapeptide based on α_1-antitrypsin residues 370 to

374 is chemotactic for human neutrophils, with maximal stimulation of about 10^{-9} M to 10^{-8} M, a potency comparable to that of the control chemotactic peptide fMLP (formyl-methionine-leucine-proline) at 10^{-8} M. The receptor is also likely to mediate the recently described chemotactic effect of α_1-antichymotrypsin-cathepsin G complexes, but not native α_1-antichymotrypsin for neutrophils (Potempa *et al.*, 1991). Previous research has already shown that antichymotrypsin-cathepsin G complexes, but not native α_1-antichymotrypsin bind to the SEC receptor (Perlmutter *et al.*, 1990).

The serpin pentapeptide sequence recognized by the SEC receptor is buried and inaccessible in crystal structures of serpins, so it has been proposed to become accessible upon complexation with the proteinase (Joslin *et al.*, 1991). Furthermore, Perlmutter and colleagues stated that cleaved serpin probably exposes this sequence as well, because cleaved α_1-antitrypsin competes with α_1-antitrypsin-elastase complexes for binding to the SEC receptor (Joslin *et al.*, 1993). Cleaved serpin and proteinase-complexed serpin do share some structural similarity, as shown by antibody preparations which recognize both these forms of antithrombin III (Björk *et al.*, 1993) and C1-inhibitor (De Agostini *et al.*, 1988) and not the native forms. However, Mast *et al.* (1991) showed that proteolytically cleaved serpins (α_1-antitrypsin, antithrombin III and α_1-antichymotrypsin) were not recognized by the serpin receptors, SR1 and SR2, suggesting that significant structural differences do exist between cleaved and complexed serpins.

1.6 Allelic Variants of α_1-Antitrypsin

Approximately 75 allelic variants (reviewed by Brantly *et al.*, 1988; Crystal *et al.*, 1989) have been identified and assigned letters corresponding to the position of migration of the α_1-antitrypsin protein in nondenaturing isoelectric focusing polyacrylamide gels (Fagerhol & Laurell, 1967; Fagerhol & Cox, 1981). This Pi, or proteinase inhibitor, classification assigns a letter

of the alphabet to each variant. The most common α_1-antitrypsin allele is designated Pi M because it migrates in the middle of the gel between the anode (pH 4) and the cathode (pH 5). Variants that migrate near to the anode are assigned the letters at the beginning of the alphabet. Restriction fragment length polymorphism (RFLP) analysis of genomic DNA (Cox *et al.*, 1985; Kueppers & Christopherson, 1978; Bamforth & Kalsheker, 1988) and direct DNA sequencing (reviewed by Nukiwa *et al.*, 1987b) have subclassified many variants by numbers or birthplace of index cases. The α_1-antitrypsin alleles (Table 1.4) can be conveniently classified on the basis of their phenotypic expression:

1) Normal alleles code for α_1-antitrypsin proteins present in normal amounts, 150 to 350 mg/dl (29-67 μM) in serum), and with normal function.

2) Null alleles in which no α_1-antitrypsin detectable in the serum can be attributed to the gene.

3) Dysfunctional alleles code for α_1-antitrypsin at normal levels but function other than as inhibitors of neutrophil elastase.

4) Deficiency alleles are associated with lower than normal α_1-antitrypsin serum levels, whereas the function may be normal or reduced.
All individuals with plasma levels below 11 μM are at risk of developing the lung disease emphysema (Gadek *et al.*, 1980; Gadek & Crystal, 1982).

Allele	Clinical disorder	Mutation	Exon site
Normal			
M1(Ala 213)	Normal	Ala^{213}GCG-ValGTG	III
M1(Val 213)		Arg^{101}CGT-HisCAT	II
M2		Glu^{376}GAA-AspGAC	V
M3		Asp^{341}GAC-AsnAAC	V
$P_{Saint\ Albans}$		Arg^{223}CGT-CysTGT	III
F			
Deficient			
Z	Emphysema plus liver disease	Glu^{342}GAG-LysAAG	V
S_{Iiyama}		Ser^{53}TCC-PheTTC	II
M_{Malton}		Phe^{52}TTC-deleted	II
$M_{Nichinan}$		Phe^{52}TTC-deleted Gly^{148}GGG-ArgAGG	II
S	Emphysema only	Glu^{264}GAA-ValGTA	III
$M_{Heerlen}$		Pro^{369}CCC-LeuCTC	V
$M_{Mineral\ springs}$		Gly^{67} GGG-GluGAG	II
$M_{Procida}$		Leu^{41} CTG-ProCCG	II
I		Arg^{39} CGC-CysTGC	II
P_{Lowell}		Asp^{256}GAT-ValGTT	III
Null			
$Null_{Granite\ Falls}$	Emphysema only	Tyr^{160}TACdeleted-5'shift-$stop^{160}$	II
$Null_{Bellingham}$		Lys^{217}AAGStopTAG	III
$Null_{Mattawa}$		Leu^{353}TTA-ins.T-PheTTT-$stop^{160}$	IV
$Null_{Procida}$		Delete 17kb (exons II-IV)	II-IV
$Null_{Hong\ Kong}$		Leu^{318}CTC-TCdel.-$stop^{334}$TAA	IV
$Null_{Bolton}$		Pro^{362}CCC-Cdel.-$stop^{373}$TAA	IV
Dysfunctional			
Pittsburgh	Bleeding disorder	Met^{358}-Ser	IV

Table 1.4 Genetic variants of α_1-antitrypsin (adapted from Crystal *et al.*, 1989). Listed are some examples of human α_1-antitrypsin variants whose sequences are known. Disease states are for the homozygous state. The relative risk of disease varies among the alleles. For details of these and other mutations see Carrell, 1986; Brantly *et al.*, 1988; Crystal *et al.*, 1989; Fabretti *et al.*, 1992.

1.6.1 Normal Variants

90-95% of all α_1-antitrypsin alleles are categorized as normal variants (Brantly *et al.*, 1988; Crystal *et al.* 1989). The four common normal alleles M1(Ala 213), M1(Val 213), M2, and M3 differ by sequential single base changes in the coding exons. M1(Ala 213) is closest in sequence to chimpanzee α_1-antitrypsin and, therefore likely to be the archetypal human allele with other alleles evolving from it (Crystal 1990).

1.6.2 Null Variants

Null variants are rather rare; the null α_1-antitrypsin alleles have a haplotypic frequency of approximately 0.1% (Laurell *et al.*, 1974). Pi null haplotypes, when inherited with either a certain deficient haplotype, such as Pi Z (see below), or with another null, put the individual at high risk for the development of emphysema (Talamo *et al.*, 1973; Garver *et al.*, 1986, Muensch *et al.*, 1986). Indeed, most null alleles were discovered in α_1-antitrypsin deficient patients associated with the Z mutation, and characterized using the Polymerase chain reaction and direct sequencing of amplified genomic DNA from the patients (Newton *et al.*, 1988; Graham *et al.*, 1989). Several alleles have been characterized at the level of their nucleotide sequence and include Pi $Null_{Bellingham}$ (Satoh *et al.*, 1988), Pi $Null_{Granite\ Falls}$ (Nukiwa *et al.*, 1987a), Pi $Null_{Hong\ Kong}$ (Sifers *et al.*, 1988). The null alleles include a variety of substitution, deletion and insertion mutations resulting in stop codons in coding exons. Thus either no α_1-antitrypsin mRNA is detectable (stop codon makes transcript unstable), or premature termination of the polypeptide occurs during mRNA translation.

The $null_{Hong\ Kong}$ (Sifers *et al.*, 1988) and $Null_{Mattawa}$ (Curiel *et al.*, 1989a) alleles code for α_1-antitrypsin (normally 394 residues) truncated near the carboxyl-terminus upto positions 333 and 375 respectively. Brodbeck & Brown (1992) constructed a series of truncated variants of α_1-antitrypsin

by oligonucleotide-directed mutagenesis, and assessed the secretory potential of the mutant proteins from transfected COS 1 cells. Truncation prior to proline at position 391 prevented movement from the endoplasmic reticulum to the Golgi apparatus, and therefore secretion. The investigators proposed that the carboxyl-terminus may serve as a signal in the intact protein for efficient secretion (Brodbeck & Brown, 1992). This carboxyl-terminal sequence is highly conserved within the serpin family, particularly Pro^{391}. Further work by Brodbeck & Brown (1994) showed that while replacing Pro^{391} with hydrophobic residues did not impair secretion significantly, other amino acids significantly restricted secretory ability. Hydrophobic residues at position 391 may therefore, impose a structural conformation on α_1-antitrypsin that maximises secretory potential (Brodbeck & Brown, 1994).

1.6.3 Dysfunctional Variant

Only one naturally occurring dysfunctional variant has been identified: α_1-AT Pittsburgh (Lewis *et al.*, 1978; Owen *et al.*, 1983) is characterized by an active site P_1Met^{358}→Arg mutation. The resulting α_1-$AT_{Pittsburgh}$ is unable to inhibit neutrophil elastase, but does inhibit a number of blood coagulation proteases, notably thrombin, kallikrein, and factor XIIa. Consequently such individuals are at risk from haemorrhage.

1.6.4 Deficiency Variants

The Pi S variant is the most common deficiency variant (Owen & Carrell, 1976a), with an allelic frequency of 2-4% in Caucasians of European descent and particularly common in South Europeans. The missense mutation results in a single amino acid substitution of G<u>A</u>A Glu^{264} to G<u>T</u>A Val. Despite serum levels of α_1-antitrypsin reduced to 50-60% of normal, S homozygotes are not at increased risk of emphysema (Carrell *et al.*, 1982). α_1-Antitrypsin mRNA transcripts of the correct length are synthesized and

at normal levels, though a fraction of the resulting protein is unstable and degraded prior to secretion (Curiel *et al.*, 1989b). Lowered serum levels of S α_1-antitrypsin may also be produced by an increased rate of clearance from the serum (Jeppsson *et al.*, 1978).

The Pi Z variant is associated with severe α_1-antitrypsin deficiency. A single nucleotide substitution (GAG to AAG) produces a substitution of a Lys for Glu at residue 342 in the α_1-antitrypsin coding sequence (Jeppsson, 1976; Owen & Carrell, 1976b; Yoshida *et al.*, 1976; Kidd *et al.*, 1983). A second mutation Val^{213} to Ala^{213}, has been identified by Nukiwa *et al.* (1986) in all Pi Z alleles studied, but this is unlikely to be involved directly in the defect because the polymorphism is also present in 20-25% of normal Pi M subjects. Some 4% of northern Europeans are heterozygous for the Z variant resulting in about 1 in 1500-2000 of the population being ZZ homozygotes (Sveger, 1976). Z α_1-antitrypsin mRNA transcripts are of the normal length and synthesized at the same rate as Pi M transcripts, but the cells secrete 10-15% of the α_1-antitrypsin secreted by Pi M cells (Mornex *et al.*, 1986).

Mixed heterozygotes of deficiency and null Pi-alleles give plasma levels determined by the independent expression of the Pi-alleles. Thus Pi SZ heterozygotes have serum levels of α_1-antitrypsin of about 35% [PiS(60/2) + PiZ(15/2)] of normal (Gadek & Crystal, 1982). Individuals at risk from emphysema have plasma concentrations of α_1-antitrypsin below about 40% of the normal concentration (Gadek *et al.*, 1980). The incidence of the clinically-important deficiency genotypes, Pi ZZ and Pi SZ, is about 1:1000 in the Northern European population (Sveger, 1976).

Restriction fragment length polymorphism analysis of the α_1-antitrypsin gene has revealed a single unique haplotype, the *Ava* II polymorphic site, in 96% of Pi ZZ individuals (Cox *et al.*, 1985), indicating a single origin for the Pi Z allele. The limitation of the Pi Z allele to Caucasians and absence in black or oriental populations (Fagerhol & Cox,

1981) indicates that the Pi Z allele has arisen recently in a northern Caucasian race, after the divergence of the races. Linkage analysis estimates that the Pi Z mutation arose almost 6,500 years ago (Cox *et al.*, 1985). Selective forces must have then increased and allowed the Pi Z allele to remain at a relatively high frequency. One mechanism that has been proposed states that the reduction in proteinase activity in Pi Z heterozygotes decreases cervical mucus viscosity and as a consequence enhances fertility by the increase in sperm migration (Kueppers, 1972).

Other rarer deficiency variants are shown in Table 1.4. These are divided into those that cause emphysema only and those that cause either emphysema or liver disease. The latter variants are of particularly significance to my work. These mutations are located either at amino acd position 342 (Z variant) or at positions 52/53 (S_{iiyama}, M_{Malton} and $M_{Nichinan}$).

1.7 α_1-Antitrypsin and Lung Disease

The first association of α_1-antitrypsin with disease was made by Laurell and Eriksson in 1960, who noted that amongst patients with early-onset pulmonary emphysema there was a high prevalence of the abnormal Z α_1-antitrypsin as measured by plasma protein electrophoresis (Laurell & Eriksson, 1963). Jeppson (1976) showed that these Pi ZZ individuals had a point mutation Glu→Lys at amino acid 342. Emphysema is a condition of the lungs characterized by abnormal permanent enlargements of the air spaces distal to the terminal bronchiole, as a result of destruction of the walls between adjacent alveoli. The type of emphysema associated with α_1-antitrypsin deficiency is panacinar (panlobular) emphysema (Figure 1.5), distinguished by damage of the alveoli and alveolar ducts and with destruction of the respiratory bronchioles as the condition progresses. In contrast, the moderate to severe degrees of emphysema observed in smokers and coal-workers exposed to carbon dust is of the centrilobular type, with the disease progressing from the respiratory bronchioles in the

upper lobes of the lung (Figure 1.5). Gas exchange of oxygen and carbon dioxide at the lung surface of patients with emphysema is impaired as a consequence of the reduction in surface area on formation of larger air sacs. The clinical manifestations of emphysema (reviewed by Crystal *et al.*, 1989; Blank & Brantly, 1994) do not appear until at least one third of the pulmonary cell lining is incapacitated. Initial symptoms are increasing shortness of breath, known as dyspnoea (Eriksson, 1964). Progressive damage to the lungs in severe cases of emphysema increases resistance to blood flow from the heart through the branches of the pulmonary artery and causes pulmonary hypertension. This strain on the heart may eventually cause right-sided heart failure. Diagnosis of emphysema is made on physical examination as a slowing of forced expiration and chest X-rays reveal areas of the lungs affected (Eriksson, 1965; reviewed by Kueppers & Black, 1974).

All individuals with α_1-antitrypsin serum levels below 80 mg/dl are at risk from developing emphysema; the highest risk in the Pi ZZ and Pi Null-Null phenotypes (see reviews Fagerhol & Cox, 1981; Carrell *et al.*, 1982; Crystal *et al.*, 1989; Blank & Brantly, 1994). Over 95% of α_1-antitrypsin deficiency related emphysema cases are Pi ZZ homozygotes. Although Long *et al.* (1984) quoted that 80-90% of Pi ZZ subjects will develop emphysema, emphysema may not develop in such a high proportion of Pi ZZ cases because the number of patients examined is disproportionate to the number of cases expected in the populations investigated (Larsson *et al.*, 1978; Tobin *et al.*, 1983). Patients with α_1-antitrypsin deficiency develop dyspnoea typically between ages 20 to 40 (Gadek & Crystal, 1982; Crystal, 1989). The onset of pulmonary symptoms is markedly accelerated by 10 to 20 years in cigarette smokers. One study by Larsson (1978) revealed that the median age at onset of dyspnoea in Pi Z smokers was 40 years, as compared to 53 years in non-smokers.

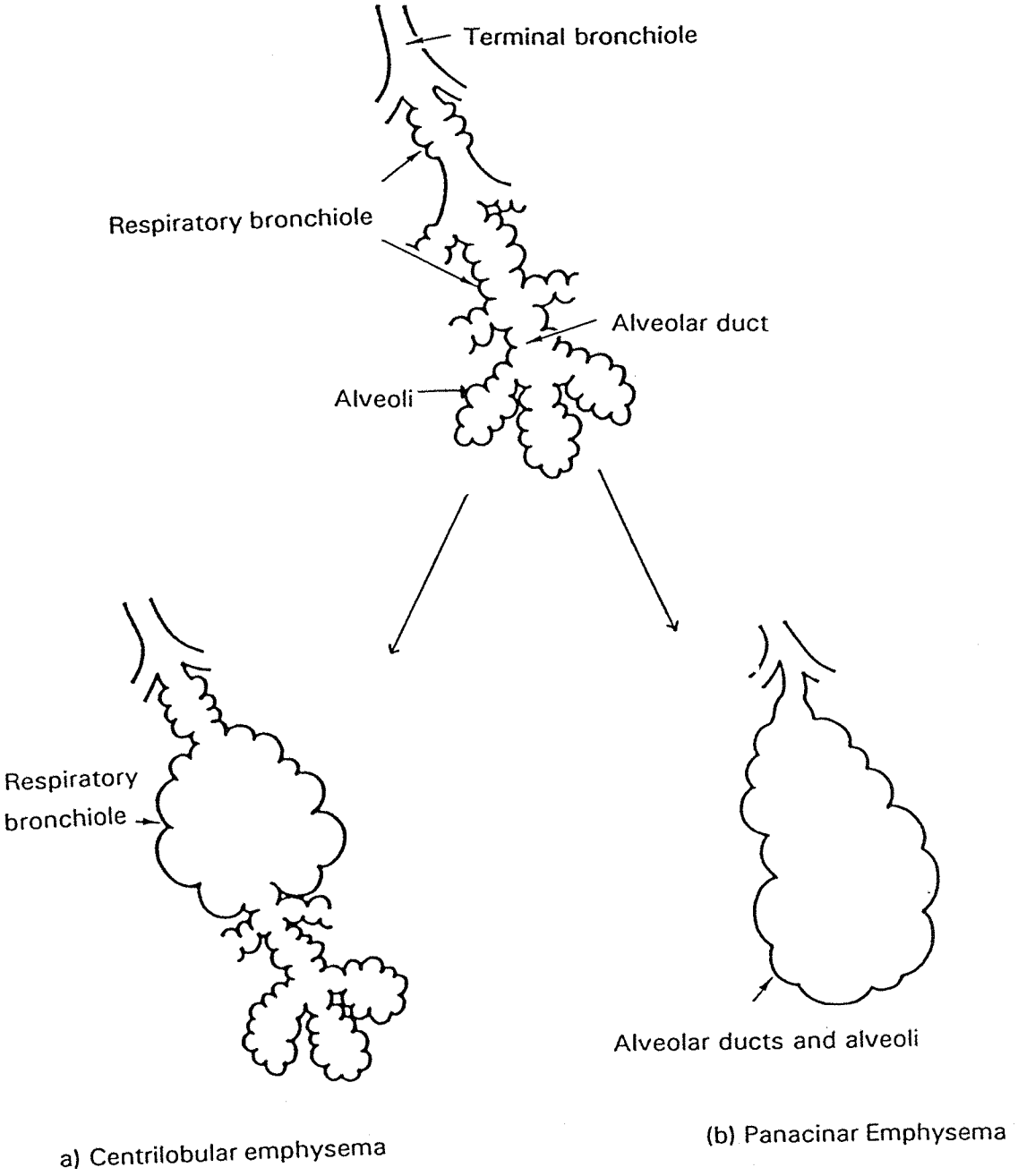


Figure 1.5 Diagrammatic model of emphysema. Emphysema occurs on destruction of the elastic walls of the lungs, and dilation of air spaces within the lungs. If the air spaces are present primarily in the respiratory bronchioles it is termed centrilobular emphysema (a). Panacinar emphysema (b) initially affects the alveolar ducts and alveoli, and then extends to the respiratory bronchioles. (modified from Cotran *et al.*, 1989)

Current evidence indicates that emphysema is due to an imbalance between proteinase (mainly elastase) and antiproteinase activity in the lungs. Any increase in elastase activity or decrease in antielastase activity will tip the balance in favour of proteolytic degradation of the elastin fibres of the lungs and advance the onset of emphysema. α_1-Antitrypsin is the predominant antiproteinase in serum and the principal cellular proteinase activity is derived from neutrophils, cells which form the first line of defense against microbes and other exogenous particles. Proteinases, including elastase and cathepsin G, are stored in granules in neutrophils and released during cell activation by chemotactic agonists, during phagocytosis, and following lysis of the cells (Weiss, 1989). Enzymes with elastase activity administered intratracheally to laboratory animals have been shown to cause emphysema-like conditions (Janoff, 1985; Senior *et al.*, 1989). This suggests, that in α_1-antitrypsin deficient individuals, any stimulus which activates neutrophils in the lungs (for example, cigarette smoking or atmospheric pollutants) may result in elastase-induced tissue destruction. Moreover, a high prevalence of emphysema is distinguished in smokers and the onset of emphysema is accelerated by 10-20 years in smokers with genetic α_1-antitrypsin deficiency (Larsson, 1978). Cigarette smoke, in particular nicotine, induces recruitment of neutrophils and macrophages to the alveoli chemotactically. Smoking also induces activation of mast cells, which release elastases in addition to neutrophil chemotactic agents (Hunninghake & Crystal, 1983; Janoff, 1985). An increased number of neutrophils are found in the alveolar lavage fluid of patients with Z α_1-antitrypsin deficiency compared to controls (Morrison *et al.*, 1987). Over and above the increase in elastase activity, oxidants in cigarette smoke and oxygen free radicals released by neutrophils inactivate α_1-antitrypsin by oxidation of the reactive centre methionine (Met^{358}) to its sulphoxide (Johnson & Travis, 1978, 1979; Janus *et al.*, 1985; Travis, 1988). The increased polarity and size of the sulphoxide ion at the active site of α_1-antitrypsin, obstructs insertion into the active site cleft of elastase, its target enzyme (Johnson & Travis, 1979; Brot & Weissbach, 1982). Indeed, the

association rate constant between neutrophil elastase and α_1-antitrypsin is reduced by more the 1,000 fold when Met^{358} is oxidized (Travis, 1988). The 'double hit concept' states that smoking leads to emphysema by both an increase in elastase activity and by an inactivation of α_1-antitrypsin antiproteinase activity (Hunninghake & Crystal, 1983). A recent study (Dziegielewska *et al.*, 1993) reported reduced levels of plasma α_1-antitrypsin in children exposed to high levels of air pollution in Poland relative to unpolluted areas of Poland and control samples within the local Southampton, UK population. Furthermore, another recent study (Hood *et al.*, 1993) has shown that nitrogen dioxide (NO_2), an air pollutant produced by burning fossil fuels and a component of cigarette smoke, significantly reduces the ability of α_1-antitrypsin to inhibit human neutrophil elastase. Thus, environmental pollutants also play a role in the development of α_1-antitrypsin deficiency, together with a genetic predisposition in some individuals.

1.8 α_1-Antitrypsin and Liver Disease

Liver disease is the major clinical manifestation of α_1-antitrypsin deficiency in children. In 1969 the prevalence of juvenile cirrhosis and neonatal cholestasis in infants with Pi ZZ phenotype was noted (Sharp *et al.*, 1969). Such patients had periodic-acid-Schiff (PAS) inclusion bodies containing α_1-antitrypsin aggregates in the RER of hepatocytes (Sharp, 1971) which strongly indicate that the protein is immature and of a high-mannose form. Among 200, 000 newborns screened in Sweden 127 were Pi ZZ phenotype and 15% of the newborn Z homozygotes developed liver disease which often leads to a fatal childhood cirrhosis (Eriksson & Larsson, 1975; Sveger, 1978). Z α_1-antitrypsin deficiency is the most common genetic disorder to cause liver disease in children (Moroz *et al.*, 1976) and the most frequent genetic disease for which patients undergo liver transplantation (Gartner *et al.*, 1984). Since only a minority of Pi ZZ neonates develop liver disease, additional factors important for the

pathogenesis of liver disease have been investigated. There is a tendency of liver disease to occur in certain families and to predominate in males (Sveger, 1976; Ghisham & Greene, 1988). Hence, additional genetic and hormonal factors have been proposed as well as a possible protective effect of breast feeding infants (Udall, *et al.*, 1985). Further work revealed an association of Z α_1-antitrypsin deficiency with chronic adult-onset liver cirrhosis (Eriksson *et al.*, 1986), and hepatocellular carcinoma (Carlson & Eriksson, 1985; Perlmutter *et al.*, 1989). Larsson (1978) noted liver cirrhosis in only 2% of 104 Pi ZZ patients aged 20-50 years, but in 19% of 142 Pi ZZ cases over the age of 50.

Lung and liver disease have rarely been observed in the same individual. It can be proposed that liver disease occurs as a consequence of the low α_1-antitrypsin serum levels rendering the liver susceptible to proteolytic attack. However, there is no evidence of liver damage in individuals homozygous for the null phenotype nor is liver disease associated with α_1-antitrypsin plasma concentrations. Liver disease is therefore not caused by deficiency of α_1-antitrypsin *per se* but by the accumulation of α_1-antitrypsin. Experiments in transgenic mice carrying the human Z α_1-antitrypsin gene have demonstrated that the mice develop acute liver necrosis and inflammation, which is related to the amount of PiZ α_1-antitrypsin accumulated in the liver (Carlson *et al.*, 1989).

1.9 Treatment of α_1-Antitrypsin Deficiency

Counselling of α_1-antitrypsin deficient subjects to avoid cigarette smoking or occupations with any form of atmospheric pollution is the most important preventative measure designed to reduce the neutrophil elastase burden in the lower respiratory tract. Purified human plasma α_1-antitrypsin is available for intravenous administration to α_1-antitrypsin deficiency individuals, although the short half-life of α_1-antitrypsin means regular administration is required (Gadek *et al.*, 1981; Hubbard & Crystal, 1988).

Possible future therapies involve delivery of pooled human or genetically engineered forms of α_1-antitrypsin directly into the lungs in aerosol formulations (Hubbard *et al.*, 1989; 1990). The efficiacy of a variety of synthetic inhibitors of elastase based upon cephalosporin have also been demonstrated (Powers & Bengali, 1986; Doherty *et al.*, 1986; Eriksson, 1991). Another approach, is the stimulation of α_1-antitrypsin synthesis by the liver by the weak androgen danazol and the oestrogen antagonist, tamoxifen (Gadek *et al.*, 1980; Wewers *et al.*, 1986; 1987). However the response in patients is too small and variable to be clinically significant. A possible complication to the increased synthesis of α_1-antitrypsin, may be an increase in the intracellular accumulation of the inhibitor protein resulting in a greater risk of liver disease. Moreover, increased α_1-antitrypsin levels will theoretically be associated with enhanced levels of α_1-antitrypsin-elastase complexes, feedback up-regulation of α_1-antitrypsin synthesis by the SEC receptor, and, in turn increased accumulation of α_1-antitrypsin. Recent advances in gene therapy research have lead to the development of viral vectors which may be used for gene delivery; such vectors could deliver the normal α_1-antitrypsin gene to deficient patients (Lemarchand *et al.*, 1992). At present, for severe emphysema lung transplantation is the only radical treatment available and liver transplantation can correct the α_1-antitrypsin secretory defect in children and adults by raising the serum α_1-antitrypsin concentration to normal levels (Hood *et al.*, 1980).

1.10 Secretory Systems

Insoluble aggregates of α_1-antitrypsin in the lumen of the hepatocyte ER was stated to be the cause of reduced α_1-antitrypsin secretion into the blood (Eriksson & Laurell, 1975; Bathurst *et al.*, 1984; Carlson *et al.*, 1989). Cox *et al.* (1986) declared that the insolubility of Z α_1-antitrypsin caused it to be retained at the site of synthesis. Sifers *et al.* (1987) showed that this Z α_1-antitrypsin could be immunoisolated from soluble cell extracts. These findings suggest that the mechanism of Z α_1-antitrypsin accumulation

involves aggregation of Z α_1-antitrypsin, culminating in the formation of insoluble inclusion bodies. α_1-Antitrypsin isolated from the hepatic inclusion bodies is incompletely glycosylated (Jeppsson *et al.*, 1975; Hercz *et al.*, 1980); the protein has immature high-mannose carbohydrate sidechains, without the sialic acid termini of secreted protein. Thus this blockage of the secretory pathway occurs at the final stage of processing in the ER, prior to entry into the Golgi complex where the high-mannose sidechains will be trimmed and modified to complex forms.

The secretory defect of the Pi Z variant has been extensively studied and chosen as a model for investigating the mechanism of intracellular retention. A variety of systems are available for expressing secretory proteins and their mutants. Important considerations in the choice of system are ease of gene manipulation, cost, and protein expression with correct post-translational modifications and biological activity.

Prokaryotic systems are less expensive but lack the post-translational modification machinery, and frequently produce intracellular aggregates of the exogenous protein in the form of insoluble inclusion bodies (Mitraki & King, 1988). Nevertheless, two groups have expressed α_1-antitrypsin in *Escherichia coli* (Bollen *et al.*, 1984, Courtney *et al.*, 1984, 1985). The protein is secreted into the periplasmic space between the outer and inner wall membrane proteins. α_1-Antitrypsin is released upon cells lysis by sonication, or homogenization, or osmotic shock methods (Bischoff *et al.*, 1992). Although α_1-antitrypsin is biologically active (Courtney *et al.*, 1984, 1985), it is unglycosylated and so there may be the possibility of antigenicity if used therapeutically. The carbohydrate moieties stabilize α_1-antitrypsin because unglycosylated α_1-antitrypsin is more susceptible to heat denaturation and has a significant reduced circulating half-life when injected into rabbits (Travis *et al.* 1985).

Yeast vectors, in particular *Saccharomyces cerevisiae*, may be used

for heterologous gene expression because it is an eukaryote and posses intracellular compartments analogous to the Golgi apparatus of higher eukaryotes (Botstein & Fink, 1988). The yeast system is a viable alternative to bacteria for efficient, high-level production of α_1-antitrypsin (Rosenberg *et al.*, 1984; Travis *et al.*, 1985; Verbanac & Heath, 1986). Even so, yeast has a tendency to hyperglycosylate secretory proteins such as α_1-antitrypsin (Moir & Dumais, 1987).

It therefore follows that higher eukaryotes must be used for efficient and authentic expression of α_1-antitrypsin. Cultured mammalian cell lines are used widely to study α_1-antitrypsin protein expression and secretion (Perlmutter *et al.*, 1985; Brantly *et al.*, 1988; McCracken *et al.*, 1989; Sifers *et al.*, 1989; Cresteil *et al.*, 1990; Ciccarelli *et al.*, 1993). The cells are transfected with α_1-antitrypsin in an eukaryotic viral expression vector; most commonly SV40 based. Transient transfection results in higher levels of expression but the protein is only expressed for 1-3 days. Stably transfected cell lines are effectively immortal. Transfection may be achieved by either DEAE-dextran-mediated gene transfer or by calcium-phosphate-mediated gene transfer; though, electroporation or retrovirus-mediated gene transfer can be employed depending on the cell type to be transfected. Human α_1-antitrypsin has also been expressed in transgenic mice to provide an animal model for the Pi Z defect (Sifers *et al.*, 1987; Carlson *et al.*, 1988, 1989). Pi Z mice developed more liver damage and inflammation than Pi M or control mice (Carlson *et al.*, 1989; Martorana *et al.*, 1993), supporting the hypothesis that Pi Z accumulation in the ER of hepatocytes causes hepatic disease.

Intracellular accumulation of human Pi Z is observed in a variety of eukaryotic cell types transfected with Pi Z cDNA constructs: *Xenopus* oocytes (Foreman *et al.*, 1984; Perlmutter *et al.*, 1985); human monocytes (Perlmutter *et al.*, 1985); COS cells (McCracken *et al.*, 1989); mouse hepatoma cells (Sifers *et al.*, 1989); Chinese hamster ovary cells (Ciccarelli

et al., 1993). These heterologous cell types provide good *in vitro* models for investigations into the molecular pathology of Z α_1-antitrypsin accumulation.

The expression system available in the Department of Physiology and Pharmacology, Southampton is the *Xenopus* oocyte system. Gurdon *et al.* (1971) first demonstrated that exogenous mRNAs were translated efficiently with the correct post-translational modifications in *Xenopus* laevis oocytes. A large variety of intracellular, secretory, and membrane-bound protein mRNAs, from viruses, plants, invertebrates, and vertebrates have been translated by *Xenopus* oocytes (Gurdon & Wickens, 1983; reviewed by Colman, 1984). *Xenopus* oocyte are widely used as a surrogate system for the expression of secretory proteins (Colman & Morser, 1979) because they are large (1-1.2 mm in diameter), easy to handle, and process natural and *in vitro* transcribed mRNAs correctly and efficiently (Krieg & Melton, 1984; reviewed by Heikkila, 1990).

Stability of the Pi Z variant in serum and the identical rate of protein translation from Pi M and Pi Z mRNA injected into *Xenopus* oocytes (Errington *et al.*, 1986; Bathurst *et al.*, 1983) suggests that the defect of α_1-antitrypsin secretion, not synthesis, is responsible for the decreased concentration in serum. The pathogenesis of the secretion defect of the Pi Z α_1-antitrypsin remains unclear. Only two other mutations, M_{Malton}, and S_{iiyama}, have an association between α_1-antitrypsin deficiency and an increased risk of liver damage (Curiel et al., 1989c; Frazier *et al.*, 1989, Seyama *et al.*, 1991). To understand the mechanism(s) of α_1-antitrypsin accumulation it is necessary to investigate the structure of normal and mutant α_1-antitrypsin molecules.

1.11 Structural Studies on the Serpins

Spatial structure of serpins (Table 1.1) have played a significant role in understanding mechanisms of serpin-proteinase interaction and the pathogenesis of serpin deficiency and disease. A number of reviews of serpin structures have been published and include: Huber & Carrell, 1989; Carrell & Evans, 1992; Gettins *et al.*, 1992, 1993; and Schulze *et al.*, 1994. The following discussion is based on the structures published to October 1994.

1.11.1 Cleaved α_1-Antitrypsin

The first serpin X-ray crystal structure determination was made by Loebermann *et al.* (1984), who resolved the crystal structure of normal (M-type) human α_1-antitrypsin cleaved at the reactive site Met^{358}-Ser^{359}, (P_1-P_1'), by chymotrypsinogen A to a resolution of 3.0 Å. In addition to cleavage at the reactive site, proteolysis of α_1-antitrypsin by chymotrpysinogen A also involves cleavage at Thr^{11}-Asp^{12}, which results in the elimination of 11 residues from the N-terminus (Loebermann *et al.*, 1982). The three-dimensional structure revealed a major conformational rearrangement takes place upon cleavage, because residues 358 and 359 which are covalently bonded in the uncleaved inhibitor were at opposite ends of the molecule, some 69 Å apart. Cleaved α_1-antitrypsin has a globular shape, folded into a highly ordered structure, with three large ß-pleated sheets (A-C), nine α-helices (hA-hI), six helical turns and three internal salt bridges (Figure 1.6 and Table 1.5). The planar A ß-Sheet is the dominant feature of the molecule, comprising of six antiparallel strands, except the short strand 1 (s1A) arranged parallel to strand 2 (s2A). The central strand of the A sheet, strand s4A is formed by the residues amino terminal (343-358) to the cleavage site at Met^{358}. All the helices are grouped together behind the lower part of the A sheet, except helix F which partially covers sheet A.

Sheet B lies perpendicular to and behind the A sheet, and has a strong right-handed twist. The lower three strands (s4B, s5B, s6B) of sheet B are hydrophobic and together with helix B form a hydrophobic core to the structure. Several hydrophobic interactions and hydrogen bonds exist between sheet A and this hydrophobic core. Strand s4B (amino acids 369-378) contains the putative recognition site (residues 370-374) for the proposed serpin-enzyme complex (SEC) receptor (Joslin *et al.*, 1991), and so the molecule must undergo a structural rearrangement upon complexation with proteinase to expose the pentapeptide sequence. The transformation is proposed by some investigators to be similar to that seen upon reactive site cleavage, because cleaved α_1-antitrypsin competes with α_1-antitrypsin-elastase complexes for binding to the SEC receptor (Joslin *et al.*, 1993).

The four stranded C ß-sheet consists of a three stranded ß-sheet formed by strands s1C, s2C, and s3C, and a two stranded ß-ribbon formed by strands s3C and s4C. Strand 1C is formed by the residues immediately following the cleavage site at Ser^{359}.

A number of crystal forms of α_1-antitrypsin have been resolved that differ in the crystal packing, chemical heterogeneity of the carbohydrate sidechains and electron density (see Table 1.1). However, the different crystal structures are very similar in structure. The atomic-resolution crystal structures of other cleaved inhibitory serpins (Table 1.1) have also been determined: α_1-antichymotrypsin (Baumann *et al.*, 1991), antithrombin III (Mourey *et al.*, 1990), equine leucocyte elastase inhibitor (Baumann *et al.*, 1992). All have a tertiary structure similar to that of α_1-antitrypsin, with the predominant feature being an antiparallel six-stranded A ß-sheet in which strand s4A contains residues amino terminal to the reactive centre cleavage site (reviewed by Huber & Carrell, 1989; Carrell & Evans, 1992).

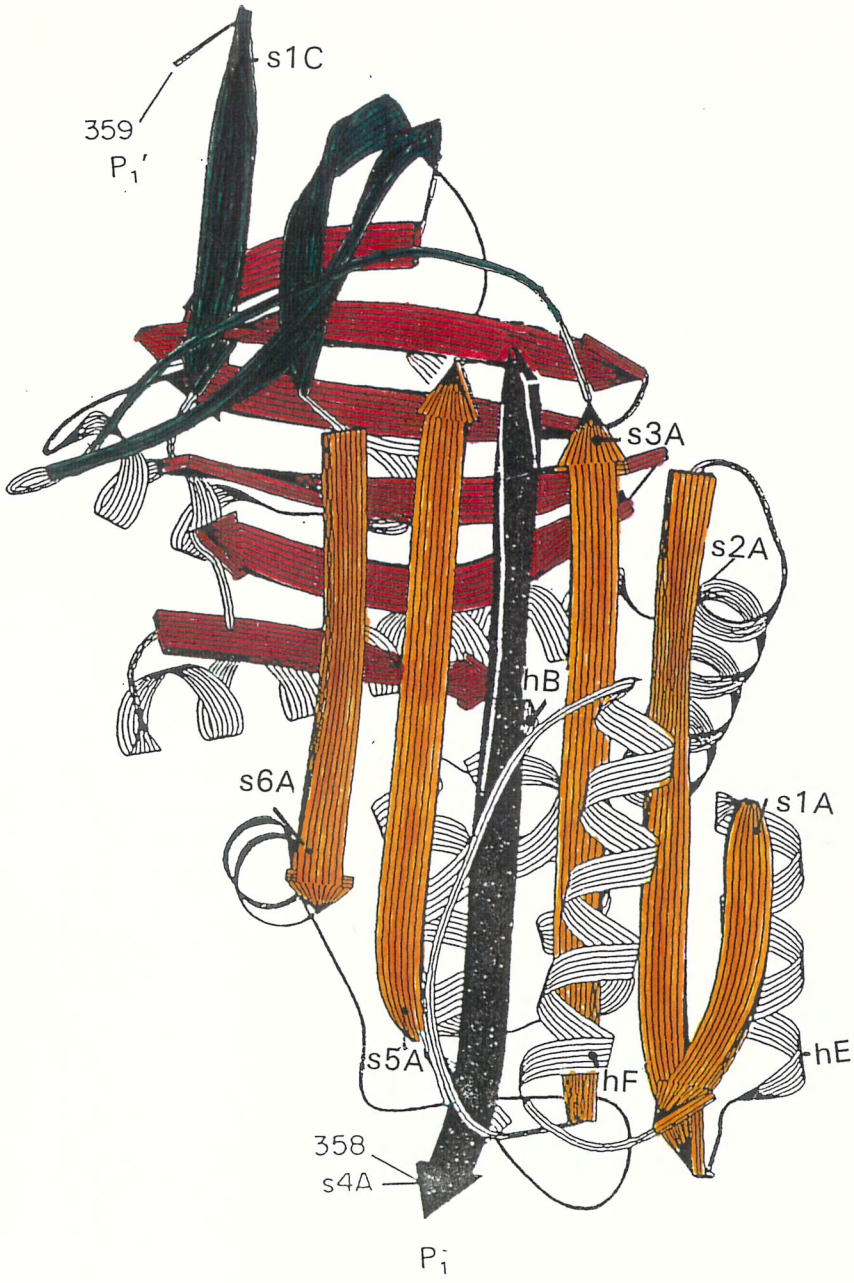


Figure 1.6 Schematic drawing of the structure of cleaved α_1-antitrypsin produced using the program, RIBBON (Priestle, 1988). Strands of ß-sheets are represented by arrows (labelled, *s*) and α-helices by helical ribbons (labelled, *h*). The region (s4A) amino terminal to the cleaved reactive centre bond (P_1-P_1') is shaded black. ß-Sheets A, B and C are coloured yellow, red and green respectively. (Adapted from Stein & Chothia, 1991)

Table 1.5 α_1-Antitrypsin: Secondary Structure Classification (from Huber & Carrell, 1989)

Helices	ß-sheet strands	Turns	Bulges
hA: 20-44	s6B: 49-53	thAs6B: 45-48	169-172
hB: 53-68	s5B: 380-389	thBhC: 68-70	171-174
hC: 69-81	s4B: 369-378	thChD: 81-88 (lh: 81)	173-176
hC1: 83-87	s3B: 247-255	thDs2A: 105-110	bs5B: 382-385
hD: 88-105	s2B: 236-245	ts2AhE: 122-127	bs5A: 329-332
hE:127-139	s1B: 228-233	thEs1A: 130-140 (lh: 139)	
hF: 149-166	s6A: 290-299	ts1AhF: 146-149	
hF1: 200-203	s5A: 326-342	thFs3A: 166-181 (lh: 166)	
hF2: 232-236	s4A: 343-356	ts3AhF1: 194-199	
hG: 259-264	s3A: 181-194	ts4Cs3C: 211-214	
hH: 268-278	s2A: 109-121	ts3Cs1B: 226-228	
hI: 299-306	s1A: 140-146	ts1Bs2B: 233-236 (lh: 236)	
hI1: 309-312	s4C: 203-212	ts2Bs3B: 244-248	
hI2: 376-380	s3C: 213-226	ts3BhG: 256-259	
hI3: 390-393	s2C: 283-289	tsHs2C: 278-283	
	s1C: 362-367	thI1s5A: 318-325	
		ts5As4A: 377-380 (lh: 380)	
		ts5Bc-ter: 389-394	

Nomenclature of the secondary structural elements is as follows: h(A-I) = helix (A-I), sX = strand X in ß-sheet (A-C), t = turn between the elements following it, b = bulge in the strand of a ß-sheet, lh = left-handed helical element.

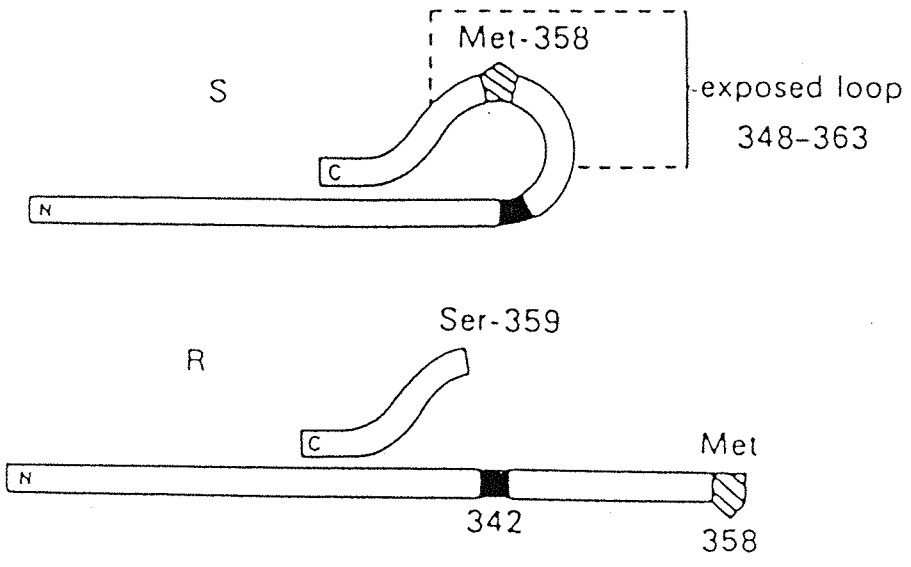


Figure 1.7 The S→R Transition. The reactive centre of α_1-antitrypsin is exposed on a loop hinged close to residue 342 (P_{17}) in a stressed (S) conformation. Cleavage by a target enzyme releases the stressed conformation to give a relaxed (R) form, with P_1 Met-358 separated from P_1' Ser-359 by 69 Å. (modified from Stein *et al.*, 1989)

1.11.2 The Stressed to Relaxed Transition

From the cleaved structure, it was clear that either strand s4A or s1C would have to adopt a different conformation in the intact structure to allow the P_1-P_1' bond to exist. From the finding that Ala^{350} is a cleavage site for a metalloprotease (Kress *et al.*, 1979) but is buried in the hydrophobic interior of cleaved α_1-antitrypsin, Loebermann *et al.* (1984) proposed that strand s4A is exposed as an external loop which is only incorporated into the A sheet after cleavage. The loop could then adopt an suitable conformation for interaction with cognate proteinase, similar to that found in the small serine proteinase inhibitor (see section 1.3.1). The investigators suggested that the sheet A is destabilized prior to cleavage in a conformation denoted as the stressed (S) state, and upon cleavage a dramatic transition to more ordered and stable, relaxed (R) state occurs (Figure 1.7). This transition has been called the S→R transition, and experimental clues confirming this increase in serpin stability upon cleavage was the dramatic increase in denaturation temperature from 56°C in native α_1-antitrypsin to 80°C in the cleaved form (Carrell & Owen, 1985; 1986). An increase in thermal stability accompanying cleavage within the exposed loop region s4A has been demonstrated in all inhibitory serpins tested, such as α_1-antichymotrypsin (Baumann *et al.*, 1991), antithrombin III (Carrell & Owen, 1986), C1-inhibitor (Pemberton *et al.*, 1989). Ovalbumin and angiotensinogen, two non-inhibitory serpins, lack the S→R change following proteolytic cleavage at sites homologous to the exposed loop of α_1-antitrypsin (Stein *et al.*, 1989). It has been proposed that cleavage of the exposed reactive centre loop is a prerequisite for the conformational change demonstrated by the S→R transition and inhibitory activity, and serpin members which during evolution have lost their role as proteinase inhibitors lack the S→R transition because it serves no useful purpose (Huber & Carrell, 1989). However the two non-inhibitors corticosteroid binding globulin (CBG) and thyroxine binding globulin (TBG) have retained the S-R transition (Pemberton *et al.*, 1988) to provide a molecular switch to modulate hormone

delivery. Cellular proteases released at sites of inflammation cleave the exposed loops of these inhibitors, resulting in decreased affinity for hormone, and therefore release of the hormone at the inflammatory site.

The S→R transition can also be characterized by increased resistance against guanidine hydrochloride induced unfolding, transverse urea gradient (TUG) gel electrophoresis, and spectroscopic techniques such as circular dichroism (CD), nuclear magnetic resonance (NMR), fluorescence emission and Fourier transform-InfraRed (FT-IR) spectroscopy. These techniques have been used to compare naturally occurring serpin conformations with experimentally induced conformations. In CD experiments, an increase in secondary structure of the cleaved serpin, is seen by an increase in negative ellipticity surrounding 220 nm (Bruch *et al.*, 1988; Schulze *et al.*, 1990). Both proton NMR (Gettins & Harten, 1988; Smith *et al.*, 1990; Perkins *et al.*, 1992) and FT-IR (Haris *et al.*, 1990; Perkins *et al.*, 1992), identify distinct spectral components which assign the greater stability of cleaved serpins to increased antiparallel ß-sheet structure. For example, a new ß-sheet band FT-IR at 1694 cm^{-1} appears for cleaved α_1-antitrypsin (Haris *et al.*, 1990) and also for cleaved α_1-antichymotrypsin and C1-inhibitor, but not for the non-inhibitory serpins ovalbumin and angiotensinogen (Perkins *et al.*, 1992). The data from both NMR and FT-IR (Hood & Gettins, 1991, Perkins *et al.*, 1992) indicate that a large number of weak hydrogen bonds in secondary structural elements become stronger in cleaved inhibitory serpins, without an alteration in overall tertiary structure. Ovalbumin and angiotensinogen, both lack the large-scale conformational change upon proteolysis as measured by the spectroscopic techniques mentioned above (Bruch *et al.*, 1988; Stein *et al.*, 1989; Gettins, 1989).

1.11.3 Cleaved Ovalbumin ('Plakalbumin')

Ovalbumin, 385 amino acids in length and 45-kDA glycoprotein, is the major protein of avian egg-white (Warner, 1954; Woo *et al.*, 1981).

Although its biological role is unknown, roles which have been proposed include the transport and storage of metal ions (Taborsky, 1974; Goux & Venkatasoubramanian, 1986) or as an amino acid store for the developing embryo (Saito & Martin, 1966). Unlike typical secretory proteins, ovalbumin lacks a classical N-terminal leader signal peptide sequence (Palmiter *et al.*, 1978; Lingappa *et al.*, 1979) even though it is secreted via passage through the endoplasmic reticulum. A hydrophobic sequence comprising of residues 50 to 68 has been proposed to act as an internal signal sequence involved in transmembrane location (Robinson *et al.*, 1986). Despite significant sequence homology (30%) of ovalbumin with α_1-antitrypsin and other inhibitory serpins (Hunt & Dayoff, 1980), ovalbumin lacks proteinase inhibitory activity (Long & Williamson, 1980; Ødum, 1987). Furthermore, although the Ala residue at the putative reactive centre suggests specificity for elastase, ovalbumin acts as a substrate rather than as an inhibitor of this enzyme (Wright, 1984). The plant protease subtilisin cleaves ovalbumin at residues P_6 Glu, P_2 Asp, as well as at the putative reactive centre P_1 Ala (Linderstrøm-Lang, 1952; Satake *et al.*, 1965) to form plakalbumin, a proteolytically nicked form of ovalbumin. From the plaque-like crystals (Miller *et al.*, 1983), hence the name, the structure of plakalbumin was determined by Wright *et al.* (1990) at a resolution of 2.8 Å (Figure 1.8). The structure closely resembles that of cleaved α_1-antitrypsin, but the location of residues P_{14}-P_1 differ in the non-inhibitor ovalbumin compared to the other inhibitory serpins. In cleaved inhibitory serpins these residues constitute the central antiparallel strand of ß-sheet A (s4A), whereas unexpectedly ovalbumin lacks the extra central strand because residues P_{14}-P_7 (P_6-P_1, or 353-358 in α_1-antitrypsin nomenclature lost due to secondary cleavage at P_7-P_6) are in a random coil conformation, exposed to solvent. Alignment of the conserved hydrophobic core regions of α_1-antitrypsin and plakalbumin revealed that the conformational transition of α_1-antitrypsin involves translation of strands s1A, s2A and s3A relative to the rest of the molecule (Wright *et al.*, 1990) presumably to allow the reactive loop to enter the A sheet. Helix F is also translated in parallel with strands s1A-s3A.

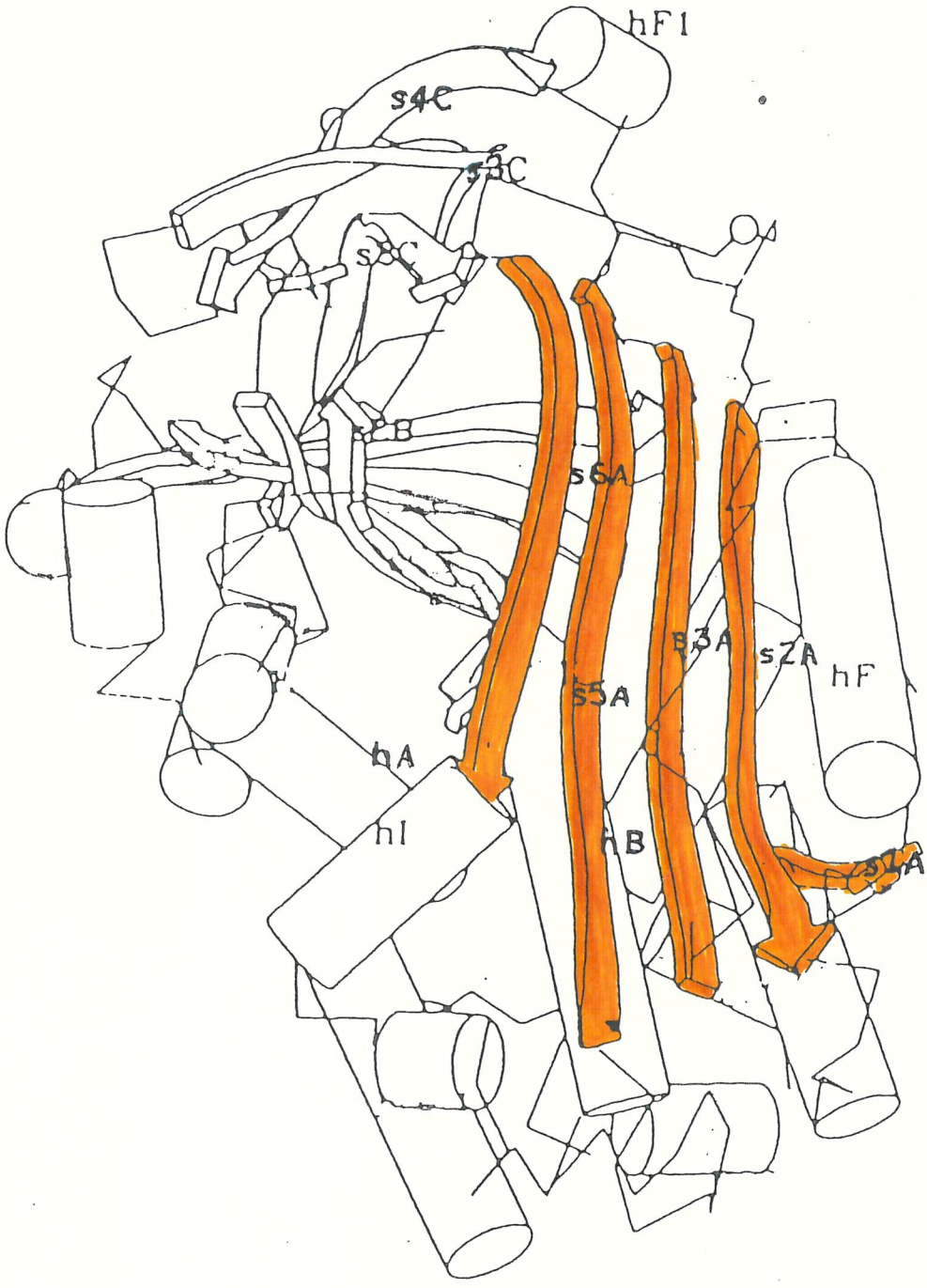


Figure 1.8 Spectroscopic drawing of the structure of plakalbumin. Strands of ß-sheets are represented by arrows (labelled, *s*) and *α*-helices by cylinders (labelled, *h*). The A sheet is coloured yellow. (From Wright *et al.*, 1990)

1.11.4 Native Ovalbumin

Until very recently (Wei *et al.*, 1994) ovalbumin was the only serpin for which a crystal structure of the native form has been published (Stein *et al.*, 1991). The 1.95 Å structure of intact ovalbumin (Figure 1.9) differs by very little from that of cleaved ovalbumin (Figure 1.8), except that the reactive centre loop residues P_9-P_1' form a 2.5 turn exposed α-helix (helix R) terminating at P_1-P_1' and joined back to the main structure at P_5'. The exposed α-helix protruding from the body of the molecule is held by two molecular stalks, each of about four residues (Stein *et al.*, 1991). The N-terminal stalk (P_{15} to P_{10}) is joined to strand s5A and the C-terminal stalk (P_2' to P_5') to strand s1C. Four molecules were present in the unit cell of the ovalbumin crystals analysed by Stein *et al.*, and in the three molecules which exhibited electron density for the reactive site loop the distances from helix R to the protein core varied by 1.7 to 3.3 Å. This was suggested to indicate high mobility for the reactive centre loop with respect to the body of the molecule.

Crystal structures of complexes formed between 'standard mechanism' serine proteinase inhibitors and their cognate proteases reveal at least eight residues of the reactive centre loop of the inhibitor are in an extended conformation (Bode *et al.*, 1986) similar to the uncomplexed inhibitor. A helical reactive centre, as seen in native ovalbumin, cannot bind to a protease active site without undergoing a major conformational change of the reactive centre loop. Even so, ovalbumin possesses cleavage sites for pancreatic elastase (Wright, 1984) and subtilisin (Linderstrom-Lang, 1952) which lie within helix R, suggesting that a mechanism exists for unfolding of helix R to form an extended active centre in some environments. However, proton NMR studies by Hood and Gettins (1991) on α_1-antitrypsin and ovalbumin showed that the extended α-helical loop of these serpins is not mobile. These observations suggest that although a number of conformations are available to native serpins, as shown by X-ray analysis,

thermodynamic interconversions between these conformations is not rapid. Residues remote from the loop may also contribute to the thermodynamics of stable complex formation.

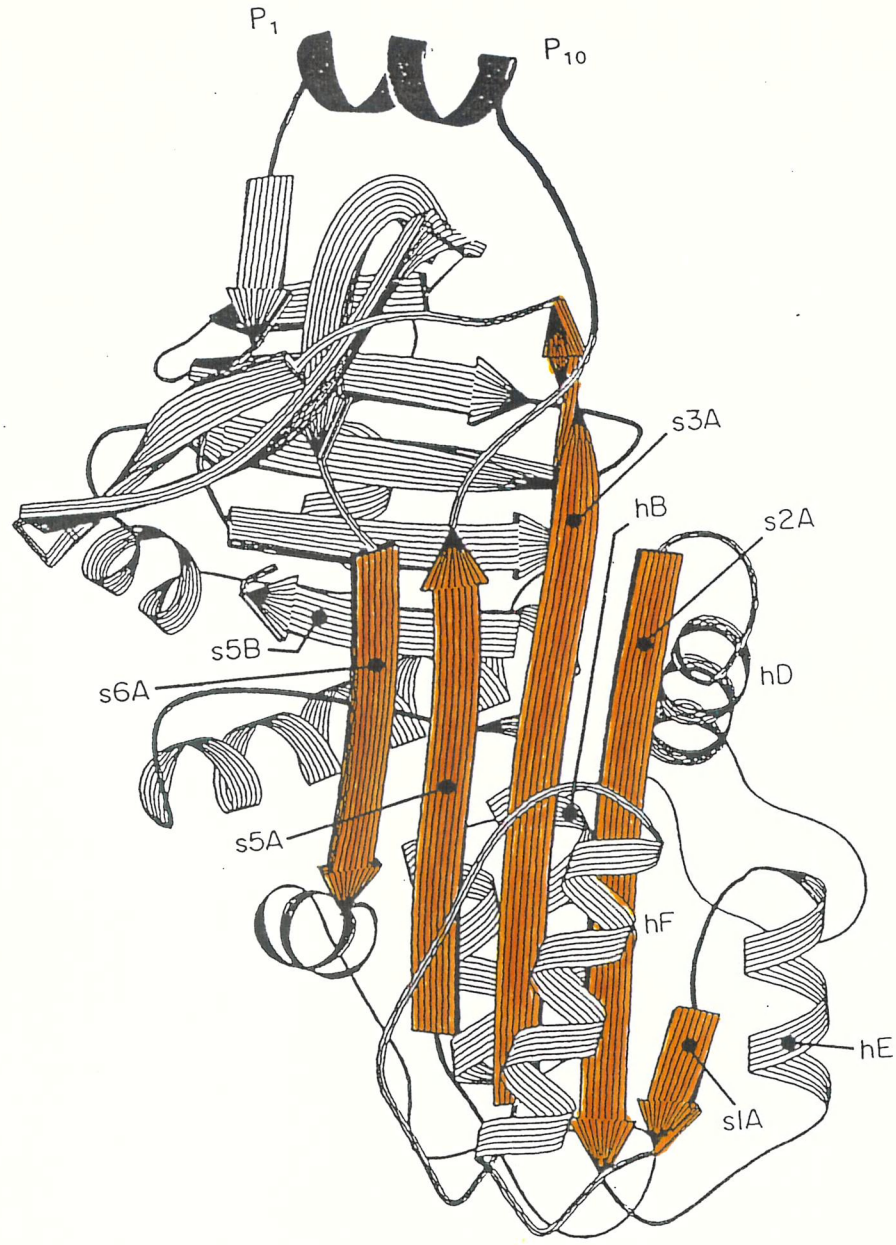


Figure 1.9 Schematic drawing of the structure of uncleaved ovalbumin produced using the program, RIBBON (Priestle, 1988). Strands of ß-sheets are represented by arrows (labelled, *s*) and *α*-helices by helical ribbons (labelled, *h*). The region homologous to the sequence inserted into the A-sheet (coloured yellow) of cleaved α_1-antitrypsin is shaded black. (Adapted from Stein & Chothia, 1991)

Figure 1.10 Reactive centre loop sequence alignment in the serpins

	Loop Hinge Residues										Exposed loop Residues												
P residue	P17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1′	2′	3′	4′	5′
amino acid	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363
Inhibitors																							
α_1-Antitrypsin	Glu	Lys	Gly	Thr	Glu	Ala	Ala	Gly	Ala	Met	Phe	Leu	Glu	Ala	Ile	Pro	**Met**	**Ser**	Ile	Pro	Pro	Glu
Antithrombin	Glu	Glu	Gly	Ser	Glu	Ala	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	**Arg**	**Ser**	Leu	Asn	Pro	Asn
Antichymotrypsin	Glu	Glu	Gly	Thr	Glu	Ala	Ser	Ala	Ala	Thr	Ala	Val	Lys	Ile	Leu	Thr	**Leu**	**Ser**	Ala	Leu	Val	Glu
Placental PA1-2	Glu	Glu	Gly	Thr	Glu	Ala	Ala	Ala	Gly	Thr	Gly	Gly	Val	Met	Thr	Gly	**Arg**	**Thr**	Gly	His	Gly	Gly
Non-inhibitors																							
Ovalbumin	Glu	Ala	Gly	Arg	Glu	Val	Val	Gly	Ser	Ala	Glu	Ala	Gly	Val	Asp	Ala	**Ala**	**Ser**	Val	Ser	Glu	Glu
Angiotensinogen	Ala	Asp	Glu	Arg	Glu	Pro	Thr	Glu	Ser	Thr	Gln	Gln	Leu	Asn	Lys	Pro	**Glu**	**Val**	Leu	Glu	Val	Thr
Corticosteroid BG	Glu	Glu	Gly	Val	Asp	Thr	Ala	Gly	Ser	Thr	Gly	Val	Thr	Leu	Asn	Leu	**Thr**	**Ser**	Lys	Pro	Ile	Ile
Thyroxine BG	Glu	Lys	Gly	Thr	Glu	Ala	Ala	Ala	Val	Pro	Glu	Val	Glu	Leu	Ser	Asp	**Gln**	**Pro**	Glu	Asn	Thr	Phe

Sequences are aligned to the α_1-antitrypsin sequence (amino acid residue number refers to that of α_1-antitrypsin). P_1-P_1' denotes the reactive-centre bond, amino acids in bold. Alignments are taken from Huber & Carrell, 1989.

1.11.5 Loop-Sheet Insertion and Inhibitory Activity

Two questions are raised by the differences in α_1-antitrypsin and ovalbumin structure, S→R transition, and function are: (1) Why does the loop of cleaved ovalbumin fail to insert into the A sheet? and (2) Is loop insertion into the A sheet a requirement for inhibitory function?

It is probable that the reactive centre loops of inhibitory serpins are non-helical in order to present an ideal substrate conformation in a similar fashion to the standard mechanism inhibitors. In the model of cleaved ovalbumin it appears that the base of the loop at the N-terminal peptide stalk is about to twist and re-enter the A ß-sheet (Figure 1.8). Modelling studies predict that the inability of the base of the reactive centre loop to insert into the A ß-sheet, and hence undergo the S→R shift, may be caused by the presence of large bulky residues (Arg/Val) at the P_{10}-P_{14} hinge region (the N-terminal peptide stalk of helix R) in ovalbumin and angiotensinogen (Wright *et al.*, 1990; Schulze *et al.*, 1990), in contrast to the conserved rows of small hydrophobic residues (Ala and Gly) in inhibitory serpins (Figure 1.10). This conservation of small hydrophobic amino acids in the hinge region, particularly at positions P_{10}, $P_{11/12}$ and P_{14} at the base of the loop, of all inhibitory serpins implies a common structure/function role for this region among the serpin family (Carrell *et al.*, 1991). These residues are orientated with their side chains facing the hydrophobic interior of the molecule (Loebermann *et al.*, 1984) and as a consequence, there is a constraint on their size and polarity if loop insertion is to occur. The absence of inhibitory activity and the S→R transition in ovalbumin and angiotensinogen can be explained by the appearance of larger and/or more polar residues in these critical positions. Schulze and colleagues (1991) constructed a recombinant α_1-antitrypsin with $P_{14}Thr^{345}$→Arg, as found in ovalbumin, that converted a reactive centre mutant of α_1-antitrypsin from an inhibitor to a substrate and also failed to undergo the S→R transition. Several natural mutants of the serpins antithrombin III and C1-inhibitor with point mutations in the hinge

region, primarily at P_{12} and P_{10}, have been identified and in most cases these are proteinase substrates not inhibitors. The P_{10}Ala→Pro mutant of antithrombin does not undergo the S→R transition and gives a complete loss of inhibitory activity (Perry *et al.*, 1989; Carrell *et al.*, 1991; Caso *et al.*, 1991). A complete loss of inhibitory activity is observed in P_{12}Ala→Glu mutant of C1-inhibitor (Skriver, 1991). A partial decrease in inhibition occurs in antithrombin Cambridge II, P_{12}Ala→Ser (Perry, 1991). Loss of inhibitory activity is attributed to the substitution of small amino acids at these sites by bulkier, and/or more polar residues (Skriver, 1991). A contradictory case has been made by Hood *et al.* (1994), who constructed a P_{14}Thr→Arg α_1-antitrypsin which retained the ability to complex with several cognate proteinases and underwent the S→R transition. A P_{12}Ala→Thr mutation converts antithrombin III from an inhibitor of α-thrombin into a substrate and prevented the S→R transiton (Ireland *et al.*, 1991). In contrast, the P_{12}Ala→Thr mutation in α_1-antitrypsin does not prevent the S→R transition or modify the inhibitory properties of the protein (Hopkins *et al.*, 1993). Thus, even though the hinge regions are well conserved between inhibitory serpins, individual serpins differ in their response to mutations in this region. Mutations in the hinge region emphasises the role of this region in controlling the partitioning of the serpins between the inhibitory complex form and the modified cleaved form (Hopkins *et al.*, 1993).

Annealing experiments involving complexation of native α_1-antitrypsin with synthetic peptides homologous to the reactive loop sequence have also demonstrated the extent of loop incorporation (Schulze *et al.*, 1992). Peptides corresponding to the P_1 to P_{14} sequence, mimicking strand s4A, could insert into the A sheet, between strands s3A and s5A, resulting in a loss of inhibitory activity of the serpin together with an increase in thermal stability, CD spectra and denaturation stability similar to that of the cleaved protein. CD spectra of the peptide-α_1-antitrypsin is consistent with the formation of a six-stranded ß-sheet (Schulze *et al.*, 1990). Analogous studies have been performed with antithrombin III (Carrell *et al.*, 1991; Bjork

et al., 1992a; 1992b) that show a similar increase in thermal stability and CD spectra changes in the complexed serpin. The extent of partial loop insertion required for inhibitory activity was investigated with peptides progressively shortened at the N-terminal end and complexed with native α_1-antitrypsin (Schulze *et al.*, 1990; Schulze, *et al.*, 1992). Peptide P_1 to P_{11} and shorter peptides did not abolish inhibitory activity suggesting that strand s4A was still able to insert into the A sheet up to residue P_{12}/P_{10} to form an ideal inhibitor loop conformation. Both the synthetic peptide insertion studies and P_{12}/P_{10} mutant results lend strong support to a structure in which residues P_{14} to P_{10} are inserted into the A ß-sheet.

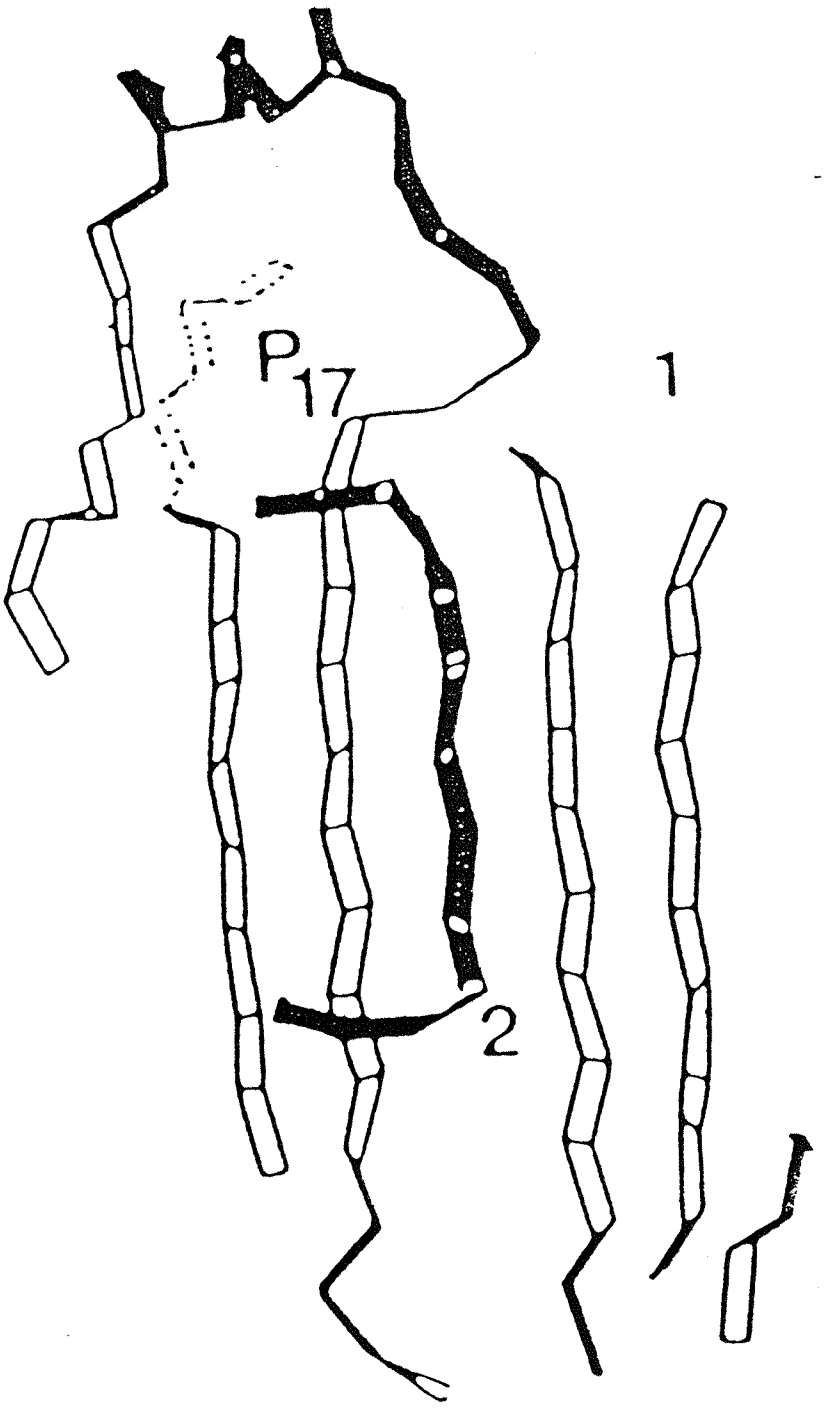


Figure 1.11 Loop-sheet polymerization involves insertion of the loop of one molecule (Number 2, shaded) into the A sheet of another (Number 1). Loop-sheet polymerization occurs spontaneously in Z α_1-antitrypsin, but M α_1-antitrypsin requires high temperatures to form polymers. (modified from Carrell & Evans, 1992).

The extent of loop insertion up to P_{12}/P_{10} is consistent with recent experiments using bacterial proteases and snake venoms that cleave different regions within the exposed loop of α_1-antitrypsin. *Staphlococcus aureus* V8 proteinase, a Glu-proteinase, cleaves the P_5Glu-P_4Ala bond of M α_1-antitrypsin but no cleavage is observed at the P_{13}Glu-P_{12}Ala bond (Potempa *et al.*, 1986; Mast *et al.*, 1992), suggesting that the P_{13}Glu is inaccessible to the protease. Papaya proteinase IV, a Gly-specific cysteine proteinase (Buttle *et al.*, 1990), cleaves at the P_{10}Gly-P_9Ala bond in M α_1-antitrypsin to form α_1-antitrypsin reduced in molecular mass by 4 kDa due to loss of the C-terminal fragment (Mast *et al.* 1992; Lomas *et al.*, 1993a). However, the P_{15}Gly is not recognized, implying that this residue is inaccessible to the protease because of insertion into the A-sheet. The susceptibility of loop residues P_{10}-P_3' to proteolytic cleavage suggested that the loop is inserted into the A sheet up to residues P_{12} to P_{10} and not further (Carrell *et al.*, 1985; Potempa *et al.*, 1986; Mast *et al.*, 1992; Lomas *et al.*, 1993a). Residues P_{16} to P_{12} are inaccessible to proteases and so must be within the A sheet. Molecular models predict that alanine P_{12} fits into a conserved pocket (Loebermann *et al.*, 1984) formed by phenylalanine residues (amino acids 51, 190, and 384 of α_1-antitrypsin) and a methionine residue (amino acid 374). Large residues at P_{12} prevent movement of the cleaved loop, strand s4A, into the A-sheet; substitution of α_1-antitrypsin P_{12}Ala for even a slightly larger threonine residue results in a significant loss of inhibitory activity (Devraj-Kizuk *et al.*, 1988).

Cleavage of the reactive centre loop of M α_1-antitrypsin by papaya proteinase IV was shown by Lomas *et al.* (1993a) to be blocked on the formation of a binary complex with a synthetic peptide analogous to the P_{14}-P_2 loop sequence of α_1-antitrypsin (Figure 1.11). The investigators propose that during binary complex formation the synthetic peptide on insertion into the A-sheet expels the reactive centre loop of M α_1-antitrypsin, forcing it to adopt a helical conformation similar to that seen in native ovalbumin. The P_{10}-P_9 bond would thus be placed at the base of the final helical turn and so

prevent proteolytic cleavage. However, Mast *et al.* (1992) showed cleavage to be unaffected by binary complex formation. Lomas *et al.* (1993a) suggest the disagreement between the results from the two groups is a consequence of the lyophilization of the α_1-antitrypsin-peptide binary complex by Mast *et al.* which opens the loop, rendering it more susceptible to proteolytic attack.

1.11.6 Latent PAI-1

Cleaved serpins such as α_1-antitrypsin, antithrombin III and α_1-antichymotrypsin are inactive because the loop is inserted further into the A sheet than is optimal for protease inhibition (Figures 1.12 & 1.13). Insertion of the loop beyond that of the inhibitory conformation was achieved for each of the inhibitory serpins, but not for ovalbumin, by exposure to mild denaturing conditions at low temperature (15°C). Treatment of the inhibitor with dilute guanidium chloride (-1 M Gu-HCl) results in complete loss of inhibitory activity, and changes in stability and CD spectra that match that of the S-R transition, and a loss of susceptibility of the serpin to proteolytic cleavage (Carrell *et al.*, 1991). Normal activity of the inhibitor can be restored by removal of the denaturing agent by dialysis and exposure to, and dialysis from, 8 M urea. The induced conformation of the inhibitors is designated as the L state (Lawrence *et al.*, 1990) because its properties are identical to those observed in an unusual latent and non-inhibitory form of the plasminogen activator inhibitor-1 (PAI-1). Goldsmith and colleagues showed with a 2.6 Å resolution structure of the latent form of human PAI-1, the complete insertion of the loop residues into the A ß-sheet as the s4A strand (Mottonen *et al.*, 1992), in a structure similar to that of cleaved serpins, but with the major difference that the P_1-P_1' bond is intact (Figure 1.13). Residues which comprise strand s1C in α_1-antitrypsin (C-terminal to the cleavage site) do not form part of sheet C in latent PAI-1; instead these residues approach strand s5B of sheet B from below the ß-ribbon of sheet C, rather than from above as in the cleaved serpin and ovalbumin structures. The structure of latent PAI-1 thus implies

the motility of sheet C, in addition to sheet A and strand s4A.Complete insertion of the loop into the A sheet implies a molecular property associated with either the reactive centre loop or the A sheet itself. Engineered mutants of PAI-1 in which the P_{17}-P_2 loop sequence had been replaced by that of antithrombin III, or a serpin consensus sequence still retained the ability to revert spontaneously to the L state, indicating this property is associated with the A sheet (Lawrence *et al.*, 1990). Recently, the X-ray structure of the latent form of antithrombin III induced experimentally has been resolved by two independent groups (Carrell *et al.*, 1994; Schreuder *et al.*, 1994). For a more detailed discussion on this structure see Section 1.13.4. Although there is no evidence that latent antithrombin occurs *in vivo*, PAI-1 is stored in blood platelets in the latent state and exists in the circulation in the active form on complexation with the plasma protein vitronectin (Booth *et al.*, 1988).

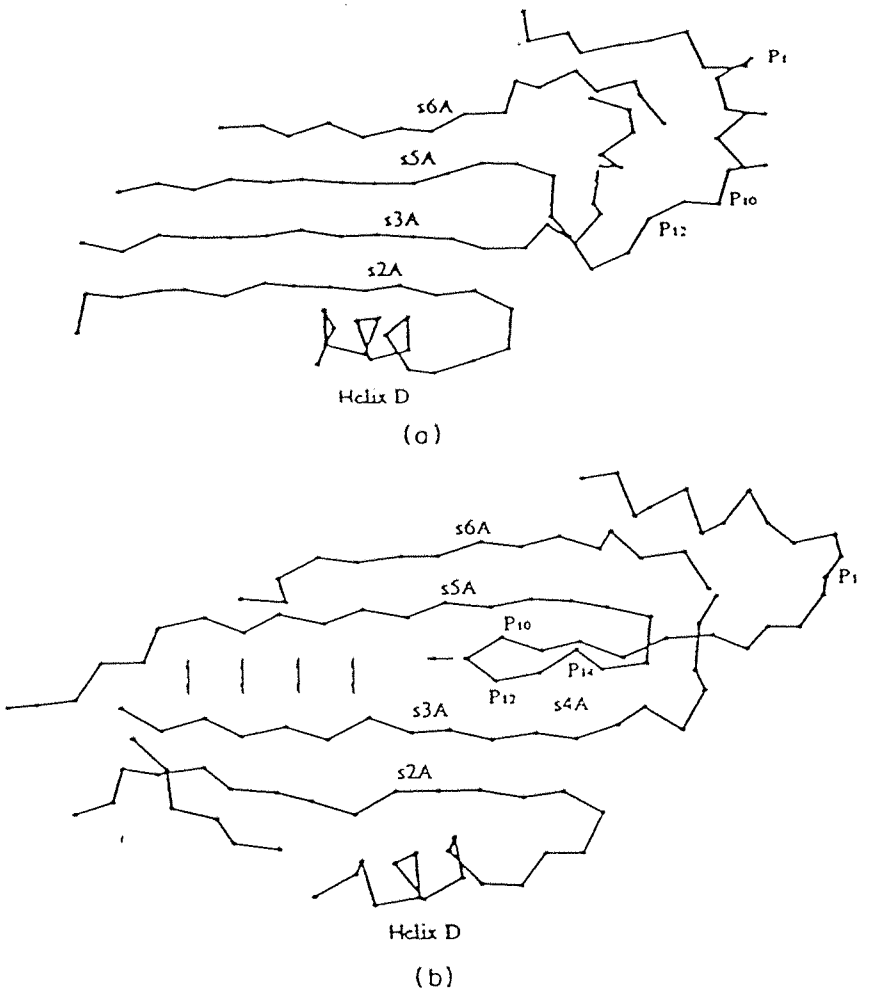


Figure 1.12 Schematic representations of the reactive centre loops of inhibitory and non-inhibitory serpins. (a) A sheet and helical reactive centre of ovalbumin and (b) the reactive centre of human antithrombin illustrating the folding of the N-terminal stalk back into the A sheet to provide an active inhibitory conformation (From Perry *et al.*, 1991).

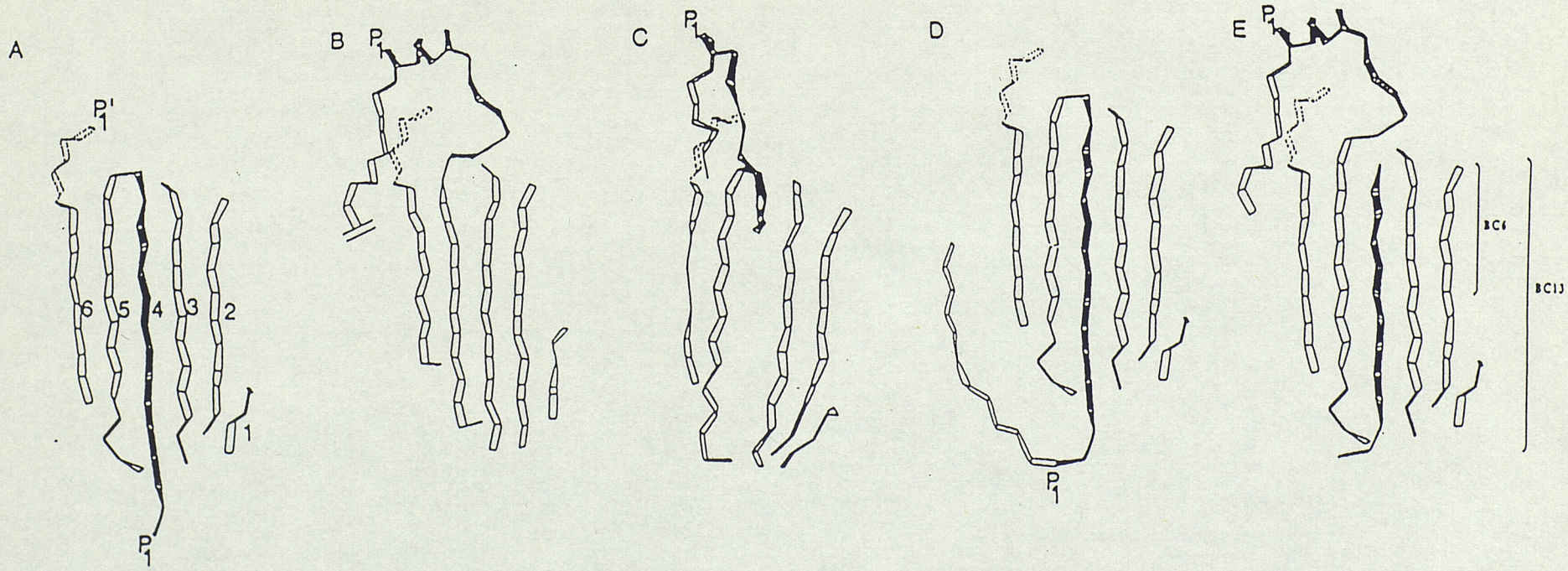


Figure 1.13 Schematic representations of the alternative conformations of the reactive centre loop with reference to the A-sheet. (A) Cleavage at the reactive centre bond, P_1-P_1' leads to incorporation of the loop into the ß-sheet A to form a six-stranded sheet and separation of P_1-P_1' residues by 69 Å. (B) Native ovalbumin has the loop exposed in an 3-turn helix. (C) The active inhibitory form is postulated to involve partial insertion of the amino-terminal portion of the loop into the sheet to allow P_3-P_3' to adopt the canonical conformation. (D) Latent (L) form is produced by full insertion of the loop into the opened A sheet, induced by mild denaturing conditions. (E) Binary complexes of serpin with reactive loop peptides (6 and 13 residue peptides shown, BC6 & BC13 respectively) expel the loop from the A sheet, forcing it into a conformation similar to that of ovalbumin. (Adapted from Carrell & Evans, 1992)

1.11.7 Native Antichymotrypsin

From the spatial structures of the cleaved and latent serpins, two models of reactive centre loop conformation of inhibitory serpins have been proposed, that both require partial insertion of the reactive centre loop into the A sheet during complex formation. The *pre-equilibrium model* requires the inhibitor to exist in an equilibrium between an inactive state in which the loop is fully exposed in a helical conformation, and in an active state with the loop partially inserted (up to P_{10}) into the A sheet (Carrell *et al.*, 1991). The target proteinase only binds to the latter state and so shifts the equilibrium towards this state. Alternatively, the *induced conformational change* model suggests that reactive loop insertion into the A sheet occurs only after the reactive site bond is recognized by target proteinase (Skriver *et al.*, 1991; Björk *et al.*, 1992b).

Just recently a native, inhibitory serpin, antichymotrypsin, has been crystallized (Wei *et al.*, 1992). More recently, the structure of this recombinant variant of antichymotrypsin, engineered to be an inhibitor of elastase by substitution of the P_3-P_3' sequence with that of α_1-antitrypsin, has been resolved and is being refined at 2.5 Å (Wei *et al.*, 1994). The reactive loop exists as an exposed distorted helical loop, the P_1 reactive site protruding away from the main body of the molecule. In comparison the reactive loop of cleaved antichymotrypsin is fully inserted into the A sheet to form strand s4A in (Baumann *et al.*, 1991). The native structure is consistent with the induced conformational change model proposed by Skriver and colleagues (1991), which states that loop insertion into the A sheet occurs only after serpin-proteinase association. A conformational change must occur to allow the P_3-P_3' residues of the loop to adopt a canonical binding conformation as observed in the smaller serine proteinase inhibitors (Bode & Huber, 1992). The authors (Wei *et al.*, 1994) speculate that concomitant to binding proteinase, the uncoiled loop partially inserts into the A sheet to form a stable complex, and only when the serpin is

released from the proteinase complex does the loop insert fully, as seen in the structures of cleaved inhibitory serpins (Table 1.1; Figure 1.6).

1.12 Loop-Sheet Polymerization

Schulze *et al.* (1990) showed that native M α_1-antitrypsin when heated at 60°C for 2 h formed polymers of high molecular mass. The investigators proposed that these polymers were formed by intermolecular complexing; with the loop of one serpin inserting into the A ß-sheet of another molecule, rather than insertion of strand s4A into the same molecule. Dimerization of native, but not reactive centre cleaved, M α_1-antitrypsin in high strength phosphate buffers was shown by neutron scattering (Haris *et al.*, 1990).

Later, Evans and colleagues demonstrated that other inhibitory serpins were able to form such loop-sheet polymers under mild denaturing conditions (~1 M guanidium hydrochloride) at 37°C (Carrell *et al.*, 1991) (NB. formation of the L state at 3-4°C). Such polymerization of M α_1-antitrypsin was blocked by addition of excess synthetic peptide BC13 derived from the reactive centre loop of antithrombin III homologous to the P_{14}-P_2 loop sequence of α_1-antitrypsin (Carrell *et al.*, 1991). The peptide BC13 incorporates into the A ß-sheet to form a stable binary complex, thereby blocking entry of other α_1-antitrypsin loops. Binary complex formation is confirmed, as detailed above, by loss of inhibitory activity, and physical properties and CD spectra (Carrell *et al.*, 1991). Polymerization is shown to be a temperature and concentration dependent process.

Subsequently, Mast *et al.* (1992) observed circular polymers of α_1-antitrypsin 6-12 units in length by electron microscopy formed with α_1-antitrypsin after proteolytic cleavage eight or nine residues upstream from the P_1 reactive site and also by heating α_1-antitrypsin at 48°C for 15 hours. Apparently polymerization results from insertion of the loop region P_8-P_1 or

P_9-P_1 of one molecule into the A ß-sheet of an adjacent proteolytically modified molecule. Polymer formation was concentration-dependent and assessed by electron microscopy and by the presence of high molecular mass bands on non-denaturing polyacrylamide gels. A single band only, of α_1-antitrypsin, was observed by SDS-PAGE implying non-covalent interactions in polymerized α_1-antitrypsin. In agreement with Carrell's work (Carrell *et al.*, 1991), polymerization was prevented by blockage of the A ß-sheet with a synthetic peptide corresponding to residues (P_{15}-P_1) of the reactive centre loop, although cleavage of the reactive loop was confirmed by SDS-PAGE and amino acid sequence analysis.

Experimenters (Mast *et al.*, 1992; Lomas *et al.*, 1992) speculated that the aggregates of Z α_1-antitrypsin observed in the hepatocytes of Pi ZZ individuals (Sharp, 1971) and high molecular weight forms in the plasma (Cox *et al.*, 1986), may be due to loop-sheet polymerization.

1.13 Mechanism of Accumulation of Z α_1-Antitrypsin

To date, the mechanism of Z α_1-antitrypsin (Glu^{342} to Lys^{342}) accumulation in the endoplasmic reticulum is not clear. The Z mutation (P_{17} residue) occurs at the junction of strand s5A at the base of the reactive centre loop. Various mechanisms of Z protein accumulation have been proposed and are discussed below.

1.13.1 Disruption of Salt Bridge

The crystal structure of M α_1-antitrypsin predicts an internal salt bridge between Glu^{342} and Lys^{290} (Loebermann *et al.*, 1984). Substitution of the positively charged Lys for Glu at position 342 in Z α_1-antitrypsin would prevent formation of this salt bridge and may change the protein folding kinetics and so hinder secretion. Site-directed mutagenesis of M α_1-antitrypsin has been used to substitute glutamic acid for Lys^{290} to disrupt the

other half of the salt bridge. Only a minor decrease in secretion was observed with this mutant from *Xenopus* oocytes (Foreman, 1987), transfected mouse hepatoma cell lines (Sifers *et al.*, 1989) and transfected mouse kidney cell lines (McCracken *et al.*, 1989). Although one groups results suggests the importance of the 290 to 342 salt bridge (Brantly *et al.*, 1989), results from several laboratories dispute this finding (Foreman, 1987; McCracken *et al.*, 1989; Sifers *et al.*, 1989). Other novel mutants support the concept that insertion of a positive residue (lysine or arginine) at position 342 results in retention of α_1-antitrypsin within the exocytotic pathway (Sifers *et al.*, 1989; McCracken *et al.*, 1991; Wu & Foreman, 1990), whereas introduction of neutral (alanine) or acidic (glutamine) residues which prevent salt bridge formation have no effect on protein secretion (Sifers *et al.*, 1989; Wu & Foreman, 1990).

1.13.2 Signal Hypothesis

Some investigators (McCracken *et al.*, 1989) have proposed that the Z mutation modifies or exposes a specific signal for protein retention or export from the endoplasmic reticulum (ER). α_1-Antitrypsin in parallel with other secretory proteins, migrates from the rough endoplasmic reticulum to the Golgi complex at characteristic rates (Lodish *et al.* 1983). The movement and localisation of α_1-antitrypsin along the exocytotic pathway can be assessed with various drugs known to act at specific sites of the secretory pathway, and by examining the glycosylation state of the protein (Figure 1.14). Endo-ß-N-glucosaminidase (Endo H), a glucosidase which cleaves the high-mannose N-linked glycoproteins at the GlcNacß1-4GlcNac linkage, can be used to monitor the movement of secretory proteins from the ER to the Golgi complex (Tarentino & Maley, 1974). Oligosaccharide moieties linked to asparagine residues of a protein remain sensitive to cleavage by Endo H until acted upon by GlcNac transferase I and mannosidase II in the medial Golgi (Farquhar, 1985). Treatment of accumulated Z α_1-antitrypsin with Endo H converts the proteins to a lower

molecular mass as detected by SDS-PAGE, confirming that the abnormal protein accumulates in a pre-Golgi compartment (Verbanac & Heath, 1986; McCracken *et al.*, 1989; Sifers *et al.*, 1989; Ciccarelli *et al.*, 1993). Recently, Le *et al.* (1990) reported that addition of a tetrapeptide KDEL sequence to the carboxyl terminus of Pi Z α_1-antitrypsin protected the protein from intracellular degradation. The KDEL sequence is a specific signal for the retention of ER-resident proteins via their recycling from a post-ER salvage compartment on recognition by KDEl-receptors (reviewed by Pelham, 1990). This implication of a post-ER but pre-Golgi degradation compartment was supported by other work (Le *et al.*, 1992; Ciccarelli *et al.*, 1993) that the localized site to be a intermediate compartment or *cis*-Golgi network (CGN), as defined by Pelham (1991). Z α_1-antitrypsin migrates to the CGN where most of it is degraded, the remainder being partially secreted and partially recycled to the ER where it accumulates. No signal for Z α_1-antitrypsin accumulation as been identified, at present.

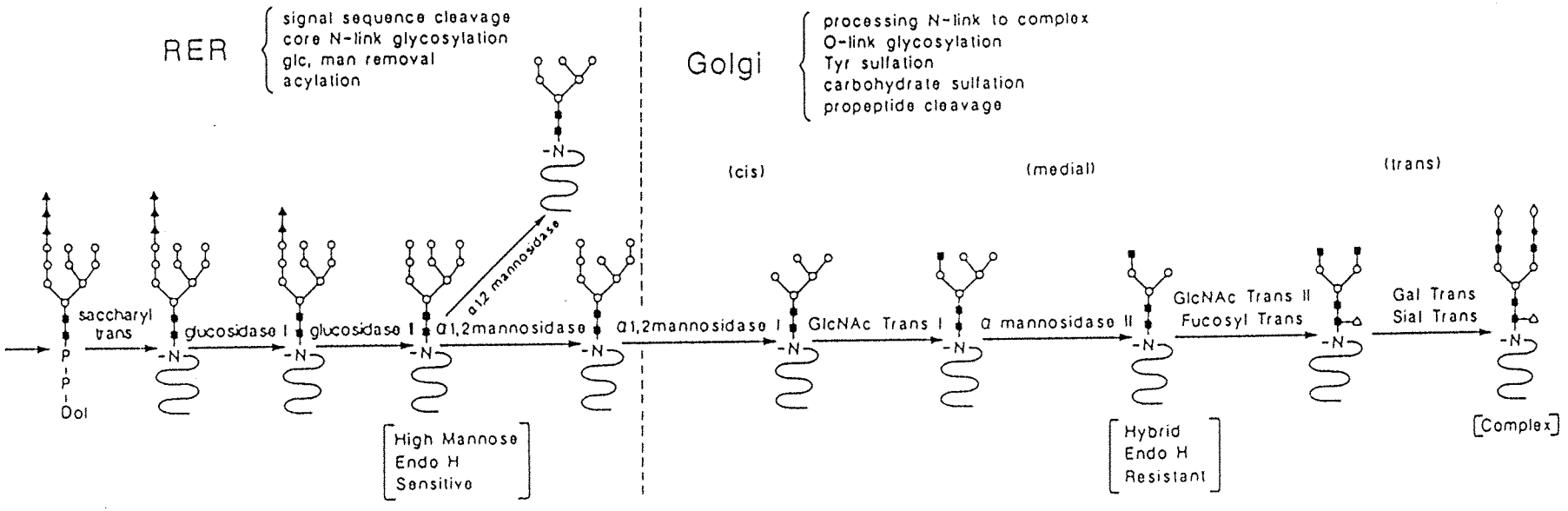


Figure 1.14 Oligosaccharide processing within the secretory pathway. The sequence of oligosaccharide processing is shown. Also indicated are the compartmentalization of the Golgi complex (cis, medial, trans) and the point of acquisition of Endo H resistanee. [Symbols: *dark triangles* = glucose; *open circles* = mannose; *squares* = N-acetylglucosamine (GlcNAc); *open triangles* = fucose; *dark circles* = galactose; *diamonds* = sialic acid (Sial). trans, Transferase; Dol, Dolichol. (modified from Kornfeld & Kornfeld, 1985)

1.13.3 Abnormal Translocation or Folding

The immunoglobulin heavy-chain-binding protein (BiP), a main ER-resident protein, interacts with many incorrectly folded polypeptides in the ER (reviewed by Rothman, 1987 and Gething & Sambrook, 1992). Immunoanalysis of BiP protein, in a variety of cell types, has not detected any association with either overexpressed Pi M α_1-antitrypsin (Sifers *et al.*, 1989) or the retained Pi Z variant (McCracken *et al.*, 1989; Graham *et al.*, 1990b; Creisteil *et al.*, 1990).

Calnexin (or p88 or IP90), a 88 kDa ER-resident calcium-binding transmembrane phosphoprotein (see review by Bergeron *et al.*, 1994) acts as a molecular chaperone for integral membrane proteins; for example, transiently binding to T-cell receptor subunits during assembly and binding more permanently to defective T-cell receptor subunits (Hochstenbach *et al.*, 1992). Recent work demonstrated calnexin bound transiently to the 52 kDa precursor of M α_1-antitrypsin as well as to a variety of other membrane and soluble glycoproteins in the ER in the human hepatoma cell line HEPG2 (Ou *et al.*, 1993). The investigators showed that the duration of interaction of calnexin with different glycoproteins is correlated to the differential transport rates, but does not account completely for the time the glycoproteins are resident in the ER because interaction with other ER factors may also be involved. Treatment with tunicamycin or other glycosidase inhibitors prevents glycoprotein presentation to calnexin and may lead to protein misfolding and aggregation or folding instead by luminal ER chaperones and export from the ER. For instance, α_1-antitrypsin folds more rapidly in the presence of tunicamycin causing protein misfolding and inhibition of the rate of protein export (Lodish & Kong, 1984). Degradation of misfolded and defective proteins in the ER may be initiated by association of the proteins with calnexin (Ou *et al.*, 1993). A number of human Endoplasmic Reticulum Storage Diseases (ERSD) are caused by the accumulation of secretory proteins in the ER (reviewed by Amara *et al.*, 1992; Callea *et al.*, 1992).

These include the major form of familial hypercholesterolaemia (type II mutant in the LDL receptor), the most common mutation (ΔF508) causing cystic fibrosis, Tay-Sachs disease as well as secretion defective mutations in α_1-antitrypsin. To date, the ER proteins which retain these defective glycoproteins have not been isolated. However, recently calnexin has been shown to co-precipitate with the $null_{Hong\ Kong}$ variant of α_1-antitrypsin (Le *et al.*, 1994). This variant protein of 333 amino acid is truncated at the carboxyl terminus due to premature termination, and is entirely retained and degraded within a pre-Golgi compartment (Sifers *et al.*, 1981). 30% of the retained $null_{Hong\ Kong}$ protein forms a 1:1 molar complex with calnexin, that dissociates in the presence of deoxycholate, suggesting hydrophobic interactions between the misfolded protein and calnexin. Calnexin is distinct from chaperones of the Hsp (Heat shock protein) family in that its synthesis is not significantly altered by stresses such as heat shock or treatment with tunicamycin (Bergeron *et al.*, 1994).

Perlmutter *et al.* (1989) observed increased synthesis of the heat shock proteins Hsp 70, Hsp 90 and ubiquitin in monocytes from Pi ZZ individuals with liver disease relative to Pi ZZ individuals without evidence of liver injury. The Hsp 70 family of proteins include proteins which assist protein translocation (Lingappa, 1989) and their activity is thought to be triggered by abnormal proteins. Hsp 70 expression is induced in *Xenopus* oocytes by microinjection of denatured proteins but not by injection of the same proteins in their native state (Ananthan *et al.*, 1986). If the mutant protein is abnormally translocated or folded a heat shock protein in the ER may recognise and bind to it. It is possible that binding may favour intracellular retention or it may initiate degradation. However, recognition and binding of abnormally folded α_1-antitrypsin by a stress protein may represent a secondary mechanism of cellular defence occurring as a consequence intracellular α_1-antitrypsin accumulation, the primary defect. Other, misfolded and unassembled polypeptides retained with the ER include unassembled T-cell receptor chains (Lippincott-Schwartz *et al.*, 1988) and

the retained H2 subunit of the asialoglycoprotein receptor (Amara *et al.*, 1989), are subjected to intracellular degradation in a similar manner to Z α_1-antitrypsin, in a pre-Golgi compartment.

1.13.4 Loop-Sheet Polymerization

The Z mutation of α_1-antitrypsin (Glu^{342} to Lys) lies at the base (P_{17} residue) of the reactive centre loop. Lomas *et al.* (1992) proposed that a change in charge (Glu to Lys) at the hinge region would prevent insertion of the loop into the gap of the A ß-sheet and, so the Pi Z variant would be more susceptible to polymerization than Pi M. Under physiological conditions at 37°C Z, but not M, α_1-antitrypsin purified from the plasma of homozygotes underwent spontaneous polymerization, as resolved by HPLC gel filtration on Q-Sepharose columns (Lomas *et al.*, 1992). The percentage of high-molecular mass material (>200 kDa) relative to native α_1-antitrypsin (52 kDa) was enhanced by increasing temperature and by increasing concentration of protein. M α_1-antitrypsin only formed polymers under denaturing conditions at high nonphysiological temperatures (Carrell *et al.*, 1991). Z α_1-antitrypsin complexed to the reactive loop peptide BC13 (Schulze *et al.*,1990) was unable to undergo loop-sheet polymerization because the A sheet was already occupied. Morphological and physical characteristics of Z α_1-antitrypsin polymerized *in vitro* are identical to tangled polymers of α_1-antitrypsin purified from hepatocytes obtained by human liver biopsies. Electron microscopy of α_1-antitrypsin polymerized *in vitro* reveals polymeric chains 4-10 molecules in length, identical to the tangle of filaments of α_1-antitrypsin from the hepatic inclusions of a PiZZ patient (Lomas *et al.*, 1992). The polymers formed failed to either complex with, or be cleaved by, bovine chymotrypsin (Lomas *et al.*, 1993a). This finding confirms that the reactive centre loop is inaccessible to protease attack, as is implied by the loop-sheet polymerization model. Polyacrylamide gel electrophoresis confirms that α_1-antitrypsin polymers formed *in vitro*, analogous to the inclusions isolated from the hepatocytes of Z homozygotes

(Cox *et al.*, 1986), are formed by non-covalent bonding and are completely dissociable to monomers by SDS in the absence of thiol reducing agents (Lomas *et al.*, 1992). Circular dichroism spectra in the near UV spectral region of Z α_1-antitrypsin (Lomas *et al.*, 1993a) support the proposal that the Glu^{342} to Lys substitution at the base of the loop allows the A ß-sheet to open to make the molecule receptive to another loop. Furthermore, Z α_1-antitrypsin complexed with a reactive-loop peptide inserted into the A sheet was unable to undergo loop-sheet polymerization (Lomas *et al.*, 1993a).

Other natural α_1-antitrypsin mutations associated with liver inclusions are α_1-antitrypsin M_{Malton} (Curiel *et al.*, 1989c; Fraizer *et al.*, 1989; Graham *et al.*, 1989) and α_1-antitrypsin S_{iiyama} (Seyama *et al.*, 1991). Both variants have mutations at positions 52 and 53 respectively which will predictably cause displacement of the B helix (Huber *et al.*, 1989) that forms the groove on which the A sheet opens and closes (Stein & Chothia, 1991). Both mutations have been postulated to disturb the structure of α_1-antitrypsin allowing opening of the A sheet and so facilitating loop-sheet polymerization (Lomas *et al.*, 1992). As for the Z variant, Lomas and colleagues subsequently isolated α_1-antitrypsin polymers 10-15 molecules in length from the plasma of an individual homozygous for the Ser^{53}→Phe mutation in α_1-antitrypsin S_{iiyama} (Lomas *et al.*, 1993b). Like polymeric Z, the polymeric form of α_1-antitrypsin S_{iiyama} was inactive as an inhibitor of bovine α-chymotrypsin and has properties of loop-sheet polymers but the polymers formed are of greater length than the Z polymers, presumably due to the more extensive degree of polymerization of the S_{iiyama} variant; 95% α_1-antitrypsin S_{iiyama} plasma was polymeric (Lomas *et al.*, 1993b).

More recently, polymers were isolated from the plasma of an $M_{Malton}/Null_{Bolton}$ heterozygote (Lomas *et al.*, 1995). These polymers are much shorter (3-5 molecules) than the Z and S_{iiyama} α_1-antitrypsin polymers and in addition amino-terminal sequencing revealed that the polymers also contained a cleaved reactive centre loop. This is presumably due to the

reactive centre loop of the final α_1-antitrypsin molecule in a chain of polymers being susceptible to cleavage by proteinases *in vivo*; polymers of S_{iiyama} are much longer and so cleavage represents a smaller fraction of the polymerized protein making detection by SDS-PAGE difficult.

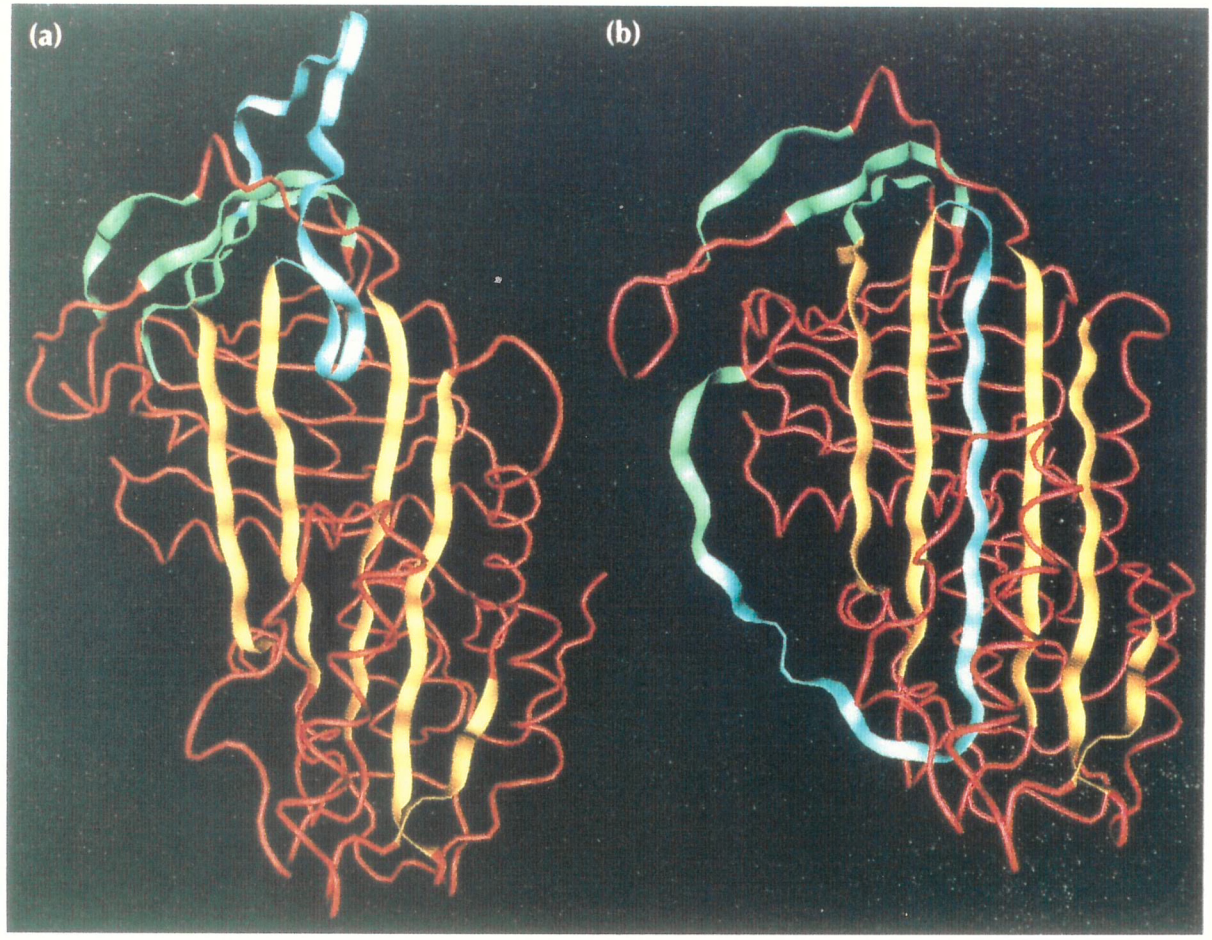
This year, Yu and colleagues demonstrated that substitution of Phe residue at position 51 by small, non-polar residues enhanced the thermal stability and decreased heat induced polymerization of M α_1-antitrypsin (Kwon *et al.*, 1994). The most efective residue at increasing the thermal stability was identified to be leucine. It is interesting to note that the position of this mutation is adjacent to the S_{iiyama} ($Ser^{53}\rightarrow Phe$) and M_{Malton} (Phe^{52} deleted) variants which form loop-sheet polymers.

Opening of the A sheet of α_1-antitrypsin provides strong evidence that loop-sheet polymerization is the common mechanism accounting for the accumulation of α_1-antitrypsin in the endoplasmic reticulum of hepatocytes. However recent structural studies suggest a related but more complex process may be involved in the loop-sheet polymerization of the serpins. Two groups (Carrell *et al.*, 1994; Schreuder *et al.*, 1994) have recently reported low resolution structures (3 Å) of dimers of antithrombin III from similar crystals. The structure of a dimer of antithrombin shows one molecule in the latent form in which the reactive loop is totally incorporated into the A sheet of the molecule. This incorporation of the loop into the A sheet in the latent molecule (L-molecule) involves the accompanying release of strand s1C from its C sheet and it is this strand that is replaced by the reactive loop of the second molecule in the dimer (Figure 1.15). The two groups disagree about the form of the non-latent molecule. Carrell *et al.* (1994) report that the second molecule is intact (I-molecule), whereas Schreuder *et al.* (1994) maintain that the reactive loop is cleaved at one or two sites. These findings suggest that an alternative to the A sheet polymerization mechanism is a reactive loop-C sheet polymerization. However, the C sheet polymerization mechanism is not incompatible with

the A sheet model since the first step in polymerization would be increased rate of refolding of the loop into the A sheet, with consequent release of the strand from the C sheet to allow sequential C sheet-reactive loop linkages.

Loop-sheet polymers have also been reported in a number of other serpin members, discussed below, but no structural aspects to polymer formation have been identified in these serpins at present. Plasminogen-activator inhibitor type 2 (PAI-2) forms polymers spontaneously under mild denaturing conditions and *in vivo* in COS cells expressing high levels of PAI-2 (Mikus *et al.*, 1993). A hinge region mutant of C1-inhibitor, P_{10}Ala→Thr, produces a dysfunctional molecule, C1-inhibitor(Mo), that which forms polymers in human plasma (Aulak *et al.*, 1993) producing type II hereditary angioedema (Levy *et al.*, 1990). Intracellular accumulation of α_1-antichymotrypsin in the liver of a patient with a Pro^{229}→Ala substitution occurs by loop-sheet polymerization (Faber *et al.*, 1993). This mutation is located in a section of the molecule that connects strand s3C and s1B and may well alter the conformation of the C-sheet.

Figure 1.15 The antithrombin dimer Schematic structural representations of **(a)** the non-latent, intact (I) molecule and **(b)** the latent (L) molecule in the antithrombin dimer (Carrell *et al.*, 1994). The reactive centre loop is shown in blue, the A sheet in yellow and the C sheet in green. **(c)** The antithrombin dimer showing the I-molecule (left) and the L-molecule (right). Regions shaded in black comprise the reactive centre loop and strand s1C of the I-molecule and their equivalent sequence in the L-molecule. Dashed lines represent regions of breaks in electron density in the crystal structure (Carrell *et al.*, 1994) (figures were kindly provided by R. W. Carrell, Cambridge)



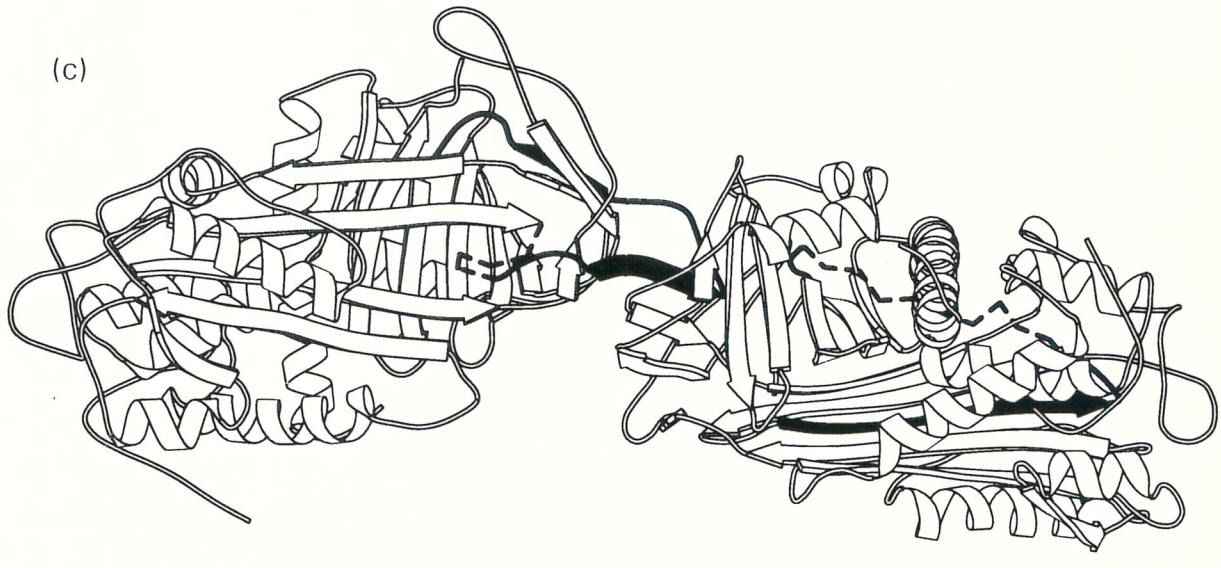


Figure 1.15

2. METHODS

2.1 Materials

DNA and RNA modifying enzymes were purchased from Promega Corporation or Boehringer Mannheim, unless otherwise indicated. [α-^{35}S] dATP (specific activity >1000 Ci.mmol^{-1}) for DNA sequencing and L-[^{35}S] methionine (specific activity >1000 Ci.mmol^{-1}) for metabolic labelling of proteins were supplied by Amersham International plc. Nucleotides and dideoxynucleotides were obtained from Pharmacia. Dideoxynucleotide sequencing was performed with Sequenase® version 2.0 (United States Biochemical Corporation). Anti-human α_1-antitrypsin was purchased from DAKO Immunoglobulins and anti-ovalbumin was a kind gift from Dr Glenn Matthews, Biochemistry Dept., University of Birmingham. All other reagents were analytical grade or better and purchased from BDH, Gibco-BRL or Sigma. Water used in PCR and RNA was obtained from a Millipore Milli R/Q® water purifier.

Basic microbiological and molecular methods were performed according to Sambrook *et al.* (1989). Plasticware (pipette tips, microfuge tubes, etc.) was autoclaved and kept sterile before use. Solutions and antibiotics were prepared in DEPC-treated water and either sterile filtered or autoclaved before use.

2.2 Standard Techniques

2.2.1 Bacterial Strains and Cloning Vectors

Recombinant DNA techniques were performed in *Escherichia coli* strain TG2 (see Sambrook *et al.*, 1989).

Cloning vectors used were pEMBL-8(+) (Dente *et al.*, 1983), the pGEM series from Promega and pSP64TRCF (Foreman, 1987) (Figure 2.1). Human α_1-antitrypsin cDNA cloned into the *Pst* I site of pEMBL 8(+) was

available (Ciliberto *et al.*, 1985). All vectors carried the gene for ampicillin resistance. The pSP645TRCF vector is a modification of the *in vitro* transcription pSP64T (Krieg & Melton, 1984) to contain a *Pst* I cloning site. The cloning site lies between the 5'- and 3'-flanking regions of the *Xenopus laevis* ß-globin sequence (includes capping site, ribosome binding site and a poly(A) tail) to allow insertion of cDNA. Thus mRNA transcribed from this vector bears the hallmarks of an endogenous well expressed mRNA, making it suitable for translation in *Xenopus* oocytes.

2.2.2 Culture of *E. coli* TG2 Cells

At first cells were cultured in LB media but low growth yield was obtained so the richer media, 2xYT was used. Recipes for 1 litre of media (broth):

LB media: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl

2xYT media: 16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl

All media was adjusted to pH 7.0-7.2 with NaOH and sterilized by autoclaving. Ampicillin was added after cooling to below 50°C at a concentration of 50 μg/ml. To prepare agar plates 15 g bacto-agar per litre is added to liquid media prior to autoclaving.

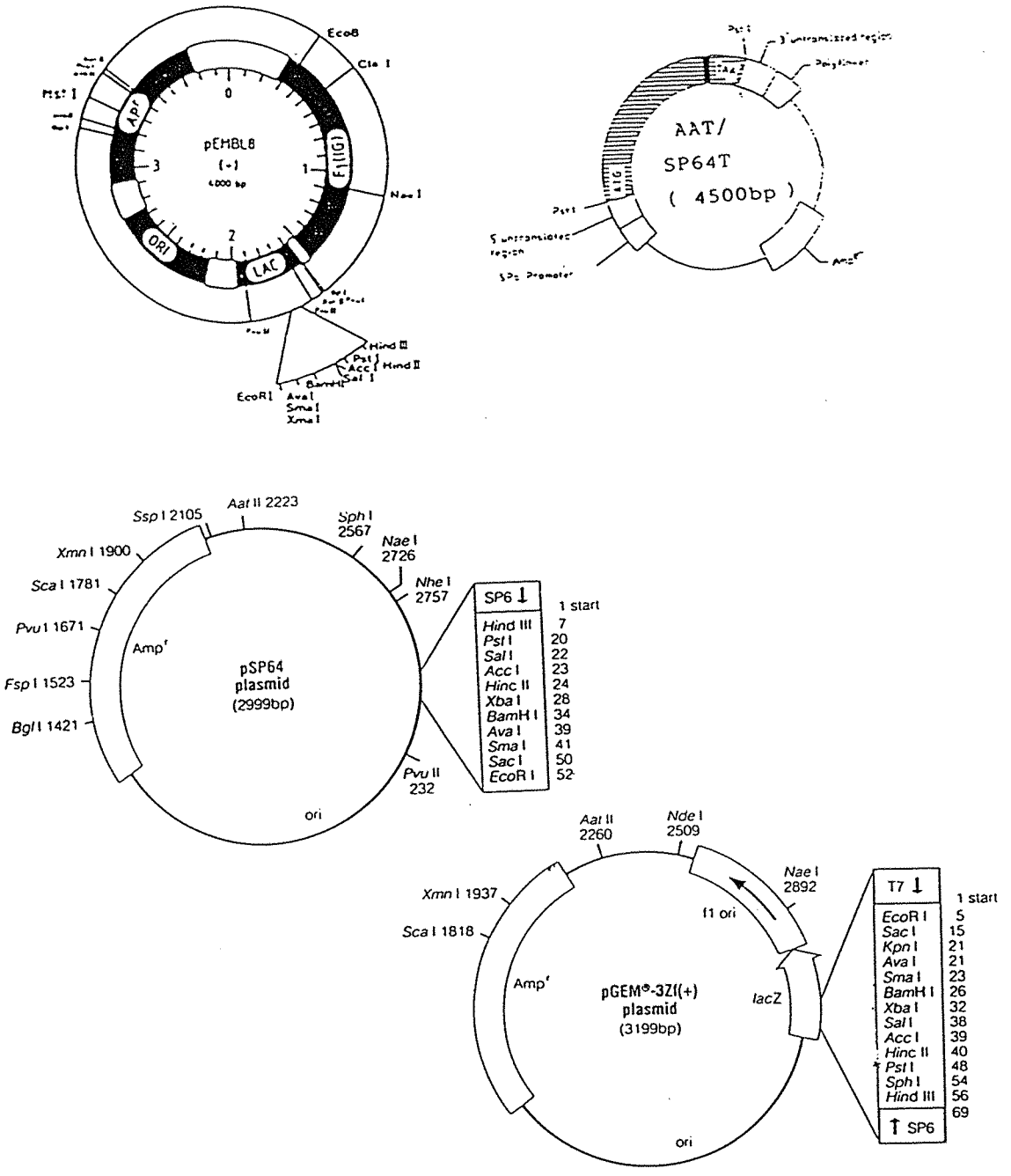


Figure 2.1 Plasmid cloning vectors used for construction and expression of mutant proteins.

2.2.3 Preparation of Transformation Competent Cells

Transformation competent cells were prepared as recorded in Sambrook *et al.* (1989), except that calcium chloride used was 100 mM $CaCl_2$ 10 mM Tris-HCl, pH 8.0. Cells were stored overnight on ice in 200 μl aliquots prior to use.

2.2.4 Transformation of Competent Cells

Competent cells are induced to take up DNA by a short heat-shock. As with the $CaCl_2$ treatment, the basis to this technique is not understood. Plasmid DNA (purified or ligation mixtures) was added to a 200 μl aliquot of competent cells, and incubated on ice for 30 minutes. No more than 10 μl (1/20th volume) DNA is added in order to maintain the $CaCl_2$ concentration and/or to prevent components of ligation mixtures interfering with DNA uptake. Cells were heat-shocked at 42°C for 90 seconds in a circulating water bath, followed by returning to ice for 2 minutes. 0.8 ml 2xYT medium pre-warmed to 37°C was added to each tube, and the mix incubated at 37°C for 45 minutes to allow time for expression of the antibiotic resistance genes.

To select for plasmid vectors carrying antibiotic resistance genes (ampicillin in my case) fractions of the mix (200 μl, 100 μl and 50 μl) were spread onto 2xYT-ampicillin plates. Plates were incubated overnight at 37°C. Transformed cells formed discrete colonies, derived from individual ampicillin resistance cells. Individual colonies were transferred into 5 ml liquid broth using sterile toothpicks and grown for further study.

2.2.5 Preparation of Plasmid DNA

5-100 ml of antibiotic containing broth was inoculated with *E. coli* containing plasmid (a single colony, or stock culture) and incubated at 37°C

overnight on a rotary shaker at 200-300 r.p.m. Plasmid DNA was isolated using the Promega Wizard™ Minipreps kit.

The quality and integrity of DNA isolated was verified by restriction enzyme analysis and 1% agarose gel electrophoresis with ethidium bromide staining. Records of gels were kept by transillumination of the gel with a UV lamp and photography onto Polaroid 667 film with an orange filter. Concentrations and purity of DNA were determined by OD_{260}/OD_{280} readings on dilutions of DNA aliquots (Sambrook *et al.*, 1989).

2.2.6 Construction of Recombinant Vectors

Plasmid vectors were digested at one locus by a single restriction enzyme, or by two at a multi-cloning site to allow insertion of target DNA with complementary ends. Vector and target DNA were digested with the appropriate restriction enzyme(s), and the DNA purified by phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). A small aliquot was analysed by gel electrophoresis to confirm complete digestion.

Digestion with a single enzyme does not allow for directional cloning (the insert can insert in either orientation) or vector DNA can recircularize. To remove 5′ phosphate groups and thus prevent recircularization of vector during ligation, the vector DNA was treated with calf alkaline phosphatase (Sambrook *et al.*, 1989).

2.2.7 DNA Sequencing

Template DNA was prepared using Promega Wizard™ Minipreps kit. Purity of the template, which is of utmost importance, was assessed by gel electrophoresis and OD_{260}/OD_{280} measurements. The double-stranded DNA was denatured by treatment with alkali for 30 minutes at 37°C, as follows:

DNA	x μl (15-20 μg)
100 mM Tris-EDTA	y μl
2 M NaOH	5 μl
Total volume	50 μl

The values of x will depend on the concentration of DNA, and y on the volume needed to bring the volume to 50 μl. The mixture was neutralized with 5 μl 3 M sodium acetate (pH 4.5-5.5) and precipitated at -70°C for 15 minutes after addition of 200 μl absolute ethanol. Denatured DNA was pelleted by centrifugation in the microfuge for 10 minutes, the supernatant removed, and the pellet dried. The DNA pellet was suspended in 6 μl sterile water, 2 μl sequenase reaction buffer, and 2 μl sequencing primer (5 pmol μl^{-1}). The sequencing primer used was complementary to a region surrounding the region of the template. The template and primer were annealed for 15-30 minutes at 37°C and then the sequencing reaction was performed using [^{35}S] dATP with Sequenase® version 2.0 (USB). 2-4 μl of sequenced template was run on 8% polyacrylamide gels at 1500-1600 Volts and the gels visualized by autoradiography onto Fuji RX film.

2.3 Site-Directed Mutagenesis

2.3.1 Principle of PCR

The polymerase reaction (PCR) (Saiki *et al.*, 1985; Mullis & Faloona, 1987) technique involves the enzymatic amplification of target DNA by use of two oligodeoxynucleotide primers hybridizing to opposite strands, and flanking the region of interest in the target DNA. Three distinct steps, performed repeatedly as a series of cycles (25-50), amplify the DNA exponentially:

1) Denaturation of the template DNA at high temperature (92°C-98°C).

2) Annealing of the primers to the DNA template (37°C-70°C). The 3'-ends of the primers point to each other.

3) Thermostable DNA polymerase extends the primers from their 3'-ends (70°C-74°C).

In order to maximise the specificity of a PCR reaction, it is essential that the primers bind to their target sites. Various factors have to be optimized to increase the specificity (one product only is desired): annealing temperature, Magnesium concentration, primer concentration, template concentration, deoxynucleoside triphosphate concentration (Innis *et al.*, 1990).

2.3.2 PCR-based Site-Directed Mutagenesis

The ability to place specific sequences on the 5'-end of the PCR primers forms the basis for a series of methods for site-specific mutagenesis, insertion/deletion mutagenesis and the generation of recombinant DNAs (Higuchi, 1989). Mis-matches in the primers can be used to introduce base changes into amplified DNA. These PCR-based procedures are more efficient, faster and less labour-intensive than traditional cloning techniques.

Mutants of α_1-antitrypsin and ovalbumin were constructed using *Taq* polymerase according to the method of Landt *et al.* (1990). Starting with a double-stranded DNA template, one single specific mutagenic primer was used in conjunction with two universal sequencing primers flanking the region to be mutated, in a dual step PCR amplification (Figure 2.2). An outline of the protocol is shown below with the mutagenic primer (3'-MP) complementary to the sense or coding strand, and the 5'- and 3'-universal primers complementary to the non-coding and coding strand respectively.

PCR - BASED SITE - DIRECTED MUTAGENESIS

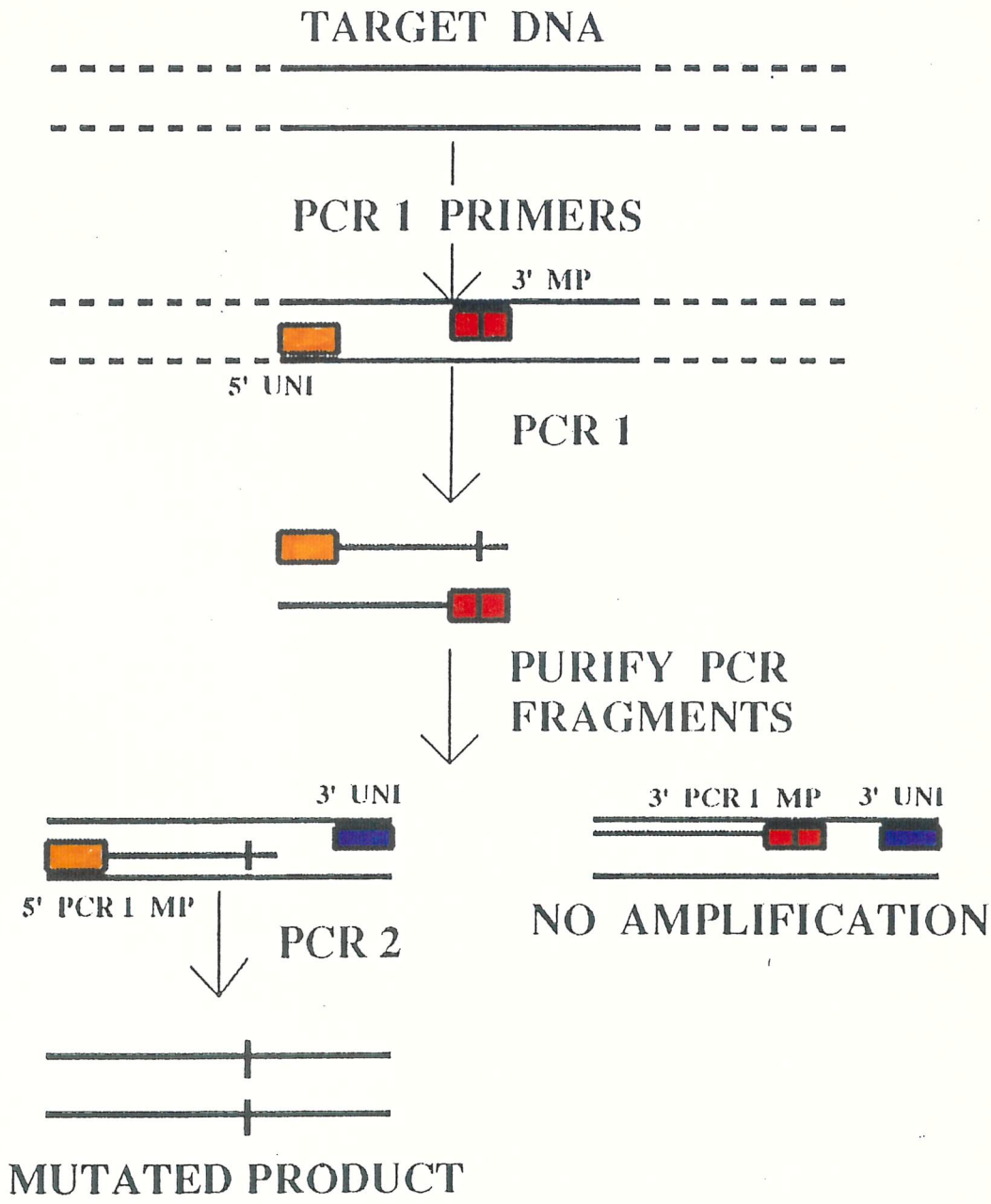


Figure 2.2 Schematic presentation of the dual-step PCR mutagenesis method (Landt *et al.*, 1990). PCR 1 involves a mutagenic primer (MP) containing the required base mis-matches and an opposite primer flanking the target gene, 5′ UNI primer in the reaction scheme shown above. PCR 2 uses the other flanking primer (3′ UNI) and the truncated gene product from PCR 1 as primers, to synthesise the full-length gene containing the desired nucleotide changes.

PCR 1 The 3'-mutagenic primer and 5'- universal primers generate a double-stranded fragment with a mutated site at one side from the double-stranded DNA template. This fragment is purified from superfluous primers and used as a primer in the second PCR step to avoid the generation of wild type.

PCR 2 The single strand from PCR 1 with the mutation at its 3'-end serves as the 5'-mutagenic primer in the second PCR, together with the 3'-universal primer to extend PCR 1 fragment in the 3' direction. The other strand remains unamplified.

2.3.3 PCR Oligonucleotide Primer Design

Oligonucleotide were designed of 15-25 nucleotides in length, a good compromise between specificity and efficiency at the reaction temperatures optimal for *Taq* polymerase. Mutations were designed so that they could not disrupt the reading frame. The universal primers contained bases coding for restriction sites to facilitate subcloning of the product into a transcription vector. The simple rules of PCR design were followed (Thein & Wallace, 1986; Lowe *et al.*, 1990) to prevent non-specific amplification.

Gratuitous addition of one nucleotide, frequently adenosine (Mole *et al.*, 1989), to the 3'-end of the strand complementary to the mutagenic primer by *Taq* polymerase during the first amplification step has been noted. The solution to this problem is to design the mutagenic primer such that its 5'-end follows the wobble position of a codon (Sharrocks & Shaw, 1992). Thus the addition of any nucleotide (if any) at the 3'-end of the complementary strand will be tolerated without an unintended amino acid residue change.

The oligonucleotides designed for PCR mutagenesis and sequencing are displayed in Tables 2.1 and 2.2. Oligonucleotides were prepared by Oswel DNA service, Edinburgh or by Dr M. A. Pickett, Southampton General

Hospital. Oligonucleotides were deprotected from their columns using concentrated ammonium hydroxide, ethanol precipitated, and then resuspended in DEPC treated water prior to storage at 4°C or -20°C. No further purification of the oligonucleotides was required prior to use as PCR primers. Concentrations were determined by UV spectrophotometry.

2.3.4 PCR Mutagenesis Procedure

The following reagents were used in PCR amplifications. Sterile DEPC-treated water, 100 mM dNTP stock (dATP, dCTP, dGTP, dTTP), *Taq* Polymerase (5 units μl^{-1}) and 25 μM $MgCl_2$ (Promega), light mineral oil (Sigma). PCR was performed on a Hybaid Thermocycler. To minimize contamination, PCR was performed in a separate workspace, with designated pipettes, using sterile techniques and minimizing handling of solutions by reducing the number of pipetting steps to a minimum (using 'master mixes'). Reactions were assembled on ice, with the enzyme added last and the mixture overlaid with 100 μl light mineral oil to prevent evaporation of the sample. Typically 20-25 amplification cycles were performed.

Generally PCR was performed in 25-30 cycles on 1-5 ng full-length template cDNA (Figures 2.3 & 2.4), 100 pmol of each primer, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase and 100 μM dNTPs. The first PCR was conducted with the mutagenic primer and the flanking universal primer of opposite orientation. An initial 5 minutes denaturation step at 93-94°C was performed on all reactions. Cycling parameters used for each mutant are displayed in Table 2.3. Each program finished with 5 minutes at 72°C to ensure the product was completely double-stranded. 3-5 μl PCR 1 product was subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide, to verify generation of a single fragment of the correct length. The product was purified using Promega Wizard™ PCR Preps to remove primers, enzyme and unincorporated nucleotides.

PCR 2 was performed on cDNA template with 5 μl (1/10 volume) of the PCR 1 product and the other universal primer as primers, using conditions as described for the first step. Products were verified by gel electrophoresis and purified as above. Fragments from the second PCR were digested with 8-12 U restriction enzyme, phenol-chloroform extracted and ethanol precipitated and washed. α_1-Antitrypsin mutants were digested with *Pst* I, and ovalbumin mutants digested with *Hin*d III. Fragments were ligated into transcription vectors for *in vitro* transcription as mentioned previously.

Primer	Sequence (5′ to 3′)	Description	Direction
AT5uni	CGTG***CTGCAG***ACAGTGAATCGACAA$_{(1)}$TG	5′-Universal with *Pst*I end	→
AT3uni	$_{(1344)}$GATC***CTGCAG***TCCAGCTCAACCCTTC	3′-Universal with *Pst*I end	←
ATP$_{11/12}$A	$_{(1105)}$ACTGAAG**T**TGT**T**GGGGCCATG	P$_{11/12}$ Ala→Val	→
ATP$_{17}$K,P$_{11/12}$A	$_{(1087)}$ACCATCGAC**A**AGAAAGGGACTGAAG**T**TGT**T**GGGGCCATGTTT	PiZ + P$_{11/12}$ Ala→Val	→
ATP$_{14}$R	$_{(1093)}$GACGAGAAGGGGA**GA**GAA	P$_{14}$ Thr→Arg	→
ATZ,P$_{14}$R	$_{(1097)}$A**GA**GAAGCTGCTGG	P$_{14}$ Thr→Arg (for Pi Z)	→
ATS$_{iiyama}$	$_{(217)}$AATATCTTCTTCT**T**CCCAGTGAGC	S$_{iiyama}$ (Ser^{53}→Phe)	→
ATM$_{Malton}$	$_{(217)}$AATATCTTCTTCCCAGTGAGCATC	M$_{Malton}$ (Phe^{52} deleted)	→
ATF^{51}→L	$_{(217)}$AATATC**C**TCTTCTT**C**CCAG	Phe^{51}→Leu (for PiM & PiZ)	→
ATS$_{iiyama}$,F^{51}→L	$_{(217)}$AATATC**C**TCTTCTT**C**CCAGTGAGC	S$_{iiyama}$ (^{53}→Phe), Phe^{51}→Leu	→
ATSeq	$_{(1202)}$TGTTCAATCATTAAGAA	Sequencing primer	←
Fwd 1	$_{(137)}$CCTTCAACAAGATCACC	Sequencing primer	→
▵4B	$_{(1044)}$GGCACCCCCTGAACTCT	Sequencing primer	→

Table 2.1 α_1-Antitrypsin PCR and sequencing oligonucleotides. The alterations as compared to α_1-antitrypsin template are indicated in bold. The position of the 5′ end of each oligonucleotide is indicated in parentheses relative to the first nucleotide of the coding region, except AT5′uni which contains the start codon ($_1$). *Pst*I restriction sites are in bold italics. Primer extension of the coding strand is indicated by → and extension of the opposite strand by ←.

Primer	Sequence (5′ to 3′)	Description	Direction
OV5uni	$_{(-35)}$CTC***AAGCTT***GCCGAAAGAC	5′-Universal with *Hin*dIII end	→
OV3uni	TGA***AAGCTT***GCCTAGATT	3′-Universal with *Hin*dIII end	←
$OVP_{17}K$	$_{(1017)}$CCTGCTT**T**ATTGATT	P_{17} Glu→Lys ("Z" Ovalbumin)	←
$OVP_{14}T,P_{11/12}A$	$_{(1034)}$GACCCT**G**CC**G**CCTCT**G**TGCCTGC	P_{14} Arg→Thr + $P_{11/12}$ Val→Ala	←
OVSeq	$_{(1097)}$AGGAATGGATGGTCAGC	Ovalbumin Sequencing Primer	←

Table 2.2 Ovalbumin PCR and sequencing oligonucleotides. The alterations as compared to ovalbumin template are indicated in bold. The numbers in parentheses refer to the position of the 5′ end of the oligonucleotide relative to the first nucleotide of the coding region. *Hin*dIII restriction sites are in boid italics. Primer extension of the coding strand is indicated by → and extension of the opposite strand by ←.

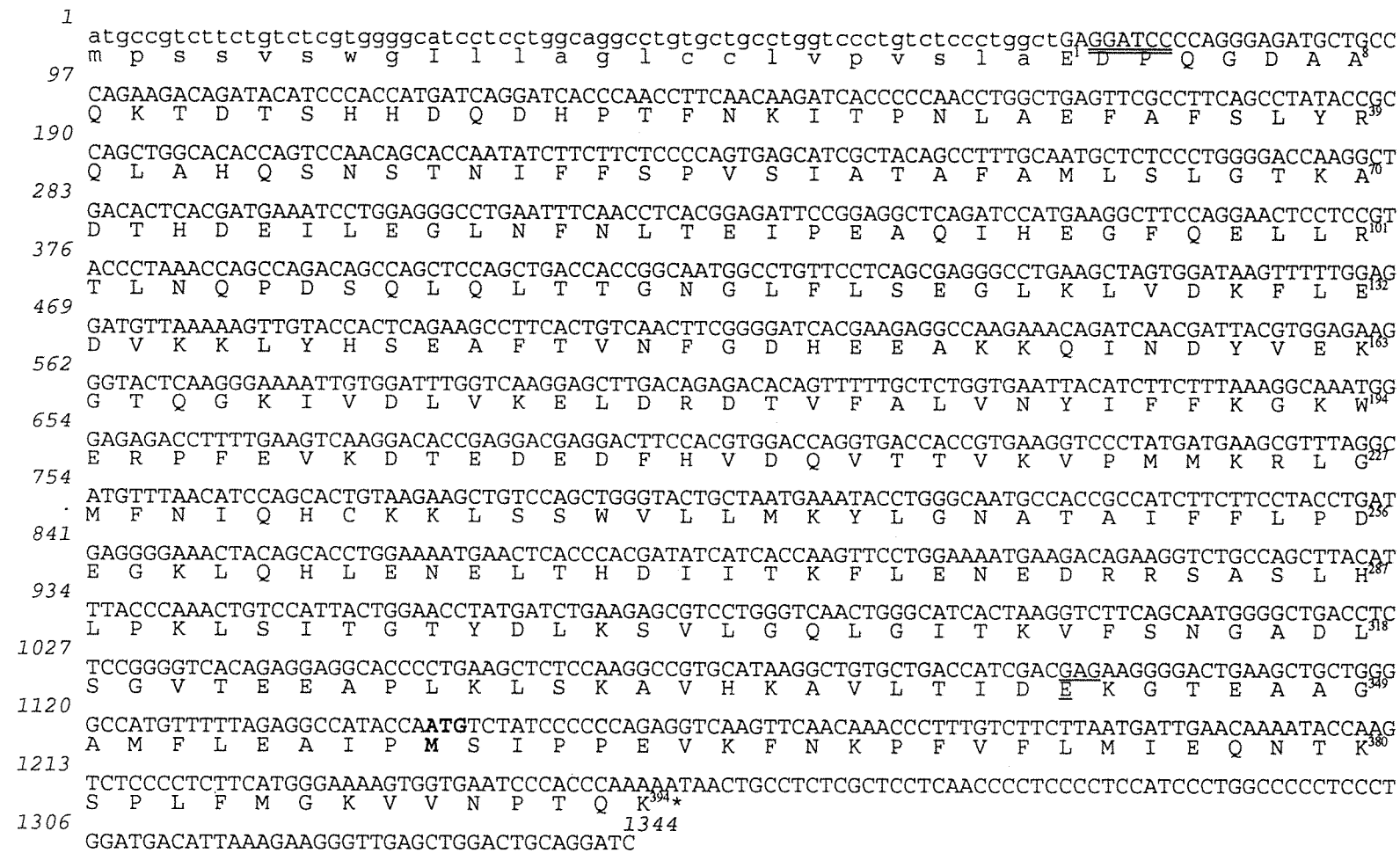


Figure 2.3 The cDNA and amino acid sequence of α_1-antitrypsin. Nucleotide sequence numbers are in italics, on the left, commencing from the initiation codon. The signal peptide is displayed in lower case print. Amino acids are numbered on the right after the signal peptide sequence. The reactive centre residue P_1 Met^{358} is displayed in bold, and the P_{17} mutation site of the Z variant ($Glu^{342} \rightarrow Lys$) is underlined. The *BamH* I site after the signal peptide sequence is double underlined.

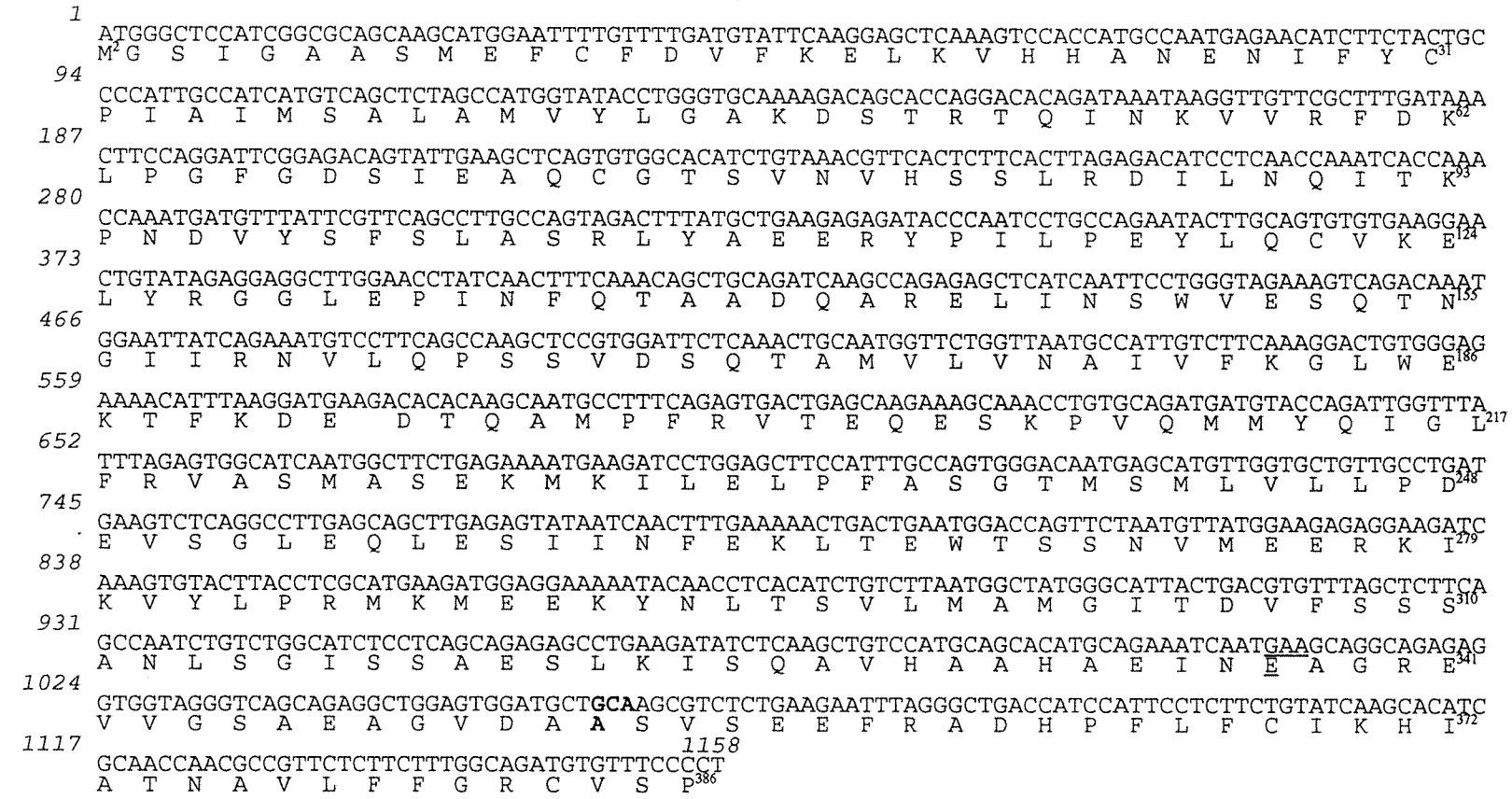


Figure 2.4 The cDNA and amino acid sequence of ovalbumin. Nucleotide sequence numbers are in italics, on the left, commencing from the initiation codon. Amino acids are numbered on the right. The reactive centre residue P_1 Ala[358], on alignment with α_1-antitrypsin, is displayed in bold, and the P_{17} site is underlined.

PCR Product	Mutagenic primer	PCR 1			PCR 2		
		D	A	E	D	A	E
PiM + $P_{11/12}$ Ala→Val	$ATP_{11/12}V$	93(1)	55(1)	72(1)	91(1)	58(1)	72(2)
PiZ + $P_{11/12}$ Ala→Val	$ATP_{17}KP_{11/12}V$	93(1)	45(1)	72(1)	94(1)	55(1)	72(2)
PiM + P_{14} Thr→Arg	$ATP_{14}R$	93(1)	45(1)	72(1)	94(1)	55(1.15)	72(2)
PiZ + P_{14} Thr→Arg	$ATZP_{14}R$	94(1)	55(1)	72(1)	94(1)	55(1)	72(2)
Siiyama	ATS_{iiyama}	93(1)	45(1)	72(1)	94(1)	60(1)	72(2)
Malton	ATM_{Malton}	93(1)	45(1)	72(2)	94(1)	55(1)	72(2)
PiM + Phe^{51}→Leu	$ATPhe^{51}$→Leu	93(1)	55(1)	72(1)	94(1)	55(1)	72(2)
PiZ + Phe^{51}→Leu	$ATPhe^{51}$→Leu$^{\#}$	93(1)	55(1)	72(1)	94(1)	55(1)	72(2)
$OvP_{14}R$→$TP_{11/12}V$→A	$OvP_{14}T, P_{11,12}A$	93(1)	45(1)	72(1)	93(1)	55(1.15)	72(1)
"Z" Ovalbumin	$OVP_{17}K$	93(1)	50(1)	72(1)	93(1)	55(1.15)	72(1)

Table 2.3 PCR amplification conditions for constructing α_1-antitrypsin and ovalbumin mutants. D:Denaturation; A:Annealing; E:Extension; temperatures in °C are indicated for each round of PCR. The numbers in parentheses represent time in minutes. The mutagenic primer used in the first PCR is also indicated. Template used is wild type α_1-antitrypsin or ovalbumin except, $^{\#}$ were PiZ template was used. 20-25 cycles were performed per reaction.

2.4 Transcription and Translation of Mutant Proteins

2.4.1 Plasmid Cloning

DNA mutants produced by PCR and the transcription vector used were digested with the appropriate restriction enzyme(s). Vector and insert were ligated, and transformed into competent cells. Colonies obtained were screened for the presence of the insert by restriction endonuclease analysis. Positive clones were tested by restriction endonuclease analysis for correct orientation of the coding region, downstream of the SP6 RNA polymerase promoter site. Individual clones were sequenced by DNA sequencing (see section 2.2.7) to verify the complete nucleotide sequence with the correct mutation was present. At least two separate clones were obtained for each mutation and stored as 15% (v/v) glycerol cultures stocks at -20°C and -70°C (Sambrook *et al.*, 1989).

Constructs were linearized with a restriction enzyme downstream of the cloned gene for *in vitro* transcription. α_1-Antitrypsin mutants cloned into pSP64TRCF were linearized with *Xba* I. Digestion products were purified by phenol/chloroform extraction and linearized DNA recovered by ethanol precipitation (Sambrook *et al.*, 1989). The integrity of the template was checked by agarose gel electrophoresis prior to transcription.

2.4.2 *In vitro* Transcription

Transcription with SP6 polymerase was performed using Promega Ribomax Transcription System, a modification of the procedure described by Krieg & Melton (1987). 50 μl transcription reactions were performed on 2 μg linearized vector DNA, according to the manufacturer's instructions (30 μM rGTP and 500 μM each of rATP, rCTP, rUTP) with the addition of 3 mM $m^7G(5')ppp(5')G$ cap structure (New England Biolabs) to produce capped RNAs suitable for expression in *Xenopus* oocytes. The significance of a cap

for synthesis of biologically active proteins is important for translation in *Xenopus* oocytes (Drummond *et al.*, 1985). Plasmid template was removed by digestion with RNase-free DNase (Promega) at a concentration of 1 unit μg^{-1}DNA. Transcribed mRNA was phenol/chloroform extracted twice, and RNA recovered by addition of sodium acetate, pH 6.5, to 300 mM and two volumes of ethanol. After precipitation at -70°C for 2-24 hours, the RNA was resuspended in nuclease-free water and aliquots of RNA transcripts were stored under liquid nitrogen.

2.4.3 *In vitro* Translation

RNAs produced *in vitro* were translated in a cell-free rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions with amino acid mixture minus methionine reaction buffer containing [^{35}S] methionine to label protein product(s). Translation products were assayed by TCA precipitation (Sambrook *et al.*, 1989) to analyze amino acid incorporation. If TCA-precipitable counts $\geq 10\times$ over background were obtained translational products were analysed by SDS polyacrylamide gel electrophoresis by the method of Laemmli (1976), followed by fluorography using 'Amplify' (Amersham International). Gels were dried and autoradiographed at -70°C on Fuji RX film. Functional mRNA should produce a strong single protein band of the expected molecular mass.

2.5 *Xenopus* Oocyte Expression System

The preparation and microinjection of *Xenopus laevis* oocytes was as described by Colman (1984). The procedure is outlined below.

2.5.1 Preparation and Microinjection of Oocytes

A female *Xenopus* frog was anaesthetized by submersion in 0.15% Tricaine solution (*ethyl*-*m*-aminobenzoate) for approximately 45 minutes. The

frog was placed ventral side up on a dissecting tray and the ovary was exposed by a incision on the posterior ventral side through the skin and abdominal wall. A lobe of oocytes was teased out with forceps, excised and placed in Barths medium (90 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM K_2SO_4, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 5 mM Hepes pH 7.5). The incision was sutured and the frog allowed to recover for 10-24 hours in distilled water. Oocytes were dissected out singly with forceps as described by Colman (1984). Mature, and healthy oocytes were selected for microinjection (Figure 2.5).

RNA was injected into oocytes using a stereo microscope, micromanipulator, hydraulic syringe and fibre optic light source. Injection needles were pulled from a capillary melting point tube to a diameter of about 50 nm, back-filled with sterile paraffin oil and connected to a micromanipulator. A 10 μl droplet of mRNA was placed on a Parafilm-coated slide stored on ice, and oocytes to be injected placed on an platform under the stereo microscope. Care was taken to prevent oocytes drying out during injection. The needle was loaded with the mRNA and approximately 50 nl (20 ng) injected into the cytoplasmic (vegetal) pole of the oocyte. Control oocytes received either water or were non-injected, with identical results. Oocytes were incubated overnight at 20°C in Barth's medium.

2.5.2 Radiolabelling and Culture of Injected Oocytes

Damaged or unhealthy oocytes were discarded and the remaining oocytes were cultured in the wells of a microtitre plate for 7 hours in batches of 4 oocytes with 25 μl Barth's medium supplemented with 0.2 $mCi.ml^{-1}$ L-[^{35}S] methionine per well. 4-6 wells (16-24 oocytes) injected with each mRNA were used per experiment. After the pulse, the radiolabelled medium was replaced with fresh unlabelled medium containing 10 mM methionine and the incubation chased overnight. Any wells showing evidence of cell lysis were discarded before further processing of the

samples.

2.5.3 Preparation of Oocyte Media and Extracts

[according to Colman, 1984]

The media surrounding microinjected oocytes was removed to tubes, avoiding damage to the oocytes, and the oocytes covered with Barth's medium to prevent drying out. The media was clarified by microfugation for 10 minutes, and the supernatant removed to a fresh tube on ice containing 3 volumes of immunoprecipitation buffer [0.1 M Tris-HCl, pH 8, 0.1 M KCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1 mM PMSF]. After centrifugation as before, the supernatant was transferred to a fresh tube for immunoprecipitation analysis.

Batches of oocytes were homogenized in 20 μl homogenization buffer [20 mM Tris-HCl, pH 7.6, 0.1 NaCl, 1% (v/v) Triton X-100, 1 mM PMSF] per oocyte. Homogenate was centrifuged for 10 minutes to remove the lipid pellet. An equal volume of immunoprecipitation buffer was added to the supernatant, mixed and centrifuged as before. The supernatant was transferred to a fresh tube for immunoprecipitation.

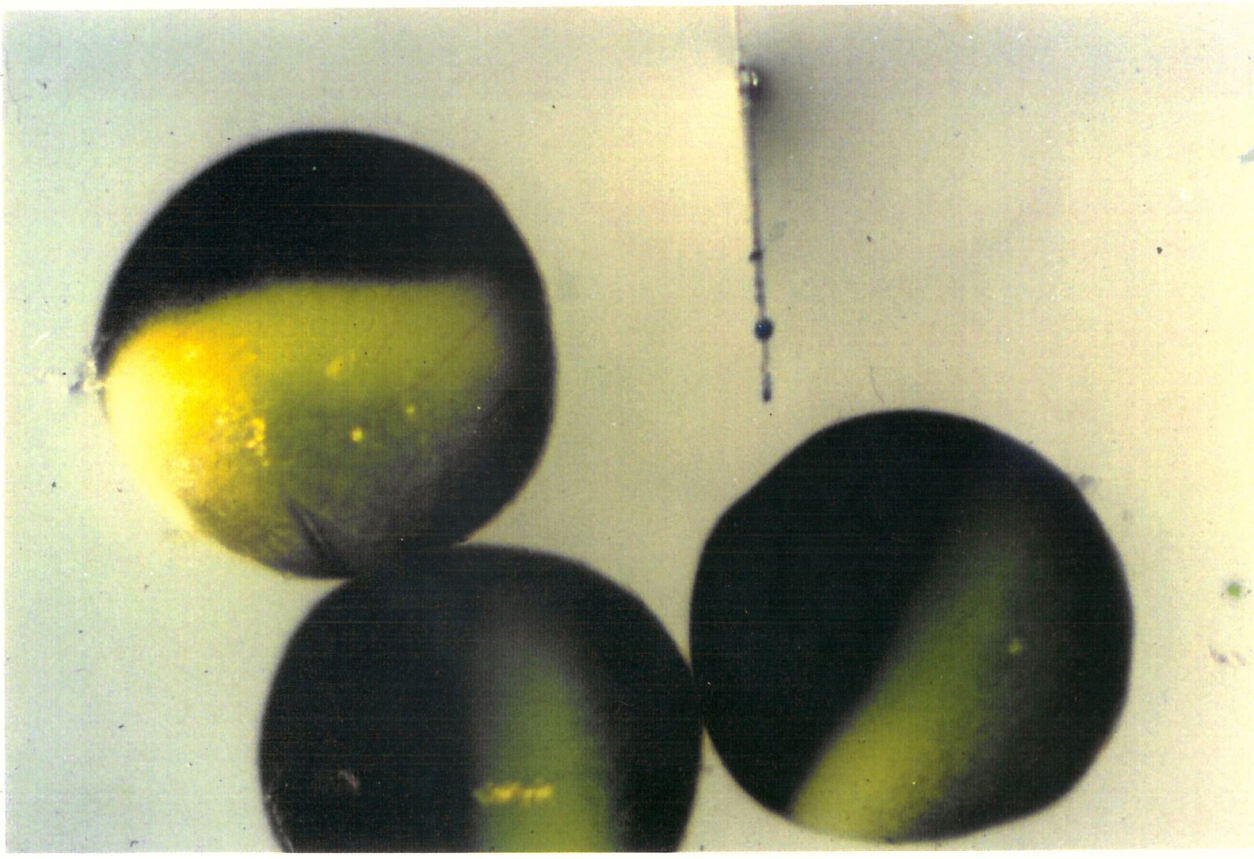


Figure 2.5 Oocytes from *Xenopus laevis*. (x50 Magnification)

2.5.4 Immunoprecipitation

Immunoprecipitation analysis of radiolabelled cells is a powerful technique which allows the synthesis and processing of a protein to be monitored. Oocyte extracts and media were immunoprecipitated as described by Foreman *et al.* (1984), and the procedure is shown below.

To the samples 4-5 μl of pre-immune serum was added and the mixture left for 30 minutes at 4°C before addition of 40 μl of a 1:1 slurry of Protein-A-agarose (Sigma) in immunoprecipitation buffer. The samples were agitated gently for 1 hour, and centrifuged for 1-2 minutes to remove the protein-A-agarose slurry. 2-3 μl of specific antiserum (for α_1-antitrypsin, anti-human α_1-antitrypsin and for ovalbumin, anti-ovalbumin) was added to each sample and the mixture incubated for 90 minutes at 4°C. After removing the supernatant, 35 μl Protein-A-agarose slurry was added and incubated with shaking for 2 hours at 4°C. The immunoabsorbed pellet obtained after centrifugation was washed three times with 1 ml aliquots of immunoprecipitation buffer, and then washed two times with 1 ml aliquots of cold water. The pellet was resuspended in 50 μl Maizel sample buffer [50 mM Tris-HCl pH 6.7, 1% (w/v) SDS, 20% (v/v) glycerol, 15 (v/v) 2-mercapto-ethanol) and boiled for 6 minutes at 95°C. After centrifugation for 1 minute, the supernatant was removed into fresh tubes and eluted proteins analysed on 12.5% (w/v) or 10% (w/v) SDS-polyacrylamide gels followed by fluorography using 'Amplify' (Amershan International plc) and autoradiography (as for *in vitro* translation analysis). Quantitation of radiolabelled proteins was performed by excising gel fragments, resuspending in 3 ml Optiphase Hi-safe scintillant (LKB) and counting on a Beckman LKB Scintillation counter.

2.6 Endoglycosidase H Digestion

After immunoprecipitation the immunoadsorbed pellet was suspended

in 60 μl of 50 mM Tris-HCl pH 5.5 containing 1% (w\v) SDS, 20% (v\v) glycerol and 1% (v\v) 2-mercaptoethanol and incubated for 5 minutes at 95°C. After cooling, tubes were centrifuged briefly and each supernatant divided into two 30 μl aliquots, one of which received 10 μl of 1 $mU.\mu l^{-1}$ endoglycosidase H (Endo H) containing 1 mM phenylmethylsulphonyl fluoride. Endo H digestion was carried out for 16 hours at 37°C (Trimble & Maley, 1984). Following this incubation, samples were subjected to SDS-PAGE and radiolabelled α_1-antitrypsin was detected as described above.

2.7 Active Site Titration of α_1-Antitrypsin

[according to Lomas *et al.*, 1993]

The active site titration of oocyte secreted α_1-antitrypsin was performed by incubating 5 μl bovine α-chymotrypsin of known active site (Kézdy & Kaiser, 1970) with increasing concentrations of oocyte secretion media (0-5 μl), and reaction buffer [0.03 M sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4] in a cuvette to give a final volume of 100 μl. The mixture was incubated for 30 minutes at 37°C prior to the addition of 0.9 ml reaction buffer containing 4 μl of substrate succinyl-L-alanyl-L-alanyl-prolyl-L-phenylalanalyl-*p*-nitroanilide(Suc-Ala-Ala-Pro-Phe-pNA; final concentration 0.16 mM) to a final volume of 1 ml. The length of incubation was at least five times the half-life for the association of enzyme with inhibitor. This was determined from the equation $t_{½} = \epsilon_o.k_{ass}$), where ϵ_o is the initial enzyme (chymotrypsin) concentration, and k_{ass} is the association rate constant of chymotrypsin with α_1-antitrypsin. The change in OD_{405} was observed over a period of 60 minutes and a graph constructed of the change in optical density/minute (OD/min) against the volume of inhibitor (oocyte secreted media). The active site of α_1-antitrypsin was determined by extrapolating the linear portion of the curve to the x-axis (Beatty, *et al.*, 1980). At the x-intercept there is no residual proteolytic activity (OD/min is zero), hence assuming a stable 1:1 chymotrypsin:α_1-antitrypsin complex, the amount of chymotrypsin titrates the α_1-antitrypsin

present.

2.8 α_1-Antitrypsin:Enzyme Complex Formation

α_1-Antitrypsin secreted into the incubation media by oocytes was incubated with porcine pancreatic elastase (Sigma) or bovine α-chymotrypsin (Sigma Type II) in reaction buffer, 0.03 M Sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4 at 37°C for 15-30 minutes. Formation of stable binary complexes and postcomplex cleaved α_1-antitrypsin was assessed by immunoprecipitation of the incubation mixture followed by SDS-PAGE.

The functional activity of intracellular M and Z α_1-antitrypsin was also assessed on diethylamine-eluted immunoprecipitates (Errington *et al.*, 1985). This involved immunoprecipitation of oocyte homogenates, not boiling the immunoadsorbed pellet but instead resuspending in 100 μl 50 mM diethylamine, lyophilizing the pellet and then resuspending in 50 mM Tris-HCl pH 7. Later studies were performed by resuspending the pellet in reaction buffer, incubating with chymotrypsin and terminating the reaction with Maizel sample buffer, prior to analysis by SDS-PAGE.

2.9 Gel Filtration Chromatography

Oocytes injected with M and Z α_1-antitrypsin and incubated overnight in L-[^{35}S] methionine media were homogenized in homogenization buffer [20 mM Tris-HCl, pH 7.6, 0.1 NaCl, 1% (v/v) Triton X-100] that lacked PMSF. Homogenates were centrifuged twice for 10 minutes to remove lipid and the supernatant applied to a Sepharose CL-6B column (Sigma; separates 10 000-4 000 000 molecular weight molecules) of 15 ml total bed volume, with a diameter of 1 cm and eluted with homogenization buffer. 50 fractions of 470 μl were collected, and from each fraction 300 μl was removed and transferred to tubes containing 3 ml Optiphase Hi-safe scintillant (LKB). Total

counts in each tube were determined in a liquid scintillation counter to ascertain into which fractions radiolabelled products elute. These fractions were then immunoprecipitated by addition of anti-α_1-antitrypsin as described in section 2.5.4. A 5 μl aliquot of each 50 μl immunoprecipitated product was spotted directly onto a Whatmann 3 MM filter and counted in a liquid scintillation counter, as above. Samples with counts above background levels were analysed by SDS-PAGE and fluorography.

2.10 Statistical Analysis

Radioactive counts were expressed as a percentage of the total immunopreipitable material which, in all experiments, exceeded 7000 cpm. Statistical significance was assessed using the Students t-test and expressed as a probability value where appropriate.

3. *XENOPUS* OOCYTE PROCESSING OF α_1-ANTITRYPSIN ACCUMULATION VARIANTS

3.1 INTRODUCTION

The Z variant (Glu^{342}→Lys) of α_1-antitrypsin is present in 4% of Northern Europeans (Laurell & Eriksson, 1963). In the homozygote it results in both an increased risk of liver disease and a plasma deficiency that predisposes to progressive lung disease (Sharp *et al.*, 1969; Sveger, 1976; Travis & Salvesen, 1983). An accompanying feature in the homozygote is the presence of inclusions of Z α_1-antitrypsin at the site of synthesis in the endoplasmic reticulum of the hepatocyte (Sharp, 1971). It has been proposed recently that the accumulation occurs due to a novel intermolecular interaction to give a loop-sheet polymerization of the variant α_1-antitrypsin with consequent disruption of its processing and secretion (Lomas *et al.*, 1992). This idea has been supported by the recent demonstration of spontaneous loop-sheet polymerization (Lomas *et al.*, 1993b) in another variant, α_1-antitrypsin S_{iiyama} (Ser^{53}→Phe) (Seyama *et al.*, 1991), which is associated with the same pattern of hepatocyte aggregates and plasma deficiency as seen in the more common Z variant. More recently, spontaneous loop-sheet polymerization has also been shown (Lomas *et al.*, 1994) in another rare deficiency variant, M_{Malton} (Phe^{52} deleted) (Curiel *et al.*, 1989c; Frazier *et al.*, 1989; Graham *et al.*, 1989), with similar liver inclusions to Z α_1-antitrypsin.

The S_{iiyama} and M_{Malton} mutants were constructed by PCR mutagenesis (Landt *et al.*, 1990) from full length α_1-antitrypsin cDNA (Ciliberto *et al.*, 1985) as described in *Methods* (Tables 2.1 and 2.3). The pattern of synthesis and secretion of Z, S_{iiyama} and M_{Malton} α_1-antitrypsin variants in *Xenopus* oocytes were investigated in comparison to that seen for normal M α_1-antitrypsin. Investigations into the site of accumulation and activity assays of secreted and accumulated were conducted as described in *Methods*.

3.2 RESULTS

3.2.1 Oocyte Processing of α_1-Antitrypsin Accumulation Variants

PCR based site-directed mutagenesis was used to reconstruct the naturally occurring S_{iiyama} (Seyama *et al.*, 1991) and M_{Malton} (Curiel *et al.*, 1989c; Frazier *et al.*, 1989; Graham *et al.*, 1989) variants of human α_1-antitrypsin (Table 2.3). To investigate the effect of both mutations on the secretion of α_1-antitrypsin PiM, PiZ, S_{iiyama}, and M_{Malton} encoding RNAs were injected into *Xenopus* oocytes. Injected oocytes were incubated in medium containing L-[^{35}S] methionine and radiolabelled α_1-antitrypsin immunoprecipitated from the incubation media and oocyte extract. Immunoprecipitated proteins were resolved by SDS polyacrylamide gel electrophoresis (Figures 3.1 and 3.2). All transcripts produced a 54 kDa intracellular species which represents a partially glycosylated form of the inhibitor. The oocyte incubation media contained a 56 kDa secreted protein which is a result of further glycosylation of the oligosaccharide moieties, presumably within the *trans* Golgi network prior to α_1-antitrypsin export. However, it was evident from the fluorograph that secretion of normal, M α_1-antitrypsin was far greater than that of Z, S_{iiyama} and M_{Malton} variants. The estimated molecular mass of secreted α_1-antitrypsin as reported by various authors, ranges over 52-58 kDa, depending on the SDS-PAGE conditions and molecular mass standards used.

To quantify differences in the extent of secretion of the α_1-antitrypsin constructs the α_1-antitrypsin secreted and α_1-antitrypsin oocyte bands were excised, counted and the experiment repeated with different batches of oocytes to eliminate, as far as possible, oocyte variation. Figure 3.3 shows the amount of inhibitor secreted expressed as a percentage of the total synthesized. The S_{iiyama} variant of α_1-antitrypsin (12.4% secreted $\pm$3.8) accumulates in oocytes to a similar degree as the Z variant (11.1% $\pm$3.3), whilst M α_1-antitrypsin (65.0% secreted $\pm$7.6) is more readily secreted from

these cells. M_{Malton} also accumulates in the oocytes, but secretion was moderately increased (19.8% secreted ± 3.1) in comparison to the Z and S_{iiyama} deficiency variants.

3.2.2 Endoglycosidase H Digestion

Secretory proteins such as α_1-antitrypsin are translocated into the lumen of the endoplasmic reticulum during synthesis although, transport-impaired human α_1-antitrypsin variants appear to be retained within the ER compartment (Sharp *et al.*, 1971; Sifers *et al.*, 1989). Endoglycosidase H cleaves the high mannose asparagine-linked oligosaccharides of glycoproteins in the ER and *cis* Golgi structures but has no effect on the terminally glycosylated oligosaccharides produced following transfer of the protein to more distal parts of the Golgi complex (Figure 1.14) (Lodish *et al.*, 1983). Thus the susceptibility of intracellular protein to Endo H modification locates the protein to an early stage of the secretory pathway. Immunoprecipitated protein was treated with Endoglycosidase H prior to analysis by SDS-PAGE to determine the intracellular location of the 54 kDa α_1-antitrypsin seen in oocytes. As shown in Figures 3.4 and 3.5, the intracellular 54 kDa species is converted to a single band of molecular mass 46 kDa on Endo H digestion. Intracellular Pi Z, S_{iiyama} and M_{Malton} proteins were sensitive to Endo H digestion indicating that the mutant proteins accumulate in a pre Golgi compartment, probably the ER. Digestion with Endo H had no effect on secreted M, Z, S_{iiyama} and M_{Malton} proteins which were terminally glycosylated. These results indicate that M α_1-antitrypsin is efficiently transported from the ER to the Golgi, and relatively little of the Z, S_{iiyama} or M_{Malton} proteins reach the Golgi apparatus.

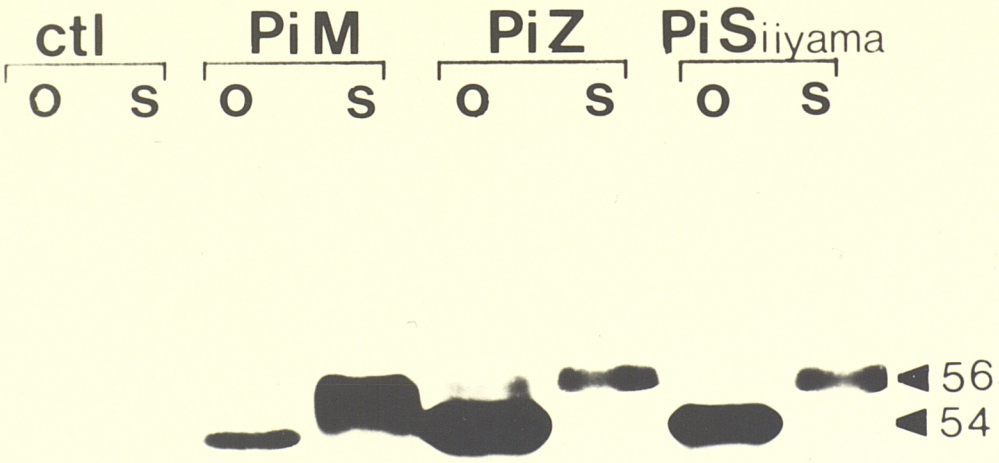


Figure 3.1 Synthesis of M, Z and S_{iiyama} antitrypsins in *Xenopus* oocytes. Twenty Oocytes were injected with messenger RNA for a given antitrypsin variant and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media then separated by SDS-PAGE as described in *Methods*. O represents oocyte extract and S represents material secreted into the surrounding medium. Control oocytes were injected with an equivalent volume of distilled water. Molecular mass was determined by the co-migration of standard protein markers.

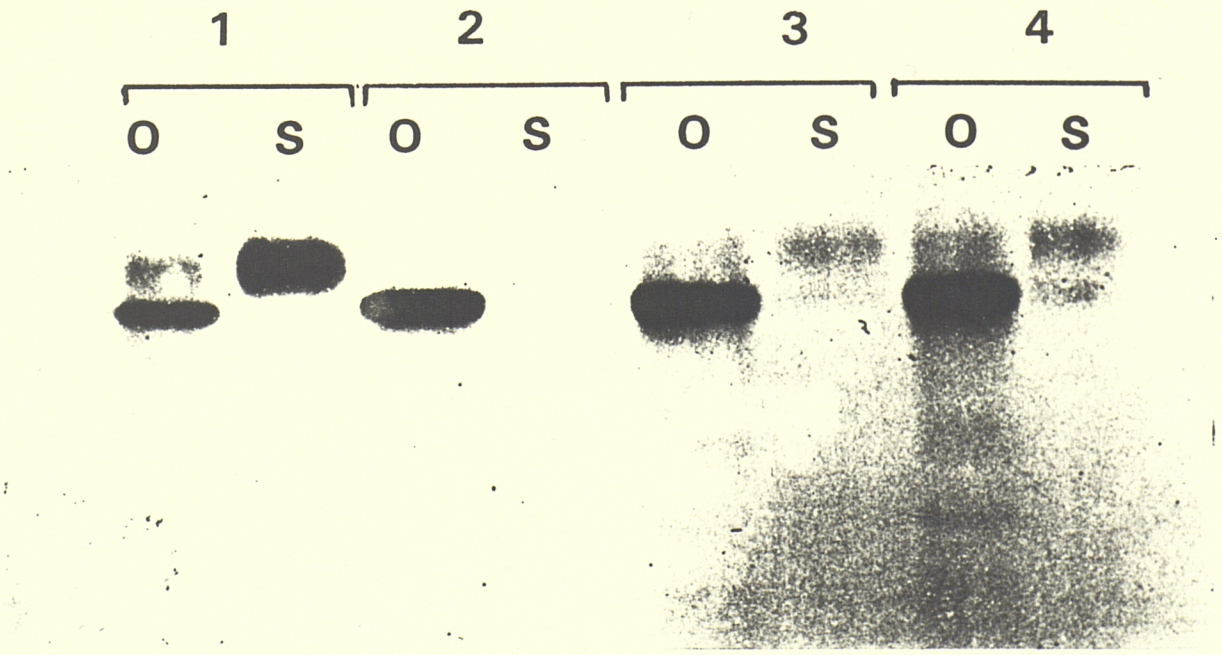


Figure 3.2 Synthesis of M, Z and M_{Malton} antitrypsins in *Xenopus* oocytes. Twenty Oocytes were injected with messenger RNA for a given antitrypsin variant and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media then separated by SDS-PAGE as described in *Methods*. 1: M α_1-antitrypsin; 2: Z α_1-antitrypsin; 3 & 4 are two individual M_{Malton} clones. O represents oocyte extract and S represents material secreted into the surrounding medium. Control oocytes were injected with an equivalent volume of distilled water. Molecular mass was determined by the co-migration of standard protein markers.

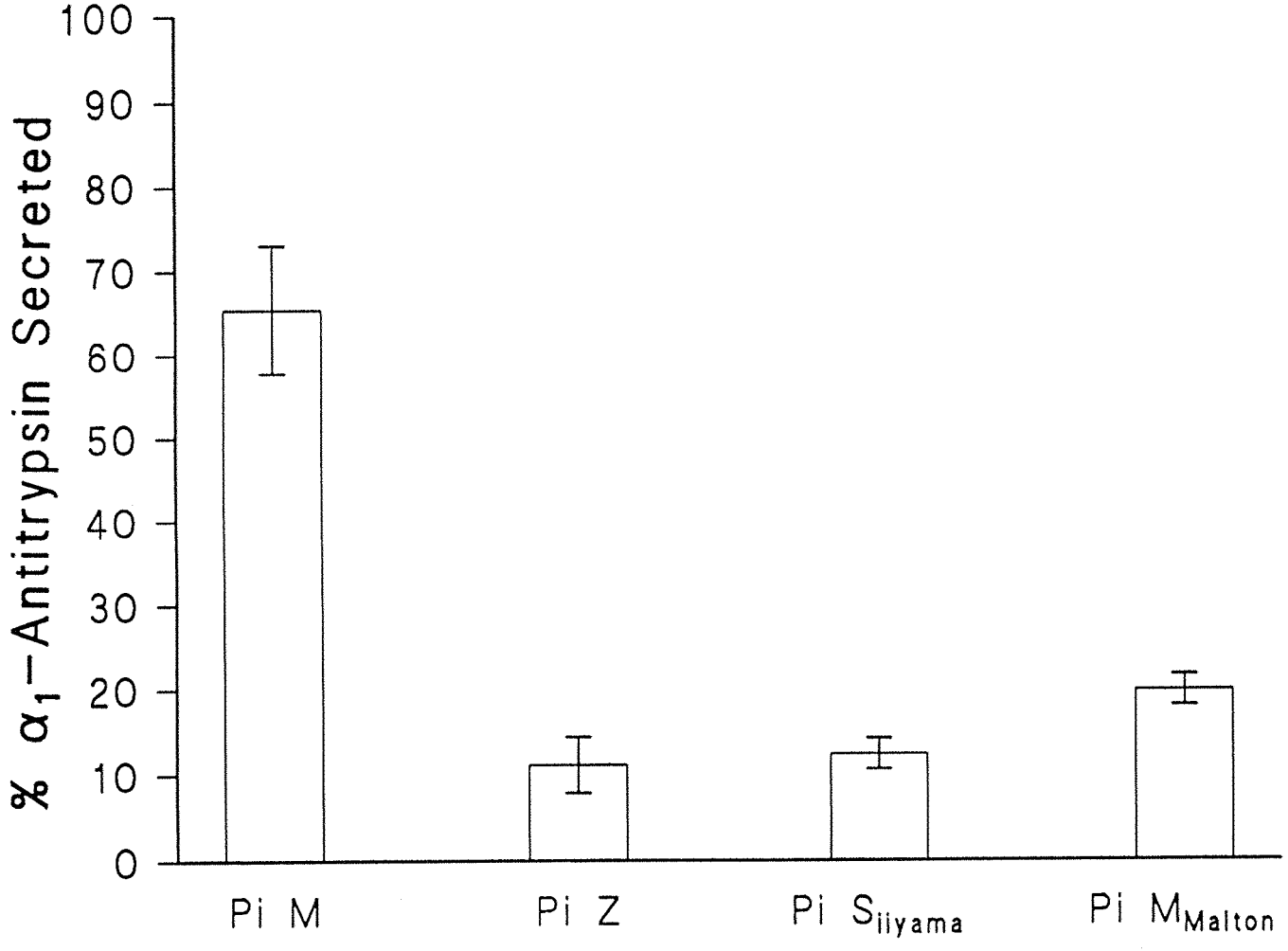


Figure 3.3 Quantitation of the relative amounts of M, Z, S_{Iiyama} and M_{Malton} antitrypsins secreted from microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Amounts of secreted antitrypsin are expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least seven different experiments using oocytes from five animals. Values shown are expressed as ± the standard error of the mean.

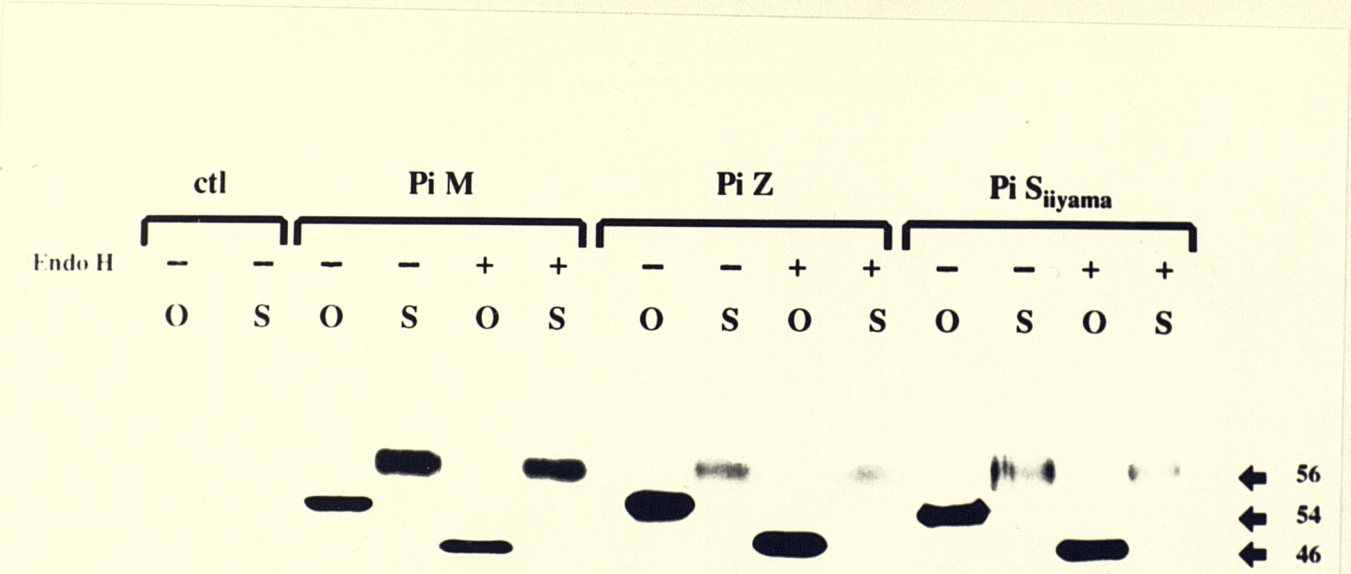


Figure 3.4 Endoglycosidase H sensitivity of M, Z and S_{iiyama} secreted and retained antitrypsins. Variant antitrypsins synthesised in oocytes were purified as before and the immunoadsorbed protein incubated with Endoglycosidase H. Digested material was separated by SDS-PAGE and compared to non-digested controls. O is immunoreactive protein from oocyte extracts and S is secreted inhibitor. Control oocytes were water injected as before.

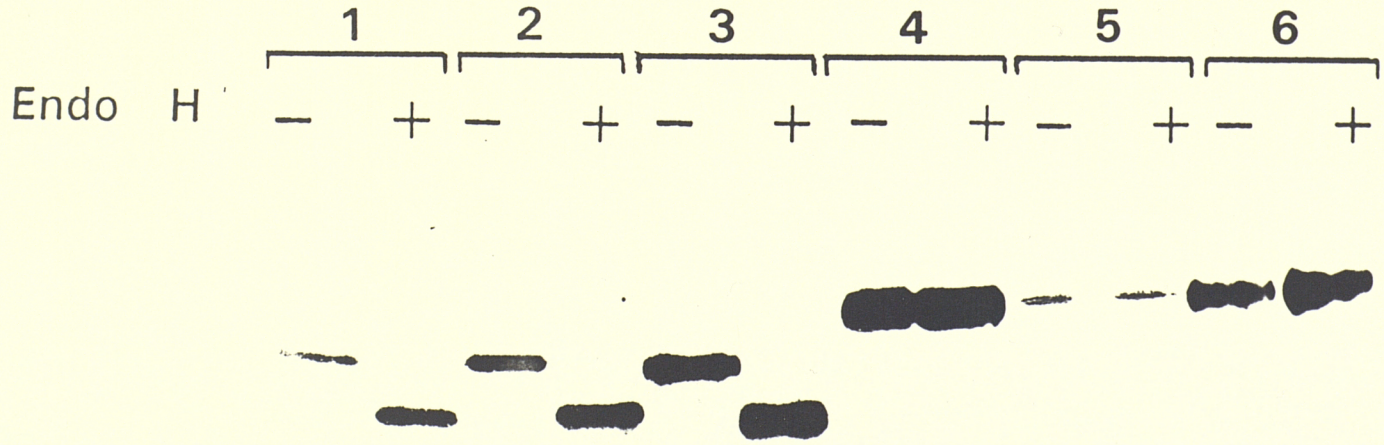


Figure 3.5 Endoglycosidase H sensitivity of M, Z and M_{Malton} secreted and retained antitrypsins. Variant antitrypsins synthesised in oocytes were purified as before and the immunoadsorbed protein incubated with Endoglycosidase H. Digested material was separated by SDS-PAGE. Lanes 1, 2 and 3 represent immunoreactive protein from oocyte extracts and lanes 4, 5 and 6 represent secreted inhibitor. Lanes 1 & 4 are M α_1-antitrypsin; lanes 2 & 5 are Z α_1-antitrypsin; and lanes 3 & 6 are M_{Malton} α_1-antitrypsin.

3.2.3 Active Site Titration of Secreted α_1-Antitrypsin

Oocytes were injected with M, Z, and S_{iiyama} mRNAs and incubated in medium containing L-[^{35}S] methionine for 48 hours. Secreted media was collected for active site titration analysis (Kézdy & Kaiser, 1970). 50 μl water injected control media was incubated with 5 μl bovine chymotrypsin (0.1 pmol active site), and reaction buffer [0.03M Sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4] in a cuvette to give a final volume of 100 μl. The mixture was incubated for 30 minutes at 37°C prior to the addition of 0.9 ml reaction buffer containing 4 μl of substrate succinyl-L-alanyl-L-alanyl-prolyl-L-phenylalanalyl-*p*-nitroanilide(Suc-Ala-Ala-Pro-Phe-pNA; final concentration 0.16 mM) to a final volume of 1 ml. The change in OD_{405} over a period of 60 minutes was 0.0119 OD/min. A control reading of 0.2578 OD/min was obtained with chymotrypsin alone in the reaction buffer. Thus the water injected controls inhibit chymotrypsin significantly, probably due to endogenous proteinase inhibitors secreted by the *Xenopus* oocytes. However, activity assays were conducted in duplicate on 3-5 μl oocyte secreted media using the conditions above and the OD/min for water injected controls subtracted from the OD/min of M, Z and S_{iiyama} α_1-antitrypsins. No difference was observed between water injected oocytes and α_1-antitrypsin Z or S_{iiyama} injected oocytes (data not shown), presumably due to lack of significant amounts of secreted α_1-antitrypsin in these mutants.

3.2.4 Functional Activity of Oocyte Processed α_1-Antitrypsin

(1) Elastase Complexes

α_1-Antitrypsin and elastase form a stable covalent complex, even when boiled in sodium dodecyl sulphate (SDS) under reducing conditions (Owen, 1975; Cohen *et al.*, 1978; Beatty & Travis, 1980). To assess the functional activity of M and Z α_1-antitrypsin synthesized by oocytes, aliquots

from the [^{35}S] methionine labelled media surrounding the oocytes was incubated with porcine pancreatic elastase at 37°C for 15 minutes. The reaction mixture was immunoprecipitated and SDS-PAGE performed to assay for complex formation. Incubation of secreted M α_1-antitrypsin with increasing concentrations of elastase resulted in complex formation and then cleavage of the reactive loop (Figure 3.6). The complex formed with M α_1-antitrypsin (Mwt. 56 kDa) and elastase (Mwt. 24 kDa) ran at approximately 80 kDa, consistent with a equimolar α_1-antitrypsin-elastase complex, and in agreement with a similar experiment conducted in *Xenopus* oocytes (Errington *et al.*, 1985). The experiment was repeated with Z α_1-antitrypsin, but because only 15% of the inhibitor synthesized is secreted, no complex formation was detected by SDS-PAGE (data not shown). Moreover, when the experiment with M α_1-antitrypsin was repeated the previous result could not be reproduced; no binary complex formation could be detected. Incubation of elastase with immunoprecipitated M and Z α_1-antitrypsin using the diethylamine extraction procedure of Errington *et al.* (1985) (see Methods) also did not result in complex formation. Later, it was decided to use the proteinase chymotrypsin instead of elastase.

(ii) Chymotrypsin Complexes

α_1-Antitrypsin was immunoprecipitated from M and Z α_1-antitrypsin messenger RNA injected oocytes, but the final α_1-antitrypsin immunoadsorbed pellet was resuspended in reaction buffer [0.03 M Sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4], and incubated with unlabelled α_1-antitrypsin (6 μg) and chymotrypsin, 40 μl total, volume for 30 minutes at 37°C. The reaction was terminated on addition of 10 μl Maizel sample buffer and analyzed by SDS-PAGE (Figure 3.7). Both intracellular and secreted M α_1-antitrypsin produced a α_1-antitrypsin band of lower molecular weight in the presence of chymotrypsin, signifying cleavage by chymotrypsin. However, oocyte retained Z α_1-antitrypsin was not cleaved by chymotrypsin. This maybe because the reactive centre loop of

intracellular Z α_1-antitrypsin is inaccessible to proteolytic attack by chymotrypsin. It is noticeable from the gel (Figure 3.7) that both M and Z α_1-antitrypsin undergo immense degradation during incubation with chymotrypsin, very little inhibitor remaining after the 30 minute incubation period.

A study was conducted to determine the concentration of chymotrypsin required to form a SDS-stable complex with unlabelled M α_1-antitrypsin, prior to reactive loop cleavage. 6 μg of purified, unlabelled human α_1-antitrypsin (kind gift from D. A. Lomas) was incubated with different ratios of chymotrypsin in reaction buffer (40 μl total volume) for 30 minutes at 37°C. SDS-PAGE analysis (Figure 3.8), reveals that 1:0.25 and 1:0.5 ratios of α_1-antitrypsin:chymotrypsin formed SDS-stable complexes. Higher concentrations of chymotrypsin resulted in cleavage of α_1-antitrypsin. Thus, future experiments on oocyte derived α_1-antitrypsin were conducted with 6 μg unlabelled α_1-antitrypsin and chymotrypsin to give a α_1-antitrypsin:chymotrypsin ratio of 1:0.5. Experiments were conducted on oocyte immunoprecipitates from Z, and S_{iiyama} α_1-antitrypsins but both unexpectedly showed cleavage of oocyte retained α_1-antitrypsin; Figure 3.9 shows a typical result from M and Z α_1-antitrypsin. It is interesting to note that on incubation of equivalent amounts of α_1-antitrypsin (lane a) with increasing chymotrypsin concentrations, M α_1-antitrypsin was more susceptible to degradation than Z α_1-antitrypsin. Very little cleaved species of radiolabelled M α_1-antitrypsin remained following treatment with chymotrypsin but more species of cleaved Z α_1-antitrypsin prevailed. In particular a species of very low molecular mass remains in all incubations of Z α_1-antitrypsin containing chymotrypsin. This may represent part of the cleaved reactive centre loop, but isolation of this species for amino acid sequence analysis as not pursued. In subsequent experiments the molarity of chymotrypsin used was varied, Triton X-100 was removed from the homogenization and immunoprecipitation buffers (data not shown) because it may disrupt α_1-antitrypsin polymers, but the result obtained previously

with chymotrypsin (Figure 3.7) that showed intracellular Z α_1-antitrypsin was inaccessible to cleavage was never reproduced.

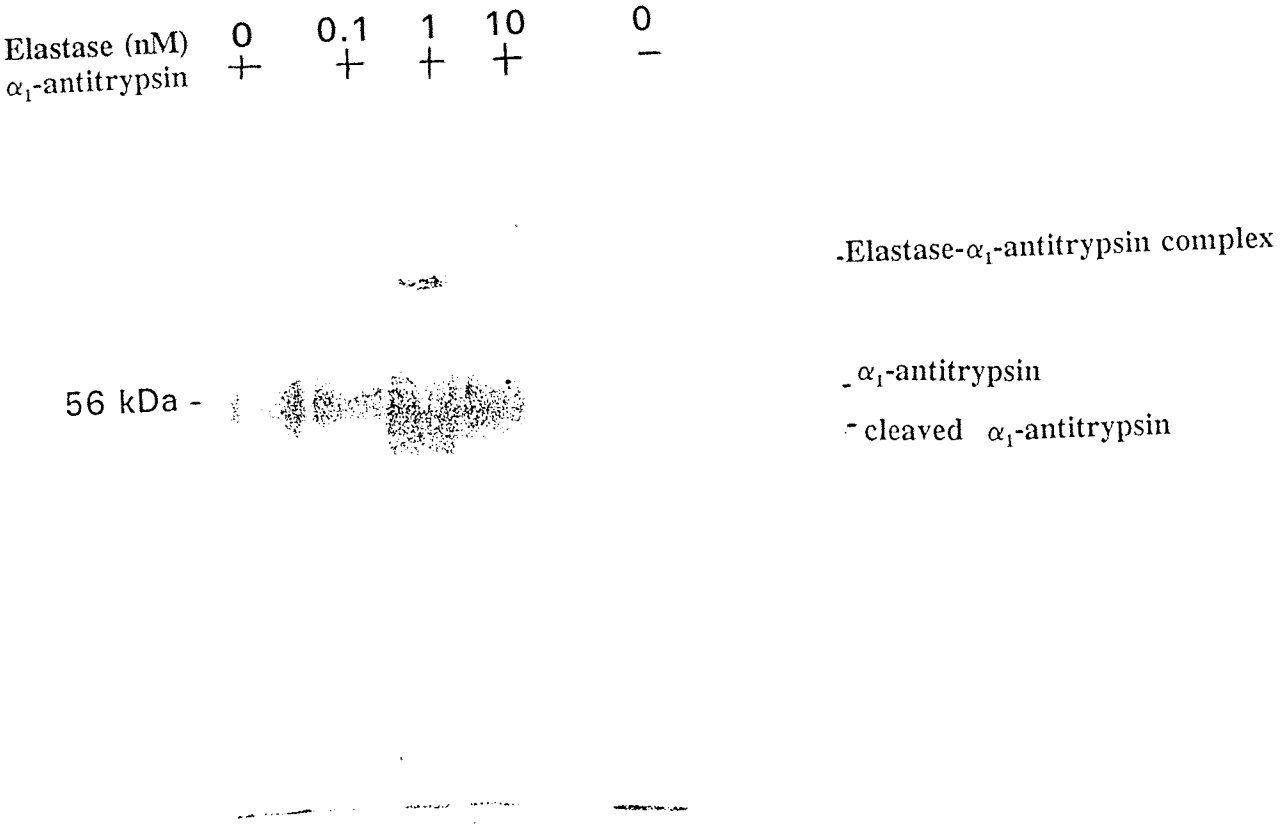


Figure 3.6 Binary complex formation of oocyte secreted M α_1-antitrypsin with porcine pancreatic elastase. 25 μl L-[^{35}S] methionine labelled media surrounding 4 oocytes injected with M α_1-antitrypsin mRNA, incubated 24 hours at 20°C, was reacted with porcine pancreatic elastase in reaction buffer (0.03 M Sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4) to give a final volume of 100 μl.The mixture was incubated for 15 minutes at 37°C prior to immunoprecipitation and separation by SDS-PAGE as described in *Methods*.

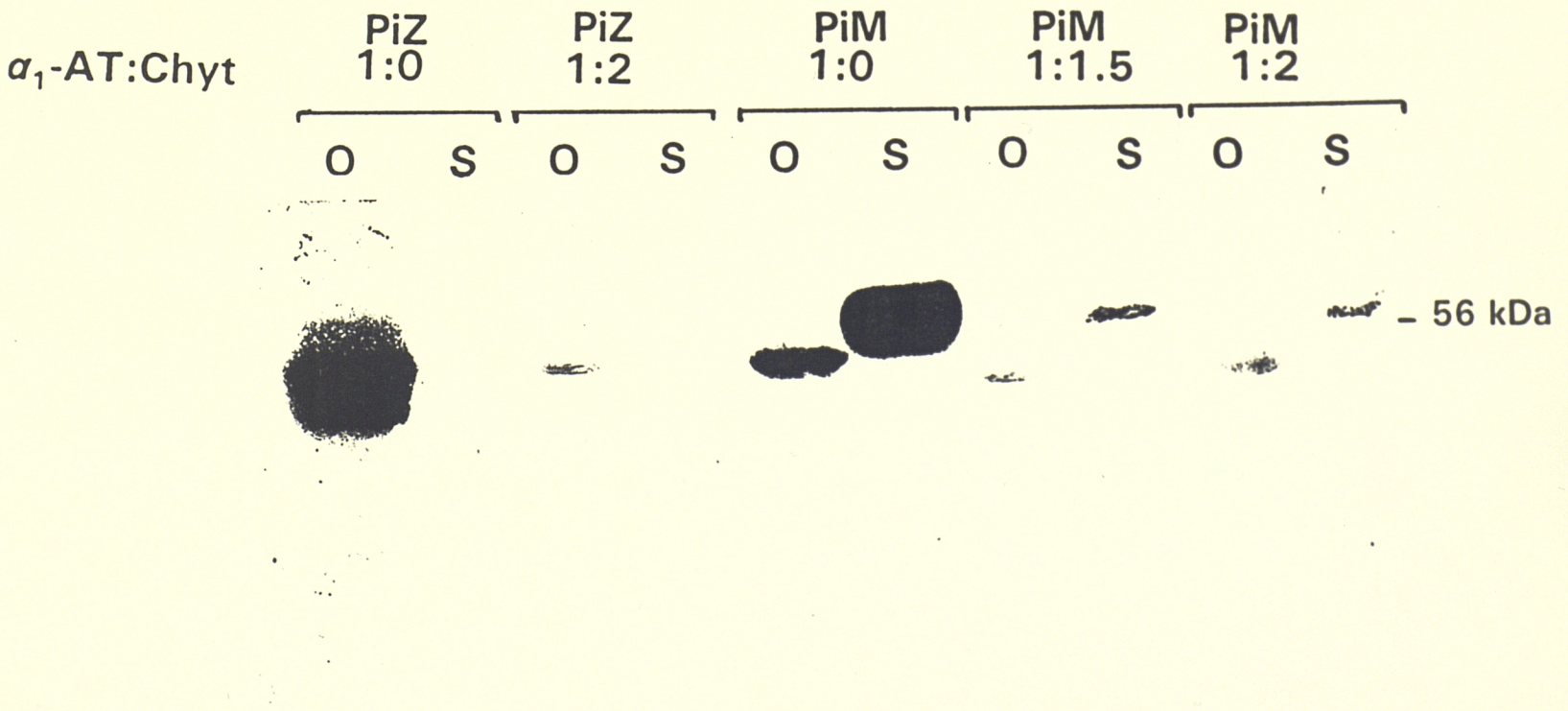


Figure 3.7 **Incubation of M and Z α_1-antitrypsin oocyte retained and secreted material with bovine chymotrypsin.** Twenty oocytes were injected with mRNA for either M or Z α_1-antitrypsin, and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell homogenates (O) and incubation media (S). The immunoadsorbed pellet was resuspended in 60 μl reaction buffer, and 20 μl aliquots were incubated with 6 μg unlabelled human α_1-antitrypsin and bovine chymotrypsin in reaction buffer (40 μl total volume) for 30 minutes at 37°C prior to addition of 10 μl Maizel buffer and analysis by SDS-PAGE as described in *Methods.* Oocyte retained M α_1-antitrypsin is cleaved at 1:1.5 and 1:2 molar ratios of α_1-antitrypsin:chymotrypsin.

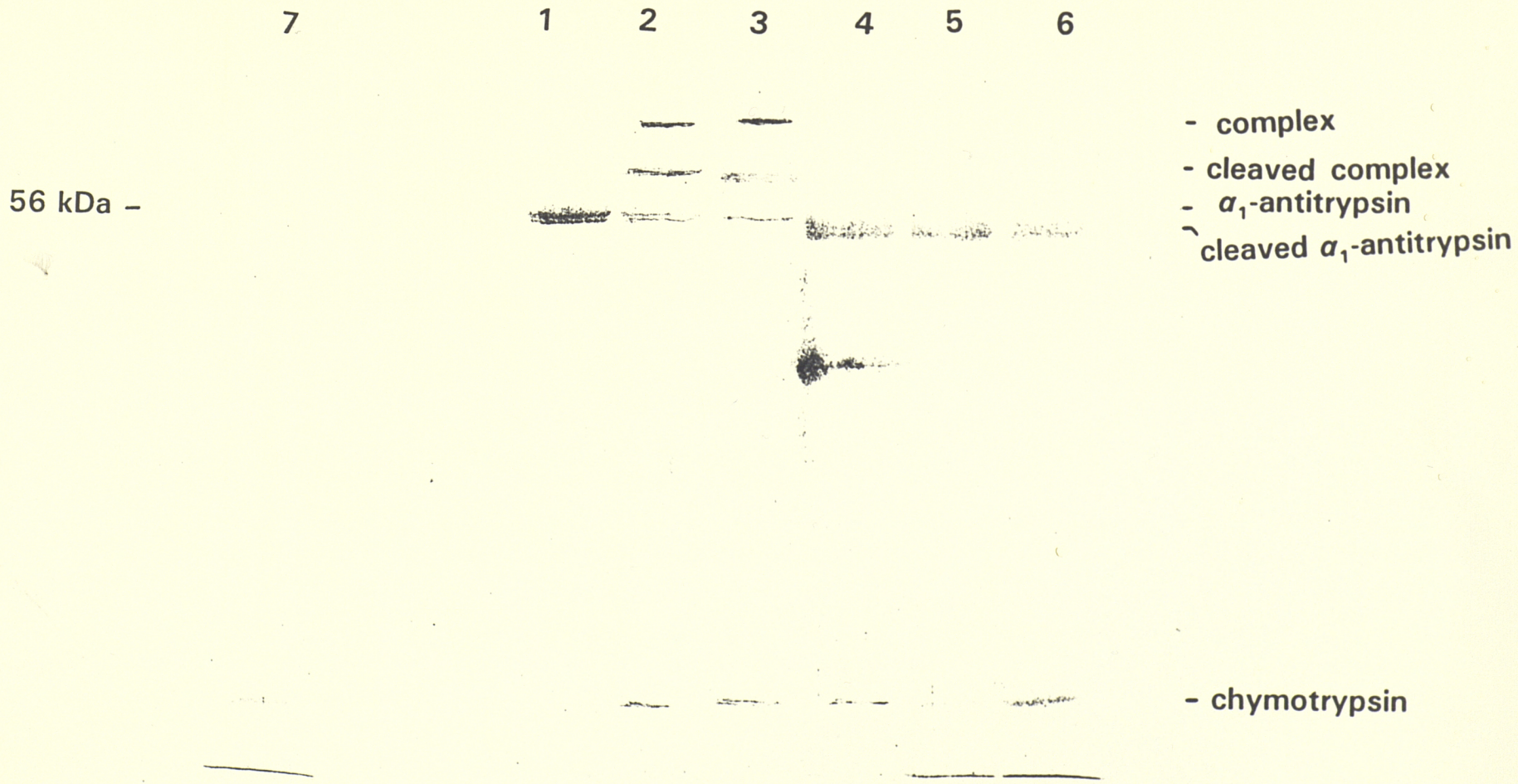


Figure 3.8 α_1-Antitrypsin-Chymotrypsin binary complex formation. 4 μg of unlabelled purified α_1-antitrypsin was incubated with bovine chymotrypsin for 30 minutes at 37°C in a total volume of 40 μl in reaction buffer. The reaction was terminated by addition of 10 μl of x5 Maizel buffer and analysed by SDS-PAGE and then Coomassie Blue staining. α_1-antitrypsin:chymotrypsin molar ratios in each lane were as follows: (1) 1:0 ratio; (2) 1:0.25 ratio; (3) 1:0.5 ratio; (4) 1:1 ratio; (5) 1:1.5 ratio; (6) 1:2 ratio; (7) 0:1 ratio.

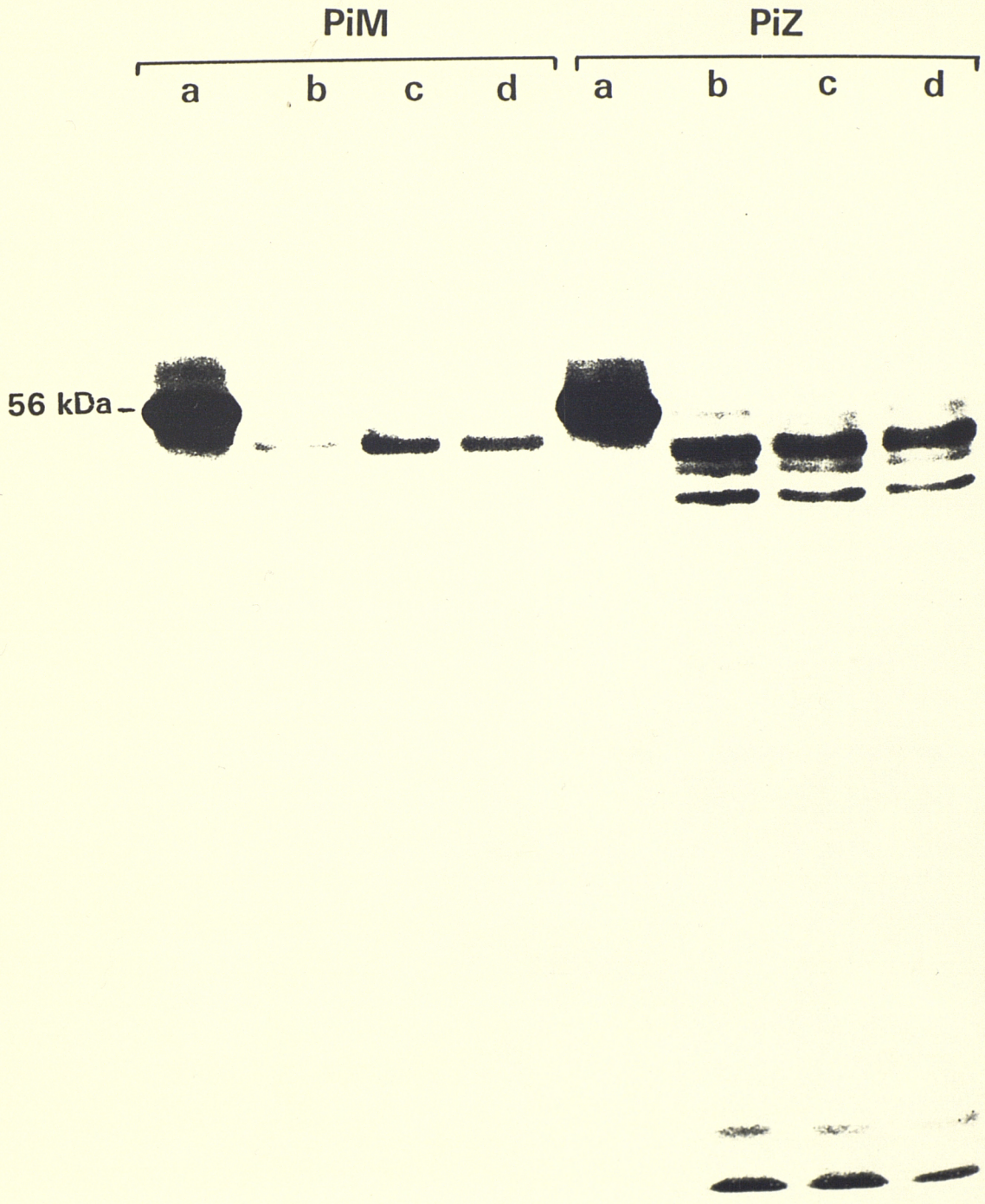


Figure 3.9 Cleavage of oocyte-retained M and Z α_1-antitrypsin by bovine chymotrypsin. Over twenty oocytes were injected with M or Z α_1-antitrypsin mRNA and incubated in L-[^{35}S] methionine. Radiolabelled α_1-antitrypsin was immunoprecipitated and the immunoadsorbed pellet resuspended in 160 μl reaction buffer (0.03 M Sodium phosphate, 0.1% (w/v) PEG 4000, 0.16 M NaCl, pH 7.4), and 40 μl aliquots were incubated with 6 μg unlabelled human α_1-antitrypsin and bovine chymotrypsin for 30 minutes at 37°C before loading on a 10% SDS-PAGE. α_1-antitrypsin:chymotrypsin ratios were as follows: lane a, 1:0; lane b, 1:0.5; lane c, 1:1; lane d, 1:2.

3.2.5 Gel Filtration Chromatography of M and Z α_1-Antitrypsin

To assess whether the intracelullar accumulations of α_1-antitrypsin in oocytes were polymeric, M and Z α_1-antitrypsin radiolabelled oocyte homogenates were applied to a Sepharose CL-6B column eluted with homogenization buffer. Radiolabelled products were eluted from the column between fractions 10 to 30. Dextran Blue (Mwt. 2 000 000) marker eluted from the column at fraction 13, so any polymeric α_1-antitrypsin would be expected to elute in the first few fractions after the void volume (after fraction 10) as noted. However no marked difference in the eluate profiles of M and Z α_1-antitrypsins was observed (data not shown). Radiolabelled material was detected between fractions 11-30. These fractions were immunoprecipitated and 5 μl aliquots were taken for scintillation counting, and protein also characterized by SDS-PAGE. Two main peaks are apparent, centred at fractions 13 and 20-23 (Figure 3.10). Each fraction contained α_1-antitrypsin of 54 kDa but the M α_1-antitrypsin bands were of greater intensity than those of Z α_1-antitrypsin (Figure 3.10), whereas immunoprecipitated oocyte extracts showed greater accumulations of α_1-antitrypsin in the Z variant relative to the M variant. This implies that perhaps the Z α_1-antitrypsin polymers for tangles and do not enter the column bed.

Later studies, used the same column but different elution buffer. The one chosen was 0.05 M Tris-HCl, 0.05 M NaCl pH 7.4 as recommended by Cox *et al.* (1986). Unfortunately again, both M and Z α_1-antitrypsin oocyte homogenates produced similar elution profiles. Further work involved application of 0.1 mg human α_1-antitrypsin to the column and also human α_1-antitrypsin polymerized *in vitro* by heating at 60°C for 2 hours. Elution of α_1-antitrypsin was monitored by spectrophotometric absorbance at 280 nm. Native M α_1-antitrypsin eluted in fractions 11 to 20 but no notable elution was obtained for polymerized M α_1-antitrypsin (Figure 3.11). Thus, α_1-antitrypsin polymers did not penetrate the column bed.

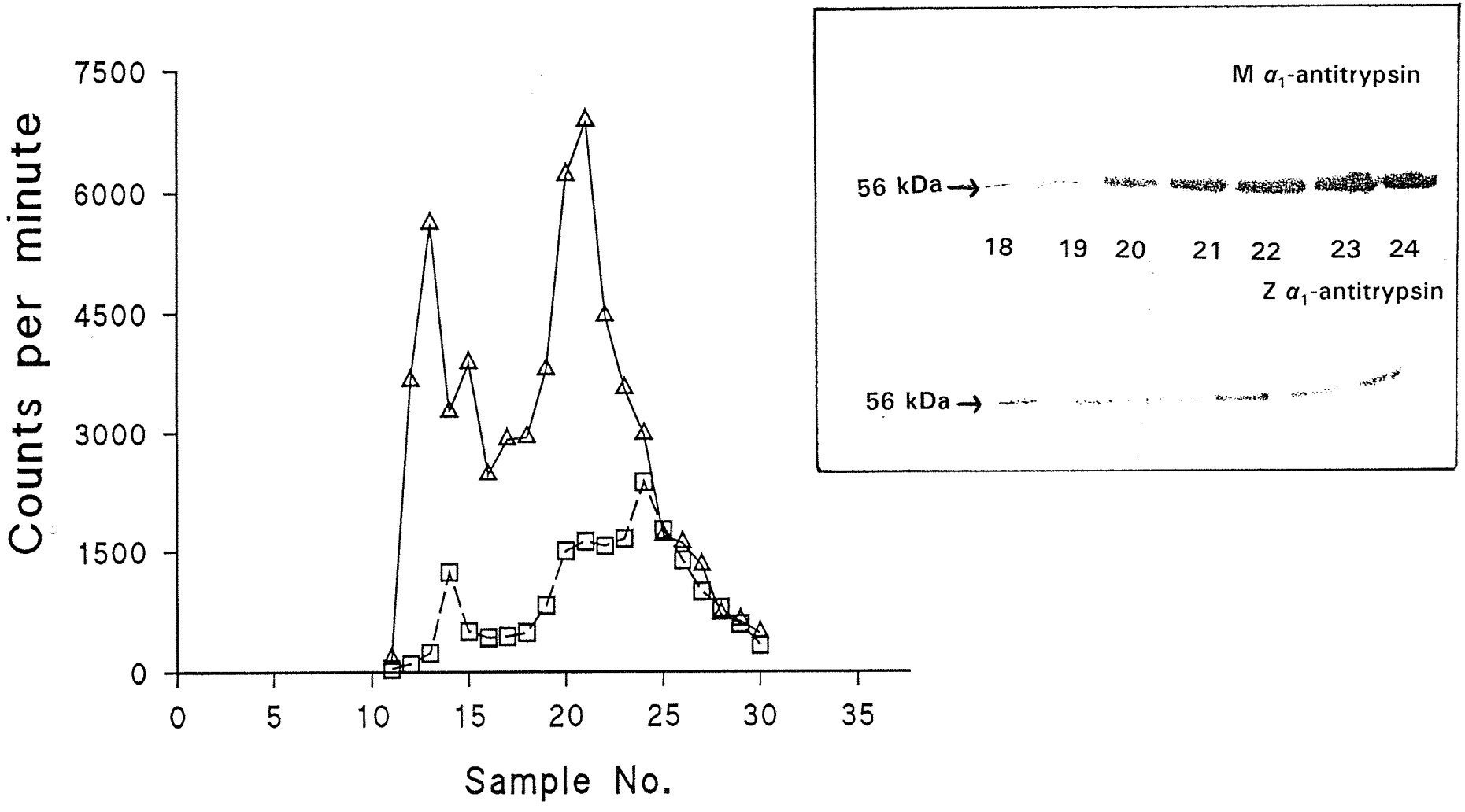


Figure 3.10 Comparison of the oocyte intracellular M (▲) and Z (□) α_1-antitrypsin eluted from a Sepharose CL-6B column, with homogenisation buffer [20 mM Tris-HCl, pH 7.6, 0.1 NaCl, 1% (v/v) Triton X-100]. 16 oocytes were injected with messenger RNA for M or Z α_1-antitrypsin and radiolabelled with L-[^{35}S] methionine. Oocytes were homogenised and centrifuged for 10 minutes twice to remove lipid, before application to the column at a flow rate of 11 ml/min. 470 μl fractions were collected. Fractions 11-30 were immunoprecipitated and 5 μl aliquots were analysed by liquid scintillation counting as described in *Methods*. the inset displays SDS-PAGE analysis of fractions (18-24) for both M and Z α_1-antitrypsin.

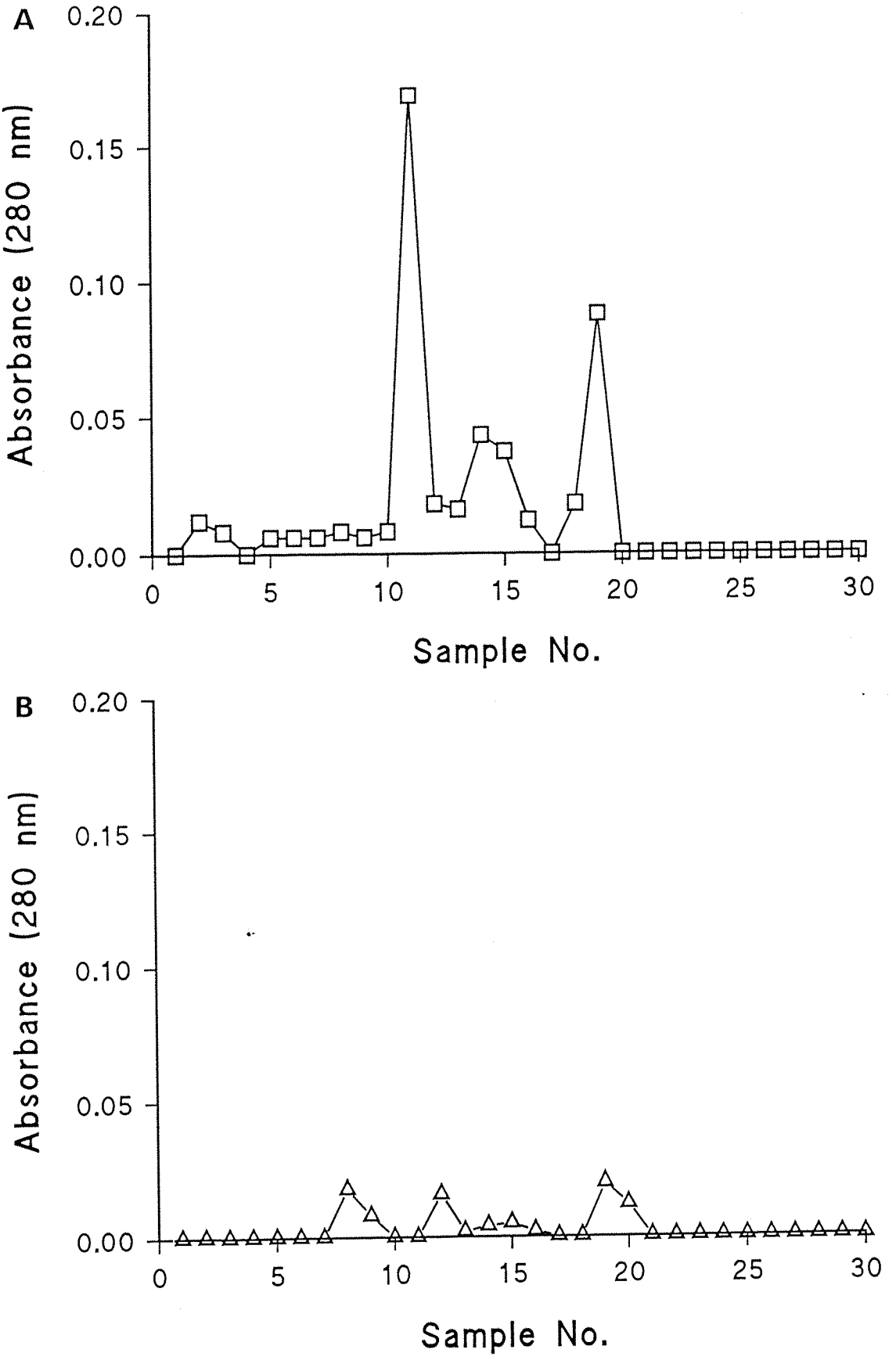


Figure 3.11 Elution of M α_1-antitrypsin (A) and *in vitro* polymerized M α_1-antitrypsin (B) from a Sepharose CL-6B column, with 0.05 M Tris-HCl, 0.05 M NaCl, pH 7.4. 0.1 mg α_1-antitrypsin was applied to the column, and fractions eluted were assessed for protein by absorbance reading at 280 nm.

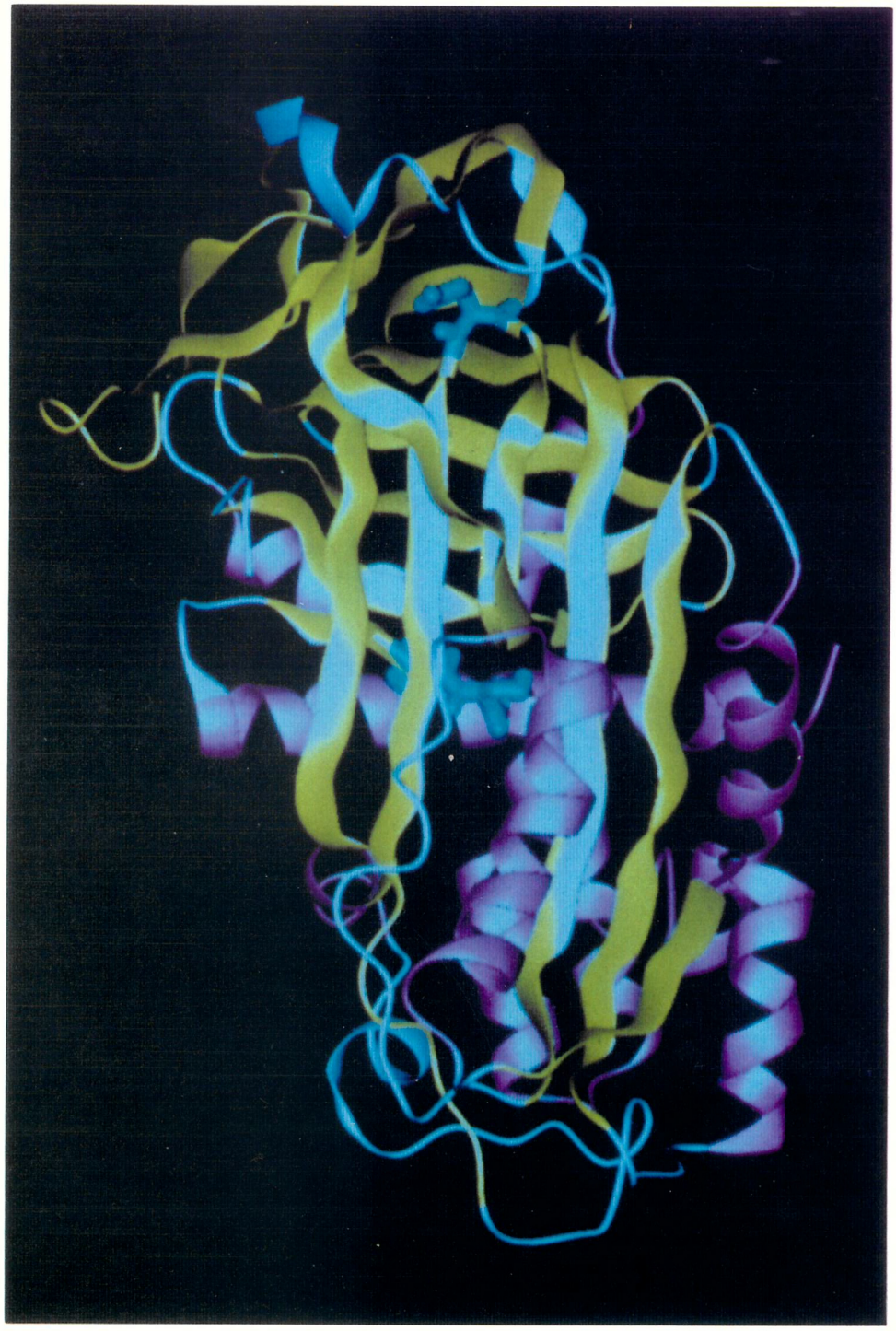


Figure 3.12 Schematic representation of uncleaved α_1-antitrypsin. ß-sheet structures are shown in yellow and α-helices in magenta. The sites of the Z, S_{Iiyama}, and M_{Malton} mutations shown in blue are predicted to hinder closing of the 5-stranded A sheet (provided by R. W. Carrell, Cambridge).

3.3 DISCUSSION

Whilst there is strong evidence that the deficiency of α_1-antitrypsin, and hence the accompanying risk of lung damage, is due to a failure in secretion rather than synthesis (Bathurst *et al.*, 1983), there is still debate as to the cause of the associated liver disease. Most adult Z homozygotes have evidence of slowly progressive liver fibrosis and 1 in 10 develop severe damage in infancy that results in a fatal juvenile cirrhosis (Sharp, 1969; Eriksson & Larsson, 1975; Sveger, 1976, 1978). Recently another variant, S_{iiyama}, was shown to have the same association with plasma deficiency and the identical histological finding of hepatocyte inclusions of mutant α_1-antitrypsin (Seyama *et al.*, 1991), together with spontaneous formation α_1-antitrypsin polymers in plasma (Lomas *et al.*, 1993b). Both the Z and S_{iiyama} mutants have amino acid substitutions, that although well separated from each other, have the same effect, that is to open the A-sheet of the molecule. The mutation in the Z of Glu^{342}→Lys is at the head of the fifth strand of the sheet (Loebermann *et al.*, 1984) and the mutation in S_{iiyama} of residue 53 in the B-helix from Ser→Phe, is such that it will sterically hinder closing of the A-sheet strands (see Figure 3.12) (Stein & Chothia 1991; Lomas *et al.*, 1993a). Moreover, recent studies (Lomas *et al.*, 1994) have revealed spontaneous loop-sheet polymer formation in a third deficiency variant, M_{Malton} (Phe^{52} deleted), that is in close proximity to the α_1-antitrypsin S_{iiyama} and also associated with similar liver inclusions and plasma aggregation. Thus, the common molecular consequence in all three deficiency mutants is that they spontaneously form polymers linked by loop-sheet bonding.

This study examined the comparative secretion of M, Z, S_{iiyama}, and M_{Malton} α_1-antitrypsins from *Xenopus* oocytes. The *Xenopus* oocyte surrogate secretory system has been shown to mimic other secretory defects (Wu *et al.*, 1990) and to accurately reflect the difference in secretion between normal M and mutant Z antitrypsin (Foreman, 1987). It was shown that Z,

S_{iiyama} and M_{Malton} mutants have an identical pattern of partial secretion of the fully processed protein, but with the majority of newly synthesized inhibitor remaining within the oocyte (Figures 3.1 and 3.2). Inadequate levels of α_1-antitrypsin were secreted from Z, S_{iiyama} and M_{Malton} variant injected oocytes to conduct activity assays and perform proteinase association rate kinetics on secreted material; furthermore, the secreted media contains substances which interfere with the active site titration assay. Endoglycosidase H analysis demonstrated that the accumulation of each variant occurs at the same stage of processing before entry to the *medial* Golgi ie. at a stage prior to the addition of the terminal complex sugars (Figures 3.4 and 3.5). The findings strongly support a common site for the accumulation of the three α_1-antitrypsin deficiency variants that results in the blockage in secretion in hepatocytes.

Incubation of M α_1-antitrypsin with increasing concentration of elastase demonstrated complex formation followed by reactive loop cleavage (Figure 3.6). M_{Malton} , S_{iiyama} and Z α_1-antitrypsin accumulations in oocytes were expected to fail to form complexes with, or be cleaved by, bovine α-chymotrypsin due to the inaccessibility of the reactive centre loop, as shown by Lomas *et al.* (1993a,b) on plasma from Z and S_{iiyama} individuals. In one experiment (Figure 3.7), it was shown that the intracellular Z variant failed to either complex with or be cleaved by bovine α-chymotrypsin, but that intracellular M α_1-antitrypsin did possess such inhibitory activity. This finding provides evidence for the occurrence of loop-sheet polymerization. However, further studies did not reproduce these findings, instead accumulated Z, S_{iiyama}. and M_{Malton} variants were cleaved by α-chymotrypsin. This may be due to disaggregation of polymerized α_1-antitrypsin at some stage during the α_1-antitrypsin isolation procedure. Homogenization and immunoprecipitation buffers lacking Triton X-100 detergent, a possible cause of polymer disruption, did not prevent accumulated α_1-antitrypsin variants from being cleaved by proteinases. Although previously, it has been shown that aggregates of Z and M_{Malton} α_1-antitrypsin cannot be disaggregated using

Triton X detergent (Cox *et al.*, 1986). Polymeric α_1-antitrypsin molecules may possibly be disrupted during the mechanical homogenization procedure.

The intracellular form of M α_1-antitrypsin was shown to be more susceptible to cleavage by chymotrypsin than the intracellular form of Z α_1-antitrypsin (Figure 3.9). This possibly implies that the reactive centre loop of Z α_1-antitrypsin is less accessible to chymotrypsin than M α_1-antitrypsin and does provide tentative evidence in support for the occurrence of Z α_1-antitrypsin loop-sheet polymerization *in vivo*.

Direct isolation of high-molecular forms of Z α_1-antitrypsin from oocyte homogenates would provide further evidence for loop-sheet polymerization. But no difference in the elution of M and Z α_1-antitrypsin from a Sepharose CL-6B column was achieved, although the column separates molecules between 10 000 - 4 000 000 molecular mass. Lomas *et al.* (1992) isolated polymers of α_1-antitrypsin, 10-15 molecules in length (>200 kDa) from the plasma of an individual homozygous for the Z mutation by HPLC on a Q-Sepharose column. Failure of *in vitro* polymerized M α_1-antitrypsin to elute from the column indicates that polymers fail to enter the column matrix, presumably forming tangles at the top of the column (figure 3.11). A former study by Cox *et al.* (1986) did isolate aggregates of α_1-antitrypsin from the plasma of Z and M_{Malton} affected individuals using a Ultragel AcA 44 column (LKB, Broma) after removal of albumin using a Sepharose CL-6B. Further studies could be conducted using a similar column (Cox *et al.*, 1986) or instead oocyte homogenates could be applied directly to native (non-denaturing) SDS with Western Blotting to detect α_1-antitrypsin polymers.

Despite technical limitations, it seems clear that the observation of *in vivo* polymerization of Z, S_{Iiyama} and M_{Malton} mutant proteins (Lomas *et al.*, 1993a,b; 1994), taken together with their identical behaviour when expressed in *Xenopus* oocytes provides evidence that polymerization does

occur in oocytes. Further support comes from the finding that liver inclusions in Z antitrypsin homozygotes are formed solely of the mutant protein (Bathurst *et al.*, 1984), in the form of tangles of polymeric fibrils (Lomas *et al.*, 1992). The consequence, in all three mutants, is the blockage in processing of all but a small proportion of the abnormal protein, to give a plasma deficiency, and in the hepatocyte increased risk of cell necrosis.

4. MUTATIONS WHICH IMPEDE LOOP-SHEET POLYMERIZATION AND THE SECRETION OF α_1-ANTITRYPSIN.

4.1 INTRODUCTION

Previous work has established that the X*enopus* system faithfully duplicates the secretory defect seen with the Z α_1-antitrypsin variant (Foreman *et al.*, 1984; Perlmutter *et al.*, 1985; Foreman, 1987) and the S_{iiyama} and M_{Malton} variants (Chapter 3). Chapter 3 highlighted the connection between loop-sheet polymerization and the secretory block. Here I examine the phenomenon of loop-sheet polymerization by three approaches to prevent entry of the reactive centre loop into the A sheet.

Firstly, the mutation Phe^{51}→Leu which stabilises M (normal) α_1-antitrypsin against polymerization (Kwon *et al.*, 1994), predictably by closing the gap between strands s3A and s5A in the A sheet, was constructed together with its chimer with Z α_1-antitrypsin (Phe^{51}→Leu, Glu^{342}→Lys) and with S_{iiyama} α_1-antitrypsin (Phe^{51}→Leu, Ser^{53}→Phe) (Tables 2.1 and 2.3). Secretion of these variants from oocytes was examined to establish, whether as predicted, polymerization is prevented by closure of the A sheet.

The second approach involved constructs of M and Z α_1-antitrypsin with amino acid substitutions in the reactive loop designed to impede loop mobility and therefore prevent refolding of the loop into the A sheet. These mutations $P_{11/12}$Ala→Val and P_{14}Thr→Arg were based on the homologous residues found in the non-inhibitory serpin ovalbumin that has an reactive loop which is immobile in the hinge region (Huber & Carrell, 1989; Stein *et al.*, 1989).

Finally, 2 mM acetylated antithrombin III-reactive centre loop peptide BC11 (Acetyl-Ser-Glu-Ala-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-OH) suspended in 50 mM Tris, 100 mM NaCl pH 7.6, was co-injected with mRNA encoding M or Z α_1-antitrypsin into oocytes (peptide BC11 was donated by R. W. Carrell, Cambridge). BC11 is homologous to the P_{14}-P_1 loop sequence of α_1-antitrypsin and readily forms complexes with α_1-antitrypsin and antithrombin

(Lomas *et al.*, 1993a). Oocytes were also co-injected with the control peptide, Neurotensin (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) (Sigma), which is not homologous to the reactive centre loop. Healthy oocytes were incubated in media containing L-[^{35}S] methionine and 80 μM peptide for 24 hours. Radiolabelled α_1-antitrypsin was immunoprecipitated from the incubation media and oocyte extracts for analysis by SDS-PAGE and fluorography, as described in *Methods*.

To target the peptide directly to the endoplasmic reticulum (ER), a construct was constructed that contained the N-terminal signal peptide sequence from α_1-antitrypsin followed by the P_{12}-P_3 reactive centre loop sequence and a C-terminal glycosylation sequence. Proteins become glycosylated in the ER at asparagine residues if they form part of an Asn-X-Ser/Thr consensus sequence, where X is any residue. However, not all such sequences are consistent in undergoing glycosylation. Hopefully, the signal sequence will target the peptide to the ER, and after signal peptide cleavage, the peptide will traverse the secretory pathway and be glycosylated. Two complementary oligonucleotides, coding for the reactive loop and glycosylation site were annealed by heating 2 μg each at 85°C, 2 minutes and cooling very slowly:

P_{12}----------P_3ASPGLYTHRstop Pst I
*GATCC*AGAAGCTGCTGGGGCCATGTTTTTAGAGGCCATAAACGGTACGTAA*T*$^{3'}$
3'*G*TCTTCGACGACCCCGGTACAAAAATCTCCGGTATTTGCCATGCATT*AGATC*$_{5'}$
*Bam*H I

Peptide sequence is in bold, glycosylation sequence is underlined, and restriction sites are in italics.

The annealed oligo was ligated into a pSP645-RCF vector containing M α_1-antitrypsin cut with *Bam*H I and *Xba* I. The *Bam*H I site follows the N-terminal signal peptide sequence (see Figure 2.3). After SP6 *in vitro* transcription, the RNA was translated *in vitro* to check the peptide was produced. Later, the peptide RNA was co-injected with M or Z α_1-antitrypsin

mRNA into oocytes and radiolabelled with L-[^{35}S] methionine for secretion analysis.

4.2 RESULTS

4.2.1 Effect of the Phe^{51}→Leu Mutation on α_1-Antitrypsin Secretion

The amino acid substitution of Phe with Leu at position 51 was constructed using PCR based mutagenesis on M and also on Z α_1-antitrypsin (Table 2.3). The double mutant [Phe^{51}→Leu, Ser^{53}→Phe] was constructed with M antitrypsin as template (Table 2.3). The effect of the Phe^{51}→Leu mutation on M, Z and S_{iiyama} antitrypsin secretion after microinjection of α_1-antitrypsin mutant mRNAs is shown by Figures 4.1 and 4.2. A significant ($p<0.005$) 3-fold enhancement of Z antitrypsin secretion was recorded in the chimer [Phe^{51}→Leu, Glu^{342}→Lys] (28.9% secreted ±4.2). Moreover, the S_{iiyama} Leu^{51} chimer was secreted (68.9% ±3.1) as efficiently as normal M antitrypsin. Therefore the Phe^{51}→Leu mutation fully reverts the secretion S_{iiyama} variant to that of the normal M phenotype and partially prevents accumulation of Z antitrypsin.

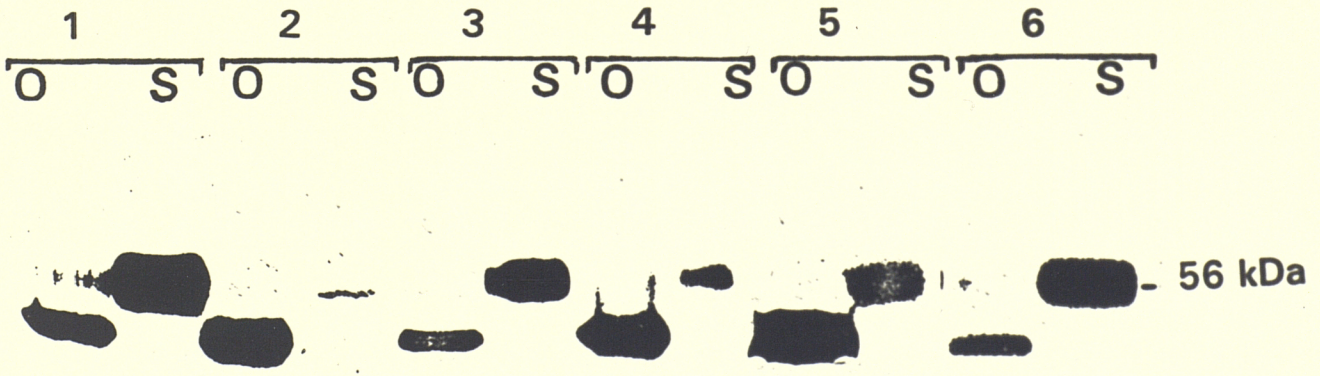


Figure 4.1 Synthesis of Phe^{51}→Leu M, Z and and S_{iiyama} antitrypsins in *Xenopus* oocytes. Twenty Oocytes were injected with messenger RNA for a given antitrypsin variant and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media then either separated by SDS-PAGE as described in ***Methods***. O represents oocyte extract and S represents material secreted into the surrounding medium. α_1-antitrypsin variants in each lane are: 1, M; 2, Z; 3, M Leu^{51}; 4, Z Leu^{51}; 5, S_{iiyama}; 6, S_{iiyama} Leu^{51}. Molecular mass was determined by the co-migration of standard protein markers.

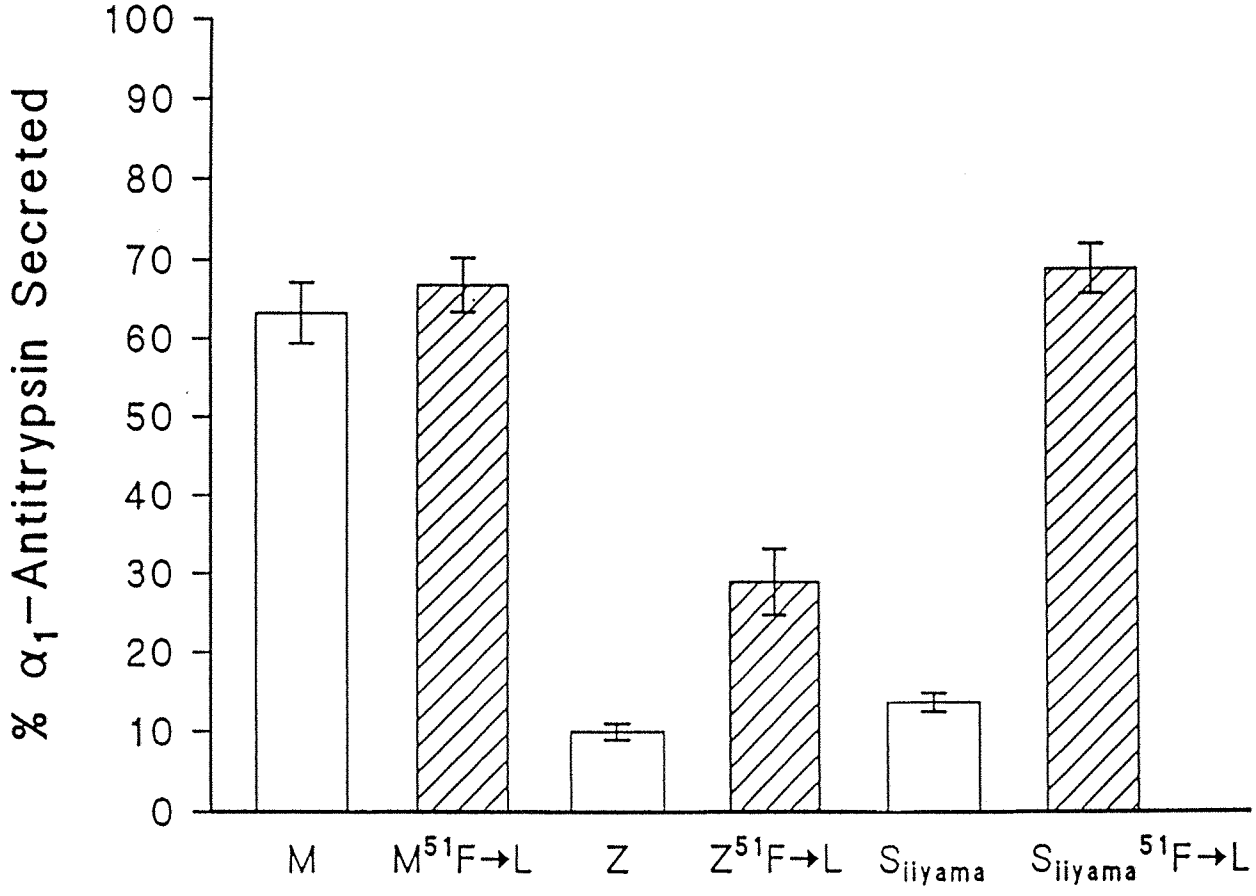


Figure 4.2 Quantitation of the relative amounts of Phe^{51}→Leu M, Z and S$_{Iiyama}$ antitrypsins secreted from microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Amounts of secreted antitrypsin are expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least seven different experiments using five different animals. Each experiment involved the labelling of at least twenty oocytes. Values shown are expressed as the arithmetic mean ± the standard error of the mean. Shaded columns denote constructs bearing the Phe^{51}→Leu change.

4.2.2 Effect of the Loop Hinge Mutants on α_1-Antitrypsin Secretion

The effect of residues P_{14}, and $P_{11/12}$ on the secretory properties of M and Z antitrypsin from *Xenopus* oocytes was examined by construction of mutant antitrypsins containing the bulkier amino acids present in the non-inhibitory serpin ovalbumin substituted at these residues; namely P_{14} Thr→Arg and $P_{11/12}$ Ala→Val (Table 2.3). Figure 4.3 and 4.4 display gel profiles of *Xenopus* oocyte processing of the P_{14} and $P_{11/12}$ mutant α_1-antitrypsins. The extent of secretion of α_1-antitrypsin as a percentage of the total inhibitor synthesized is presented in Figure 4.5. M P_{14} Arg shows a significant ($p<0.001$) reduction in secretion (41.7% ±2.3) compared to normal M antitrypsin (62.4% ±3.1). No significant difference ($p=0.54$) was obtained between Z antitrypsin (12.4% ±1.3) and the Z P_{14} Arg double mutant (13.7% ±1.3). The accumulation defect observed with Z antitrypsin is partially corrected if the $P_{11/12}$ alanines are substituted for the larger valine residues as indicated by the increased secretion of Z $P_{11/12}$ Val (25.2% ±2.7) compared to Z antitrypsin ($p<0.005$).

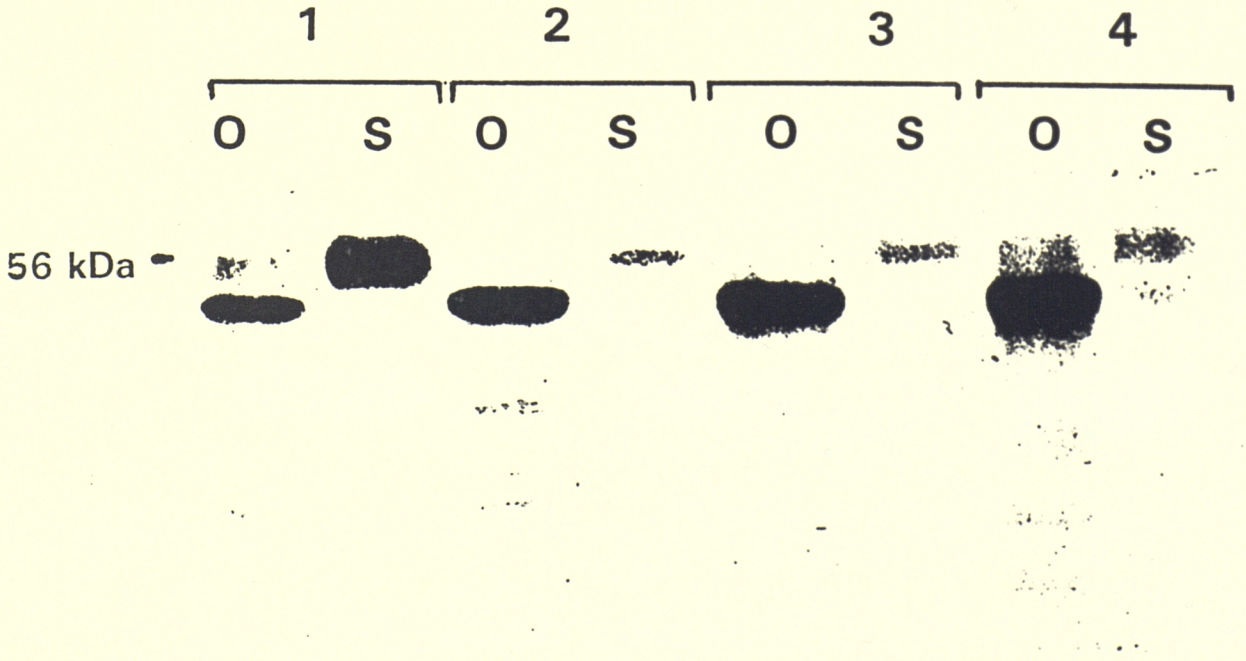


Figure 4.3 Synthesis of M, Z, M P_{14} Arg, and Z P_{14} Arg α_1-antitrypsins in *Xenopus* oocytes. Twenty Oocytes were injected with messenger RNA for a given antitrypsin variant and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media then separated by SDS-PAGE as described in *Methods*. O represents oocyte extract and S represents material secreted into the surrounding medium. Lanes: 1, M α_1-antitrypsin; 2, Z α_1-antitrypsin; 3, M P_{14}Arg α_1-antitrypsin; 4, Z P_{14} Arg α_1-antitrypsin. Molecular mass was determined by the co-migration of standard protein markers.

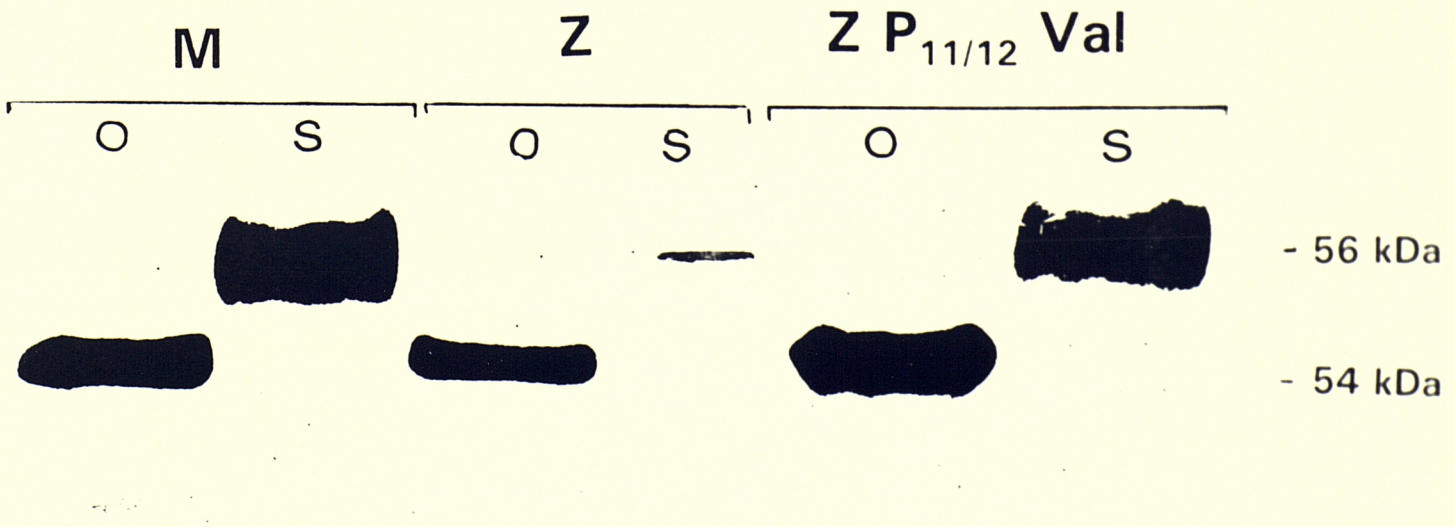


Figure 4.4 Synthesis of M, Z, and Z $P_{11/12}$ Val α_1-antitrypsins in *Xenopus* oocytes. Twenty Oocytes were injected with messenger RNA for a given antitrypsin variant and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media then separated by SDS-PAGE as described in *Methods*. O represents oocyte extract and S represents material secreted into the surrounding medium. Molecular mass was determined by the co-migration of standard protein markers.

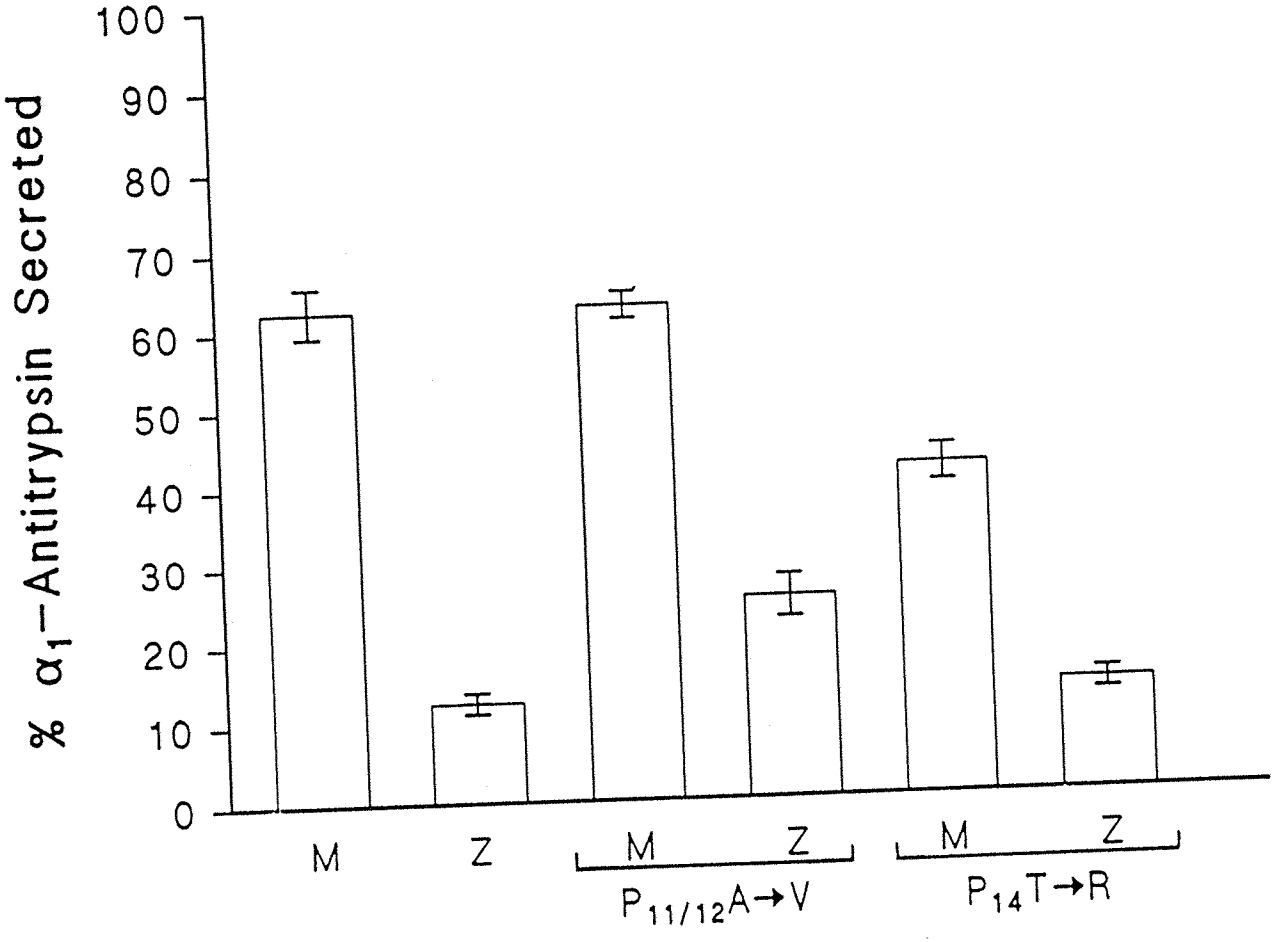


Figure 4.5 Quantitation of the relative amounts of $P_{11/12}$Val and P_{14}Arg loop hinge M and Z α_1-antitrypsins secreted from microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Amounts of secreted antitrypsin are expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least four different experiments using five different animals. Each experiment involved the labelling of at least twenty oocytes. Values shown are expressed as the mean ± the standard error of the mean

4.2.3 Effect of Reactive Loop Peptide on α_1-Antitrypsin Secretion

Figure 4.6 displays the gel of the effect of the reactive loop peptide BC11 on the secretion of M and Z α_1-antitrypsin. The extent of secretion was quantitated by scintillation counting of excised bands (Figure 4.7). Peptide BC11 had no effect on the secretion of either M α_1-antitrypsin (68.0% secreted in presence or absence of BC11) or Z α_1-antitrypsin (11.2% secreted without BC11, 9.8% secreted with BC11 present). However, the experiment does not demonstrate if the peptide has entered the endoplasmic reticulum to be available to insert into the A sheet of Z α_1-antitrypsin and prevent loop-sheet polymerization. Messenger RNA encoding peptide P_{12}-P_3 attached after the signal sequence of α_1-antitrypsin was constructed and co-injected into oocytes together with M or Z α_1-antitrypsin. Unfortunately no effect on the secretion of α_1-antitrypsin was observed with this ER targeted peptide. Again it is not known if the peptide enters the compartments of the secretory pathway.

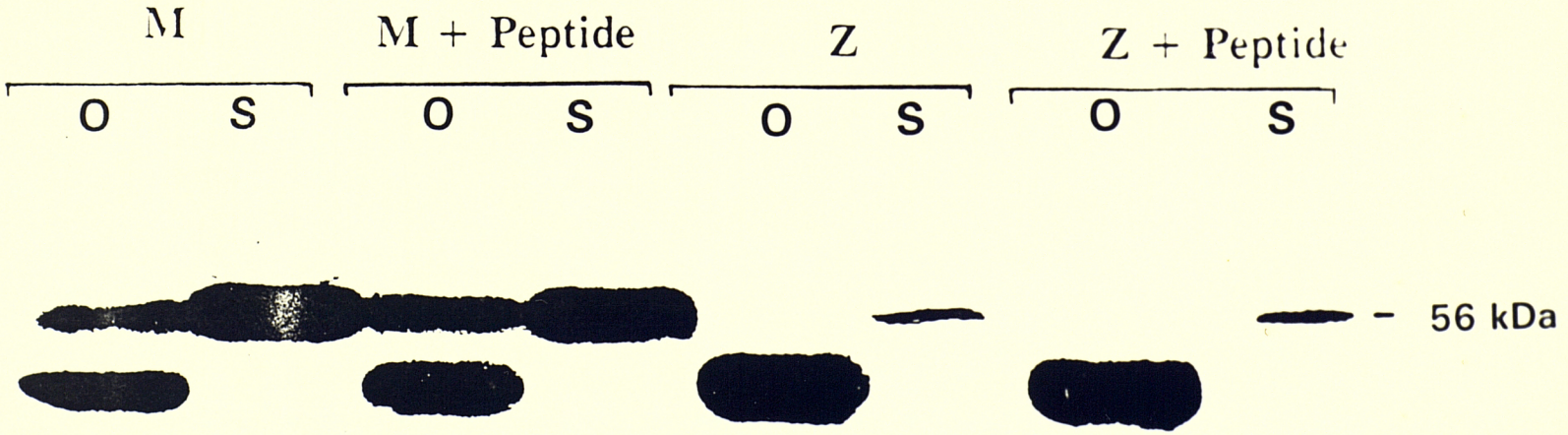


Figure 4.6 Effect of reactive loop peptide BC11 on the synthesis of M and Z α_1-antitrypsins in *Xenopus* oocytes. Twenty oocytes were injected with messenger RNA encoding M and Z antitrypsin variants with and without 2 mM BC11 peptide, and radiolabelled with L-[^{35}S] methionine. Newly synthesised proteins were immunoprecipitated from cell extracts and incubation media, then separated by SDS-PAGE as described in *Methods*. O represents oocyte extract and S represents material secreted into the surrounding medium. Molecular mass was determined by the co-migration of standard protein markers.

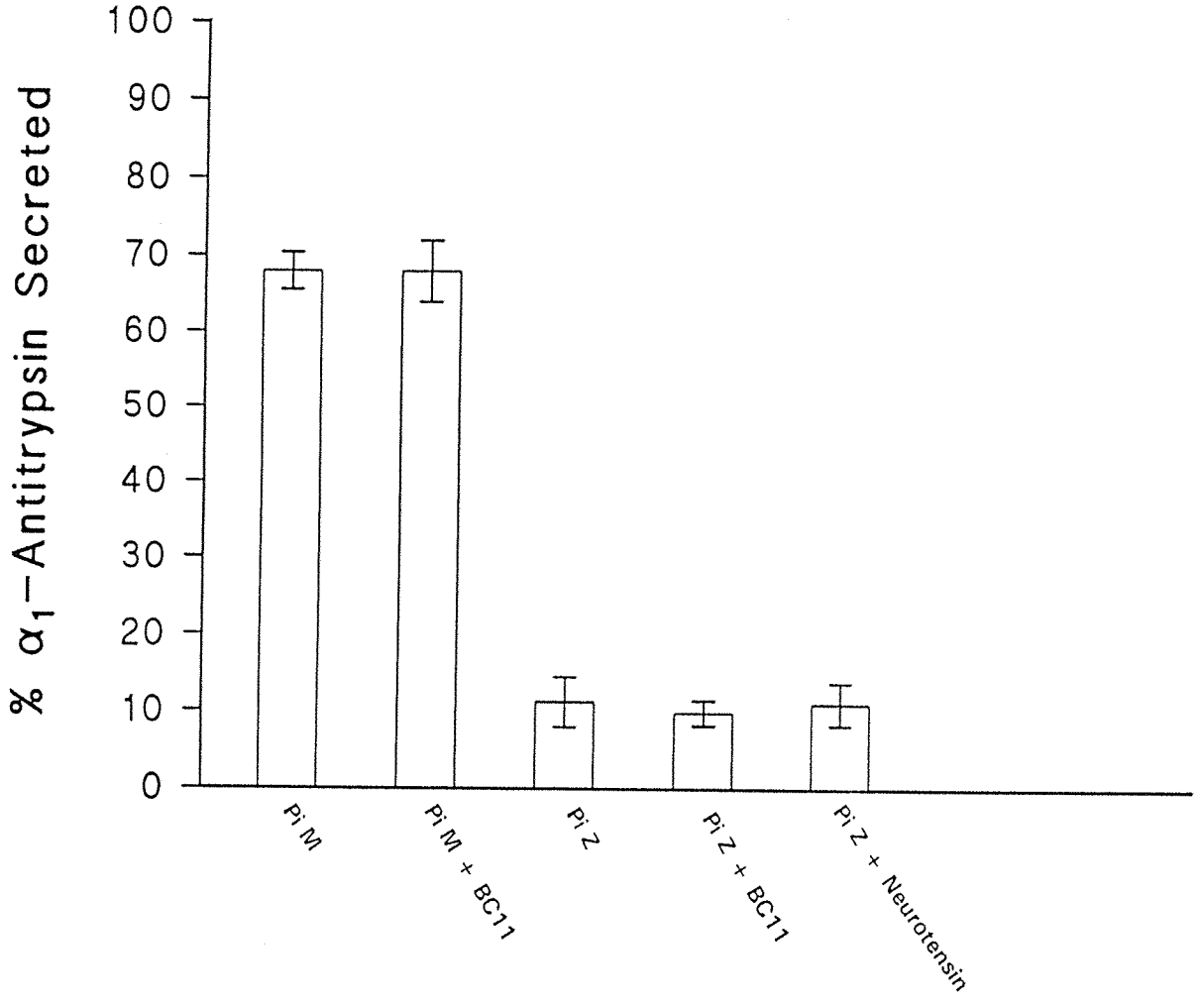


Figure 4.7 Quantitation of the effect of BC11 peptide on the relative amounts of M and Z α_1-antitrypsins secreted from microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Amounts os secreted α_1-antitrypsin are expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least six different experiments using oocytes from six animals, Values shown are expressed as ± the standard error of the mean.

4.3 DISCUSSION

One in ten Z homozygotes develop severe liver damage in infancy and most adults with this genotype have evidence of slowly progressive liver fibrosis (Eriksson & Larsson, 1975; Sveger, 1978). The liver disease arises as a direct result of the intracellular accumulation of mutant inhibitor, seen as inclusion bodies within the endoplasmic reticulum of the hepatocytes (Eriksson & Larsson, 1975). Recently another variant, α_1-antitrypsin S_{iiyama}, was shown to have the same association with plasma deficiency and the identical histological finding of hepatic inclusions of mutant inhibitor (Seyama *et al.*, 1991; Lomas *et al.*, 1993b). Both the Z and S_{iiyama} mutants have amino acid substitutions, that although well separated from each other, have the same effect, that is to open the A sheet between the third and fifth strands (Figure 3.12) and thus promote the process of loop sheet polymerization (Lomas *et al.*, 1992).

Having demonstrated the validity of the *Xenopus* oocyte secretory system for the expression of Z, and S_{iiyama} mutant α_1-antitrypsins (Chapter 3), investigations into the phenomenon of loop sheet polymerization were conducted by three approaches involving the prevention of entry of the reactive centre loop into the A sheet. Firstly, mutations to close the gap between strands s3A and s5A in the A sheet, then mutations in the loop-hinge region which prevent entry into the sheet due to steric hindrance and finally by reactive centre loop peptide insertion *in vivo*.

Secretion and Sheet Accessibility- The S_{iiyama} variant ($Ser^{53}\rightarrow Phe$) is located in the B helix which underlies the A sheet and provides a surface on which strand s3A slides in order for the sheet to open (Stein & Chothia, 1991). The substitution of the bulky, aromatic side chain at this position is thought to lock the sheet in the open conformation and thereby promote loop sheet polymerization (Stein & Chothia, 1991, Lomas *et al.*, 1993b). Recently, Yu *et al.* have reported that substitution of the Phe residue at position 51 by

aliphatic, non-polar residues enhances the thermal stability and decreases heat induced polymerization of wild type M α_1-antitrypsin (Kwon *et al.*, 1994). Leucine was the most effective residue in increasing thermal stability. Here it is shown that the stabilising properties of this change at position 51 have an ameliorating influence on the Z and S_{iiyama} secretion defective mutations (Figure 4.2). This effect, however is not equivalent for both dysfunctional proteins; secretion of the Z/Phe^{51}→Leu double mutant is increased nearly three-fold whereas secretion of S_{iiyama}/Phe^{51}→Leu is equal to M α_1-antitrypsin (a greater than five-fold increase). The increased effect on S_{iiyama} (Ser^{53}→Phe) may simply be a matter of proximity, in that removal of Phe^{51} may correct an aberrant conformation of the B helix induced by the introduction of Phe^{53} and thus allow closure of the A sheet. The location of the Z mutation, at the hinge of the reactive centre loop, may influence both sheet opening and loop mobility. Changes at position 51 are liable to reverse the former but not the latter, hence only a partial correction of the Z secretory defect.

Secretion and Loop Mobility- A profound structural transformation from a stressed [S] conformation to a more ordered, heat stable and relaxed [R] state is observed upon reactive centre cleavage of inhibitory serpins (Carrell & Owen, 1986). This S→R transition is dependent upon the insertion of the mobile reactive centre loop into ß sheet A after cleavage of the P_1-P_1' peptide bond. Inappropriate insertion of the intact loop into ß sheet A is a feature of the polymerization of aberrant serpins, but partial insertion of the uncleaved loop into the ß sheet is thought to facilitate formation of the canonical form of the active inhibitor (Engh *et al.*, 1990; Carrell *et al.*, 1991; Skriver *et al.*, 1991). The reactive centre loops of all inhibitory serpins are characterized by the conservation of small hydrophobic amino acids, particularly at positions P_{10}, $P_{11/12}$ and P_{14} at the base of the loop (Carrell *et al.*, 1991). These residues are orientated with their side chains facing the hydrophobic interior of the molecule (Loebermann *et al.*, 1984) and as a consequence, there is a constraint on their size and polarity if loop insertion

is to occur. The absence of inhibitory activity and the S→R transition in ovalbumin and angiotensinogen can be explained by the appearance of larger and/or more polar residues in these critical positions (Stein *et al.*, 1989). Similarly, several natural mutants of antithrombin III, C1-inhibitor and other serpins have been identified with point mutations at positions P_{12} and P_{10}, and in most cases these are proteinase substrates not inhibitors (Devraj-Kizuk *et al.*, 1988; Perry *et al.*, 1989, 1991; Carrell *et al.*, 1991; Skriver *et al*, 1991). For example, the P_{10}Ala→Pro mutant of antithrombin fails to undergo the S→R transition and has no inhibitory activity but maintains the ability to bind heparin (Perry *et al.*, 1989; Carrell *et al.*, 1991).

Schulze *et al.* (1991) have shown that substitution of P_{14}Thr by Arg converts a reactive centre mutant of α_1-antitrypsin from an inhibitor to a substrate that fails to undergo a detectable conformational change, presumably because the mutation prevents normal loop sheet interaction. A contradictory case has been made by Hood *et al.* (1994), who constructed a P_{14}Thr→Arg α_1-antitrypsin which retained the ability to complex with several cognate proteinases and underwent the S→R transition. Annealing experiments involving complexation of native serpins with synthetic peptides homologous to the reactive loop sequence have also demonstrated the extent of loop incorporation (Schulze *et al.*, 1992). Peptides corresponding to P_1-P_{14} inserted into the A sheet, resulting in a loss of inhibitory activity of the serpin together with an increase in thermal stability similar to that of cleaved serpin. Peptide P_1-P_{11} and shorter peptides retained inhibitory activity suggesting that strand s4A can insert into the A sheet up to residue P_{12}/P_{10} to form an ideal inhibitor loop conformation.

While the effect of such mutations on the thermal stability and inhibitory activity of normal serpins are well established their influence on polymerization and secretion have yet to be explored. The secretion of Z α_1-antitrypsin from oocytes was unaltered by the replacement of P_{14}Thr by Arginine. This may mean that aggregation is unhampered by partial

exclusion of strand 4A from the sheet, alternatively the gap between strands s3A and s5A may be wider as a result of the lysine residue at position 342 and thus able to accommodate the larger and more polar arginine side chain. The results suggest that the arginine residue at P_{14} does not prevent loop-sheet polymerization and the resulting polymerization of the Z variant, and are in general agreement with the findings of Hood *et al.* (1994) that such a change in the normal inhibitor is compatible with the S→R transition and the maintenance of inhibitory function. Nonetheless, the P_{14}Arg mutation may have more profound structural consequences in addition to its effect on loop mobility since the secretion of M type α_1-antitrypsin P_{14}Thr→Arg was significantly reduced, but not to the level observed for Z α_1-antitrypsin. Alanine to valine substitutions at P_{11} and P_{12} were more effective in overcoming the block in secretion imposed by the Z mutation, causing a two-fold increase in export of this mutant α_1-antitrypsin (Figure 4.5). This finding may indicate the relative stretches of the loop which are involved in polymerization; the region from P_{12} approaching the reactive centre rather than those residues towards the hinge region. A naturally occurring mutation of the human C1-inhibitor with P_{12}Ala→Glu was not an effective inhibitor and did not undergo the S→R conformational change but also showed no tendency to polymerise (Skriver *et al.*, 1991). The mutants described in this chapter affect the interaction between the mobile loop and ß sheet A, the initial step common to both A sheet and C sheet models for serpin polymerization (Carrell *et al.*, 1994; Schreuder *et al.*, 1994), however the degree of partial loop insertion may be different for the two models (see section 1.13.4). The A sheet polymerization model would require the space between strands s3A and s4A to remain substantially free for intermolecular association while the C sheet model demands incorporation of more distal parts of the loop in order to displace strand 1 from the C sheet. Replacement of the alanines at positions P_{11} and P_{12} with valines may hinder this more extensive incorporation of the reactive loop, although single replacement with threonine at position P_{12} does not prevent M type α_1-antitrypsin from undergoing the S→R transition (Hopkins *et al.*, 1993). Thus

the increase in secretion of Z α_1-antitrypsin with valine residues at positions P_{11} and P_{12} is compatible with, but does not confirm, the C sheet model of polymerization.

Although the mutants described in this study do not provide definitive support for either the A sheet or C sheet models what is clearly demonstrated is the relationship between loop mobility, polymerization and *in vivo* secretion.

Secretion and Peptide Insertion- *In vitro* studies have shown that addition of an excess of synthetic peptide homologous to the reactive loop sequence of α_1-antitrypsin can prevent polymerization of α_1-antitrypsin (Schulze *et al.*, 1990; Carrell *et al.*, 1991; Lomas *et al.*, 1993a). Peptide insertion into the A sheet forms a binary complex that bars the reactive centre loop of another α_1-antitrypsin molecule. The ability of the reactive loop peptide BC11, to inhibit loop-sheet polymerization of α_1-antitrypsin *in vivo* by blocking the A sheet during the passage of α_1-antitrypsin along the endoplasmic reticulum was assessed in the *Xenopus* oocyte system. An initial attempt involving direct injection of the acyl peptide into the oocyte along with mRNA coding for Z α_1-antitrypsin did not have any effect on Z α_1-antitrypsin accumulation. This could be due to a number of reasons, such as degradation of the peptide prior to entry into the ER; but the peptide was acetylated in the manner of many intracellular proteins to decrease susceptibility to degradation. Another possible reason is that the peptide does not enter the ER to achieve insertion into the A sheet. An attempt was made to target the peptide to the ER by attaching a N-terminal signal sequence to the peptide and a glycosylation site to ensure secretion through the Golgi complex. Unfortunately, this signal-sequence containing peptide had no effect on the secretion of Z α_1-antitrypsin. Further work which could be pursued to target the ER, could involve addition of a C-terminal KDEL sequence, a signal responsible for ER localization of lumenal proteins (Pelham 1990; 1991), and so retain the peptide in the lumen of the ER.

The passage of the peptide through the secretory apparatus could be monitored by iodination of the peptide and subcellular fractionation of oocytes by sucrose density gradients (Colman, 1984). The intracellular location of labelled peptide could be determined, but clean well-defined fractions cannot be obtained because of the large amounts of lipid and yolk granules in the oocytes (Colman, 1984). Gradient fractions are divided into cytosolic and membrane bound components. If peptide is found in the membrane bound compartments it would suggest peptide entry into the ER/Golgi complex.

The prevention of loop-sheet polymerization by reactive loop peptides *in vitro* has implications for therapeutic intervention *in vivo* in patients affected with α_1-antitrypsin induced liver disease. Peptide targeting to the hepatocytes could involve conjugation to bile salts (Mills & Elias, 1991) or even possibly delivery of the gene coding for the peptide. Furthermore, it is important to know whether the A sheet or the C sheet model accounts for serpin polymerization for meaningful therapeutic intervention. Differentiation between the two models could be made by studying pathological variants and constructing mutants in the A sheet and C sheet. Loop-sheet polymers have been identified in other serpins (Aulak *et al.*, 1993; Faber *et al.*, 1993). It is interesting to note that one of these mutants is a loop hinge mutant, the C1-inhibitor P_{10}Aa→Thr, which forms polymers that can be detected in human plasma (Aulak *et al.*, 1993).

5. OVALBUMIN LOOP HINGE MUTANTS

5.1 INTRODUCTION

Alignment of the reactive centre loop motif of serpins shows a consensus sequence of small side chains in the hinge region, P9-P15 (Figure 1.10). This sequence allows a degree of flexibility in the structure essential for insertion of the base of the loop strand into the A-sheet (Carrell *et al.*, 1991). Ovalbumin diverges from the consensus sequence, as does angiotensinogen another non-inhibitory serpin, with a bulky arginine residue at P14 replacing threonine, and two valines at P12 and P11 replacing alanines. By PCR-based site directed mutagenesis (Landt *et al.*, 1990) an ovalbumin mutant ($OvP_{14}R,P_{11/12}A$) was constructed by replacing P_{14} arginine with threonine and the P_{11} and P_{12} alanines by valines to produce a sequence conforming to the serpin reactive centre loop consensus sequence (Tables 2.2 and 2.3; Figure 5.1). These substitutions were designed with a view to increase loop mobility and allow the altered ovalbumin to undergo structural rearrangement of the reactive centre loop and hence, induce inhibitory activity. Physical changes typical of the S-R conformational changes can be tested for by thermal stability and CD spectral shift changes (Gettins, 1989; Stein *et al.*, 1989).

	P_{17} P_1
α_1-antitrypsin	E K G T E A A G A M F L E A I P M
Ovalbumin	E A G R E V V G S A E A G V D A A
$OvP_{14}T,P_{11/12}A$	E A G **T** E **A A** G S A E A G V D A A
"Z" Ovalbumin	**K** A G T E A A G S A E A G V D A A

Figure 5.1 Loop sequence (P_1-P_{17}) of ovalbumin mutant constructs aligned to the corresponding sequences of M (normal) α_1-antitrypsin and wild type (normal) ovalbumin. Ovalbumin mutagenesis sites are in bold.

The secretory properties of the ovalbumin mutant in *Xenopus* oocytes was also assessed, to determine the relationship between loop mobility (ability to undergo S→R change) and secretion. Another ovalbumin mutant

constructed involved replacement of the well conserved P17 glutamic acid by lysine at the hinge region of ovalbumin, corresponding to residue to 342 of α_1-antitrypsin. This construct is a ovalbumin homologue of Z α_1-antitrypsin, known here as "Z" ovalbumin ($OvP_{17}K$) (Tables 2.2 and 2.3; Figure 5.1). Substitution of non polar residues in α_1-antitrypsin at position 342 does not cause an impairment in secretion of the protein indicating that the secretion defect is a result of the bulky, positively charged lysine residue rather than the absence of the carboxyl group of glutamic acid (Sifers *et al.*, 1989; Wu & Foreman, 1990; McCracken *et al.*, 1991). The secretory properties of "Z" ovalbumin in *Xenopus* oocytes in comparison with wild type ovalbumin was examined to establish the significance of this mutation. The mutation, P_{17}Glu→Lys, in Z α_1-antitrypsin occurs at the turn between strands s4A and s5A of the A sheet (Loebermann *et al.*, 1984) and is proposed to sterically hold open the A sheet between strands 3 and 5 (Figure 3.12) and thereby promote loop-sheet polymerization (Lomas *et al.*, 1992).

5.2 RESULTS

5.2.1 Secretion of Ovalbumin Mutants

The two ovalbumin mutants, "Z" ovalbumin and $OvP_{14}Thr,P_{11/12}Ala$, were constructed by PCR-based mutagenesis using wild type ovalbumin as template (Table 2.3 and Figure 5.2). The 1.3 kb PCR products (lanes 2 and 4) were cloned into the *Hin*d III site of the plasmid vector pSP64 downstream of the SP6 promoter. Three independent "Z" ovalbumin clones were obtained and only one clone of $OvP_{14}Thr,P_{11/12}Ala$. After full-length sequencing of these ovalbumin variants they were *in vitro* transcribed as described in *Methods*. mRNAs were translated *in vitro* by the reticulocyte lysate system and subjected to SDS-PAGE, to confirm that ovalbumin protein was produced (Figure 5.3A). Wild type and "Z" ovalbumin produced protein bands of similar size and intensity (lanes 1 and 2); however, in

comparison the $OvP_{14}T,P_{11/12}$ construct produced very weak bands.

Oocytes were then injected with the same concentrations of ovalbumin wild type or mutant mRNAs and the oocytes cultured for 24 hours in the presence of L-[^{35}S] methionine. Radiolabelled ovalbumin was immunoprecipitated from the incubation media and oocyte homogenates, and submitted to SDS-PAGE followed by fluorography (Figure 5.3B). The loop mutant of ovalbumin, $OvP_{14}T,P_{11/12}A$, did not produce any ovalbumin (data not shown), although the reasons for this lack of protein production were not clear. Wild type and "Z" ovalbumin produced two intracellular ovalbumin bands of molecular weight 45-46 kDa. These bands represent glycosylated derivatives of ovalbumin, and similar results have been obtained previously with ovalbumin mRNA injected *Xenopus* oocytes (Colman *et al.*, 1981). Of the two ovalbumin polypeptides secreted into the media, the lower molecular mass species is of stronger intensity than the larger ovalbumin species. The fluorograph and quantitation by band excision and scintillation counting (Figure 5.4) shows that a relatively small amount of wild type ovalbumin is secreted (20.0% $\pm$1.4). In the case of "Z" ovalbumin even less of the serpin, a 50% decrease compared to wild type, was secreted (9.0% $\pm$1.0).

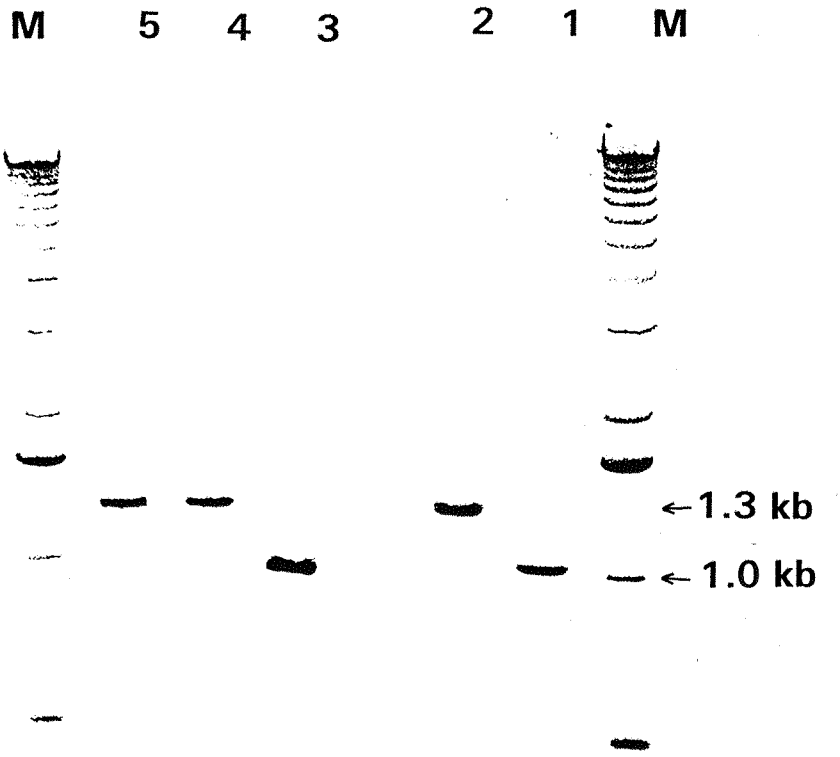


Figure 5.2 Agarose-gel electrophoresis of ovalbumin PCR mutagenesis products. Products of the first PCR (lanes 1 and 3) of size 1 kb and the second PCR (lanes 2 and 4) of size 1.3 kb were analyzed by electrophoresis in a 1% agarose gel in TBE buffer containing 0.5 μg ethidium bromide/ml. Lanes 1 & 2: Ovalbumin P_{17}Glu→Lys; Lanes 3 & 4: Ovalbumin P_{14}Arg→Thr, $P_{11/12}$Val→Ala. Lanes 5: 1.3 kb ovalbumin wild type cDNA insert; M: 1-kb DNA ladder (Gibco BRL, Life Technologies).

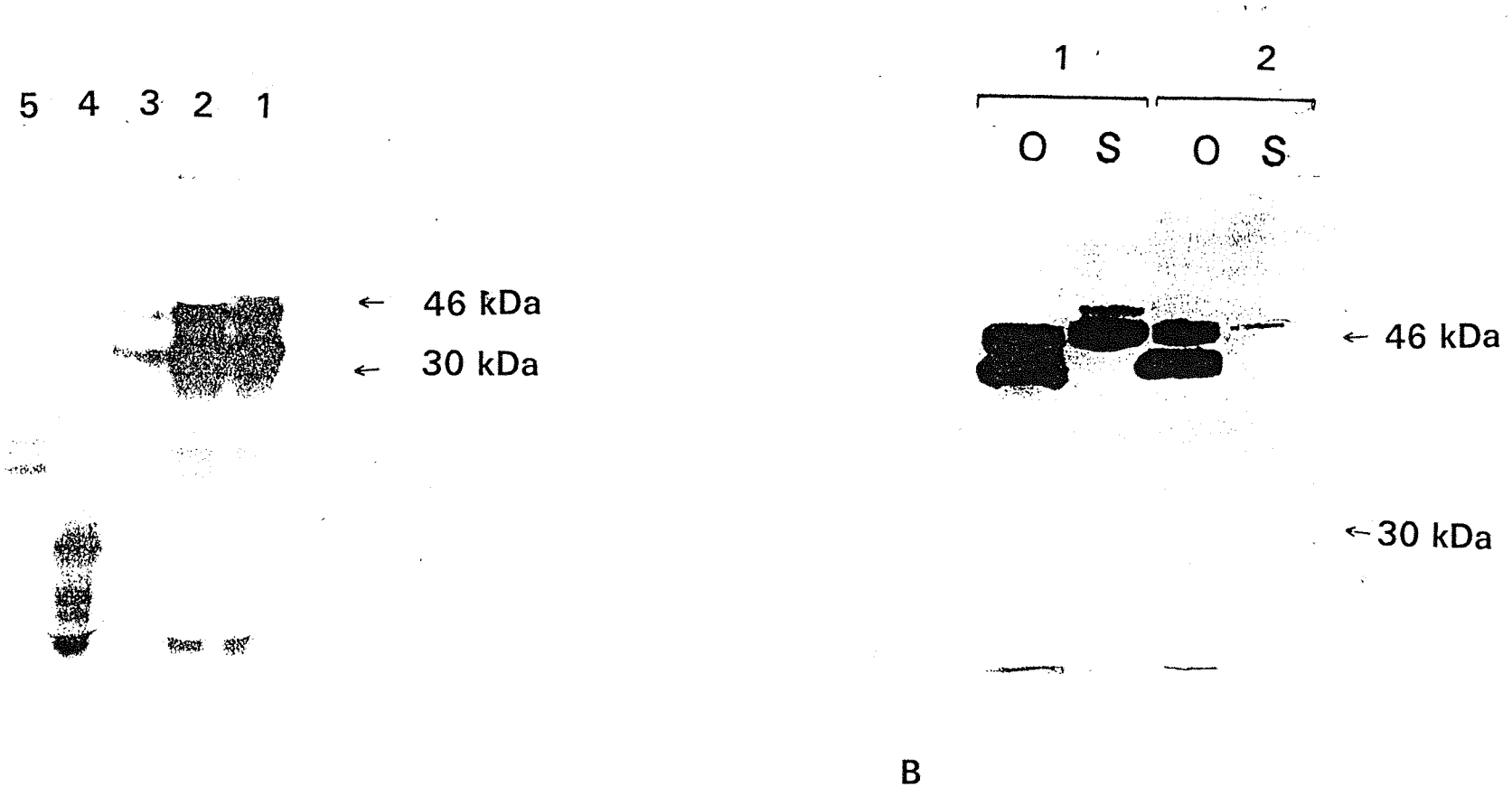


Figure 5.3 *In vitro* and *in ovo* translation of wild type and mutant ovalbumins. (A) Ovalbumin mRNAs were translated in a rabbit reticulocyte cell-free system in the presence of ^{35}S methionine and analyzed by SDS-PAGE as described in *Methods*. Lanes: 1, wild type ovalbumin; 2, "Z" ovalbumin; 3, $OvP_{14}Thr,P_{11/12}Ala$; 4, control RNA; 5, no RNA - control. (B) Twenty oocytes were injected with ovalbumin mRNA and incubated in media containing ^{35}S methionine. Labelled protein was immunoprecipitated from cell extracts and incubation media, and subjected to SDS-PAGE as described in *Methods*. O represents oocyte extract; S represents secreted material. Lanes: 1, wild type ovalbumin; 2, "Z" ovalbumin.

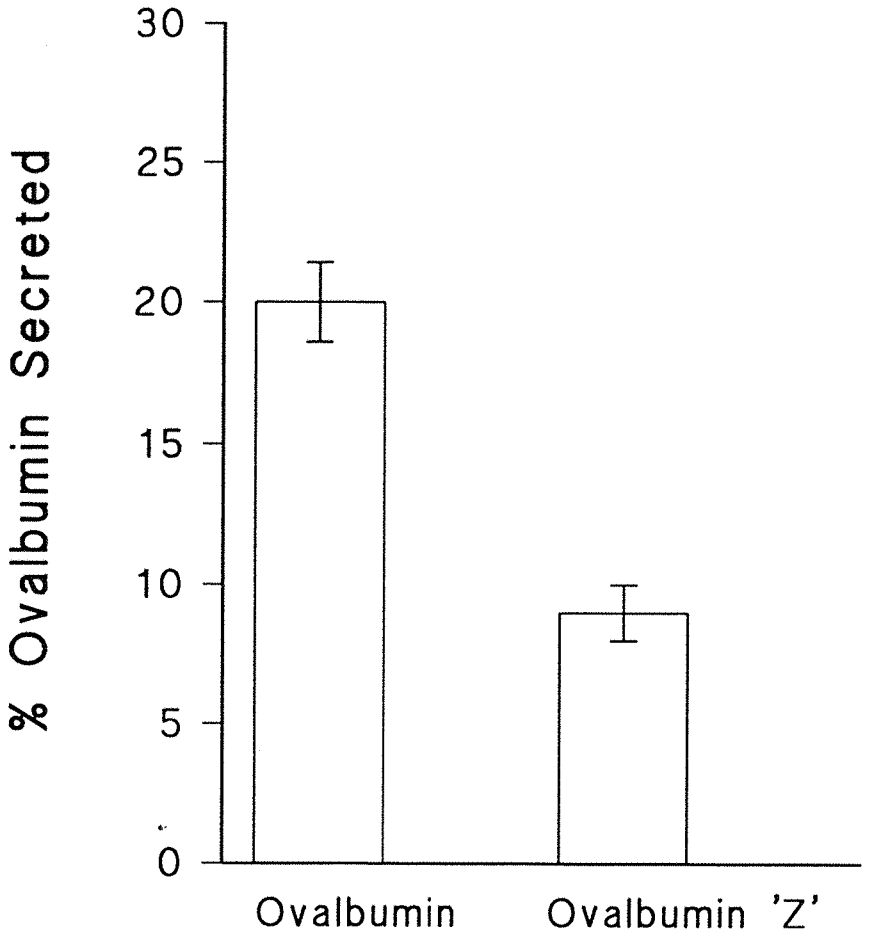


Figure 5.4 Quantitation of the relative amounts of wild type and "Z" ovalbumin secreted from microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Amounts of secreted ovalbumin are expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least four different experiments using three different animals. Each experiment involved the labelling of at least twenty oocytes. Values shown are expressed as the mean ± the standard error of the mean.

5.3 DISCUSSION

Ovalbumin is a non-inhibitory member of the serpin family that despite the failure to undergo the S→R serpin conformational change (Stein *et al.*, 1989), typical of inhibitory serpins, shares a 30% sequence and tertiary structure homology with α_1-antitrypsin (Hunt & Dayhoff, 1980). The S→R transition of inhibitory serpins is accompanied by incorporation of the reactive loop into the A sheet to form the central strand, s4A, of the six-stranded antiparallel ß-sheet (Loebermann *et al.*, 1984). The failure of ovalbumin, and one other non-inhibitory serpin angiotensinogen, to undergo the S→R transition is attributed to the presence of large and/or more polar residues at positions $P_{11/12}$ and P_{14} (Figure 1.10) in the base of the loop (Stein *et al.*, 1989). All inhibitory serpins have conserved small hydrophobic amino acids in this region that are believed to form a mobile hinge which facilitates partial entry of the loop into sheet A to develop the canonical form of the active inhibitor (Engh *et al.*, 1990; Carrell *et al.*, 1991; Skriver *et al.*, 1991). A large number of studies have demonstrated that point mutations in the hinge region inhibitory serpins convert the proteinase inhibitors into proteinase substrates and prevent the S→R transition (Perry *et al.*, 1989; Carrell *et al.*, 1991; Caso *et al.*, 1991; Skriver *et al.*, 1991). This study involved construction of a mutant of ovalbumin with point mutations in the hinge region, converting P_{14} arginine to threonine and P_{11} and P_{12} valines to alanines (OvP_{14}T,$P_{11/12}$A), to produce a loop sequence in consensus with the inhibitory serpin loop motif (Figure 5.1). This attempt to convert ovalbumin into a proteinase inhibitor and allow it to undergo the S-R conformational changes, such as increased thermal and denaturation stability was not successful. Unfortunately, the OvP_{14}T,$P_{11/12}$A mutant was not expressed in *Xenopus* oocytes, whereas it was translated *in vitro* by the reticulocyte lysate cell-free system, albeit not as efficiently as wild type ovalbumin (Figure 5.3A). No undesired mutations were found in the complete sequence of the ovalbumin variant, so perhaps the P_{14}Thr and $P_{11/12}$Ala hinge mutations have a profound structural consequences on either

mRNA stability, protein folding or translocation *in ovo*. These possibilities were not investigated because more pressing experiments pursued.

The P_{17} glutamic acid residue is strongly conserved among the serpins, inhibitory and non-inhibitory, implying an important structural-functional role (Huber & Carrell, 1989). The naturally occurring Z α_1-antitrypsin variant has the mutation P_{17}Glu→Lys (Jeppsson, 1976), that lies at the turn between strands s4A and s5A (Loebermann *et al.*, 1984), acting as a hinge for partial insertion of the loop (s4A) into the A sheet upon binding to a proteinase or after reactive loop cleavage (Ogushi *et al.*, 1987). The major consequence of the mutation is a defect in secretion that results in the intracellular accumulation of the inhibitor (Sharp, 1971; Bathurst *et al.*, 1983). It has been proposed that the Z mutation at the hinge of the reactive loop impedes refolding of the loop into the A sheet so holds the A sheet in an open conformation to allow the loop of another Z α_1-antitrypsin molecule to insert, with the sequential formation of loop-sheet polymers (Lomas *et al.*, 1992; 1993a). The importance of the hinge region of ovalbumin corresponding to P_{17} of α_1-antitrypsin was examined by the replacement of the well conserved glutamic acid at this position with lysine, thus creating a "Z" ovalbumin. The secretory properties of this mutant were compared with wild type ovalbumin to ascertain the significance of this mutation. Wild type ovalbumin synthesized by oocytes was secreted very poorly; only 20% of the total ovalbumin synthesized was present in the incubation media (Figure 5.4). Ovalbumin lacks a classical N-terminal signal peptide sequence (Lingappa *et al.*, 1979; Palmiter *et al.*, 1978), but instead has a uncleaved internal N-terminal signal sequence between residues 50 and 66 that is implicated in transmembrane location (Robinson *et al.*, 1986). Passage into the lumen of the endoplasmic reticulum is a prerequisite for secretion and any ovalbumin in the cytosol is not secreted (Lingappa *et al.*, 1979; Colman *et al.*, 1981). Thus, miscompartmentalisation of some of the ovalbumin synthesized results in the low levels of secretion. The construct ovalbumin "Z" was poorly secreted, a 50% decrease in secretion compared

to wild type, so no secreted material could be collected to conduct activity and stability assays. Nevertheless, it was clear that the "Z" ovalbumin secretory profile mirrors that of Z α_1-antitrypsin, in that the P_{17}Glu→Lys mutation specifically restricts the secretory activity of the serpin in *Xenopus* oocytes. Time constraints prevented progression with experiments designed to investigate whether the secretory blockage of ovalbumin P_{17}Glu→Lys occurred by the mechanism of loop-sheet polymerisation, as proposed for the intracellular accumulation Z α_1-antitrypsin (Lomas *et al.*, 1992). Similar experiments to those conducted in chapter 3, for example sensitivity to Endoglycosidase H, would have been conducted to monitor the movement and site of accumulation of ovalbumin along the secretory pathway. If loop-sheet polymerization is promoted by the mutation P_{17}Glu→Lys in "Z" ovalbumin, it implies that the reactive loop of ovalbumin can insert into the A sheet despite the bulky loop residues, particularly at positions P_{14}, P_{11} and P_{12} (Stein *et al.*, 1989). Hence in contrast to work in previous chapters on α_1-antitrypsin loop mutants, it seems that the loop of ovalbumin can insert into the A sheet to produce polymers. But we have not considered the difference in A sheet structures between α_1-antitrypsin and ovalbumin, which will have implications on the mechanism of polymerization of the two proteins.

Other possible mechanisms that may explain the defect in "Z" ovalbumin secretion are increased mRNA or protein degradation, or abnormal posttranslational processing/transport. The *Xenopus* system provides an ideal system for investigating these mechanisms in detail. For example, the kinetics of mutant ovalbumin secretion can be monitored by pulse-labelling ovalbumin microinjected oocytes in ^{35}S labelled media and then chasing in the presence of unlabelled media for various time periods. SDS-PAGE analysis of immunoprecipitated chase cell lysate and incubation medium samples allows the time course of secretion to be determined. The relative stability of "Z" ovalbumin mRNA compared to wild type could be assessed by microinjection of ^{32}P labelled ovalbumin mRNAs followed by RNA

extraction and analysis by formaldehyde gel electrophoresis and autoradiography (Sambrook *et al.*, 1989) at various time intervals.

Time was not available to conduct heat stability and inhibitory capacity assays in order to determine whether the ability to undergo the S→R transition had been bestowed on the mutant ovalbumins. High levels of protein are required for analysis and could be achieved by cloning the ovalbumin cDNAs into a high expression vector under the control of an inducible promoter, for example the yeast pKV50 vector (Delta Biotechnology Limited) which is induced by galactose.

6. GENE EXPRESSION WITHOUT CLONING: EXPRESSION-PCR

6.1 INTRODUCTION

Standard methods of protein expression from plasmid DNA involve cloning into a plasmid vector containing a bacteriophage promoter, transcription in the presence of the appropriate RNA polymerase and *in vitro* translation (Krieg & Melton, 1984; Melton *et al.*, 1984). These methods are labour intensive, time consuming, and require a specialized transcription vector, cloning, plasmid isolation and linearization, and limited by the restriction enzyme cloning sites in the vector. Previous work in this study has used the vector SP64T (Krieg & Melton, 1984), modified to contain a single *Pst* I cloning site flanked by 5′- and 3′ untranslated regions of the *Xenopus laevis* ß-globin gene which include the ribosome binding site, capping site and poly(A) tail (Foreman, 1987). This vector produces mRNA that is well expressed in oocytes because it bears the signals that distinguish it as endogenous mRNA.

Previously it has been shown that by incorporating a bacteriophage promoter into a 5′-amplimer, PCR amplified templates can be transcribed directly without the need for cloning (Browning, 1989). Following this technique termed expression-PCR, 5′ oligonucleotide primers for α_1-antitrypsin and ovalbumin were designed to contain the bacteriophage SP6 promoter. These primers were used in a single PCR reaction as the 5′ amplimer together with the downstream 3′ universal primer of α_1-antitrypsin or ovalbumin (Tables 2.1 and 2.3) to amplify the P_{17}Glu→Lys variants of both α_1-antitrypsin and ovalbumin previously produced by PCR mutagenesis. Wild type template was also prepared by a single PCR reaction with the 5′-SP6 primer and the downstream 3′ universal primer amplifying wild type template. DNA templates produced were purified using Promega Wizard™ PCR Preps and transcribed using the Promega Ribomax transcription system as reported in *Methods*. RNA produced was translated *in vitro* in a rabbit reticulocyte lysate cell-free system in the presence of L-[^{35}S] methionine according to the manufacturers specifications (Promega). Labelled proteins

were analyzed by SDS-PAGE on 10% resolving gels and radiography. Translated proteins were immunoprecipitated to determine if proteins produced *in vitro* were biologically active. Functional RNAs were then microinjected into *Xenopus* oocytes to compare the secretory properties of PCR-template transcribed RNA to RNA transcribed using from template in the vector pSP645RCF.

6.2 RESULTS

6.2.1 Ovalbumin Expression-PCR

Initial experiments involved the construction of wild type ovalbumin and "Z" ovalbumin using the primer OVSPUNI (see Table 6.1) in a single PCR reaction with OV3uni primer (Table 2.2). Reaction conditions were 20 cycles consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, followed by a 5 min incubation at 72°C. 100 pmoles of each primer and 2.5 units of *Taq* polymerase were used in a reaction volume of 100 μl, using reaction buffer recommended by the manufacturer (Promega). PCR products were analyzed by 1% agarose gel electrophoresis as described in *Methods*, purified and then resuspended in 50 μl DEPC treated RNase-free water. 2-5 μg of the PCR DNA template was transcribed and translated *in vitro* using reticulocyte lysates. Translated proteins were immunoprecipitated and submitted to SDS-PAGE analysis. As shown in Figure 6.1 the antibody against ovalbumin recognized the ovalbumin translation products produced by expression-PCR.

Ovalbumin mRNAs produced by expression-PCR were microinjected into *Xenopus* oocytes which were incubated in media containing ^{35}S methionine for 24 hours. Immunoprecipitation and SDS-PAGE analysis did not produce any radiolabelled ovalbumin, even though the RNAs were well expressed *in vitro*. Using ^{32}P UTP in the Promega Ribomax transcription mix, radiolabelled RNA as produced and analyzed by formaldehyde denaturing gel

electrophoresis (according to Sambrook *et al.*, 1989) to characterise the RNA. Figure 6.2 displays an autoradiogram of ovalbumin transcripts, revealing only one major RNA species in both ovalbumin transcripts constructed by expression-PCR that were of identical length to ovalbumin RNA transcribed from ovalbumin cDNA in the plasmid vector pSP64.

Primer Name	Primer Sequence (5′ - 3′)	Base Pairs
ATSP6UNI	ACG**ATTTAGGTGACACTATA**GAATAGACAGTGAATCGACAATG	43
OVSP6UNI	ACG**ATTTAGGTGACACTATA**GAATAAAGCTTGCCGAAAGAC	41
ATSP6UNI2	GGCCAAGCTTCG**ATTTAGGTGACACTATA**GGTTTTATTTTTAATTTTCTTTCAAATACTTCCAGTGAATCGACAATG	78

SP6 Promoter — UTL from AMV

Table 6.1 5′-primer sequences used in expression-PCR reactions of α_1-antitrypsin and ovalbumin. The SP6 promoter sequence in each primer is shown in bold and the untranslated leader sequence (UTL) from alfalfa mosaic virus (AMV) is underlined.

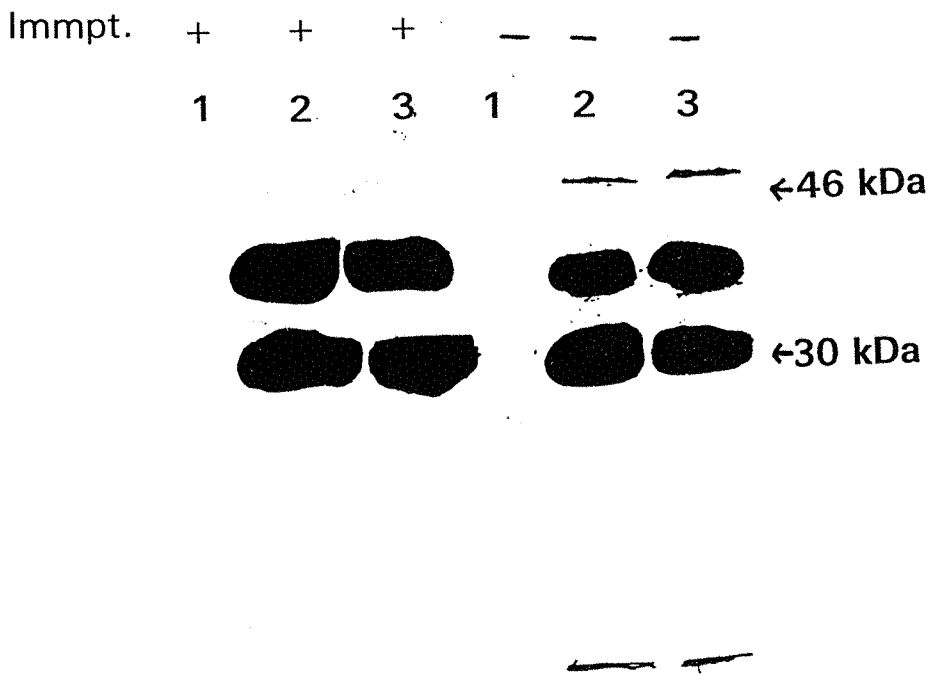


Figure 6.1 *In vitro* translation and immunoprecipitation of wild type and "Z" ovalbumin. Ovalbumin mRNAs were transcribed from PCR templates generated by ovalbumin expression-PCR, and then translated in a rabbit reticulocyte lysate system (Promega) in the presence of ^{35}S methionine. Translation products were immunoprecipitated by anti-ovalbumin and analyzed by SDS-PAGE and autoradiography as described in *Methods*. Lane 1, no RNA (control); 2, wild type ovalbumin RNA; 3, "Z" ovalbumin RNA. Immpt. indicates immunoprecipitation. Molecular mass was determined by co-migration of standard markers.

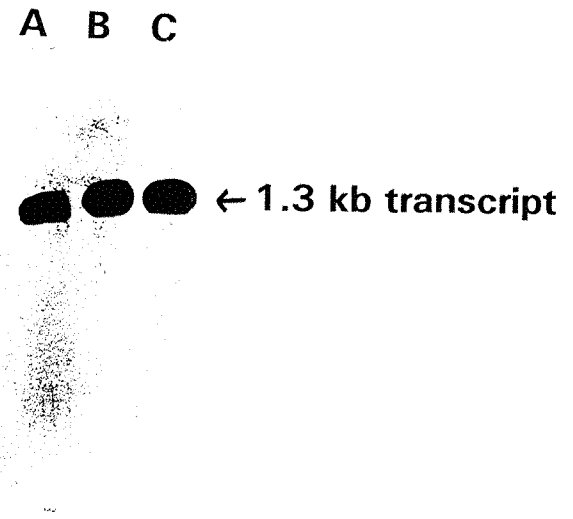


Figure 6.2 Analysis of ovalbumin transcripts. ^{32}P labelled RNA transcripts of wild type (lane B) and "Z" ovalbumin (lane C) were produced from expression-PCR template (SP6OVUNI primer) and analyzed on a 1% agarose denaturing formaldehyde gel in 1x MOPS/EDTA buffer, followed by autoradiography (according to Sambrook *et al.*, 1989). Lane A is ^{32}P labelled ovalbumin RNA produced from wild type ovalbumin cloned in the vector pSP64.

6.2.2 α_1-Antitrypsin Expression-PCR

In experiments analogous to those conducted for ovalbumin, expression-PCR was used to produce normal α_1-antitrypsin and Z α_1-antitrypsin. Initially primer ATSP6UNI (Table 6.1) containing a bacteriophage SP6 promoter was used as the 5'-amplimer and the resulting PCR template transcribed *in vitro* as described in *Methods*. However, the transcripts were not translated efficiently in rabbit reticulocyte cell-free translation system. This maybe because the major difference between vector transcribed RNA and expression-PCR transcribed RNA is that the former contains 5'-untranslated leader sequences (UTL) which increase the translation efficiency. Subsequent experiments were conducted with the primer ATSP6UNI2 that contains an untranslated leader sequence (UTL) from the coat protein mRNA of the alfalfa mosaic virus (AMV) between the bacteriophage SP6 promoter and the AUG initiation codon (Table 6.1). The UTL sequence from AMV has been reported to increase the efficiency of translation of *in vitro* transcribed products as much as 35-fold (Jobling & Gehrke, 1987). Figure 6.3 displays the results from the microinjection of wild type and Z α_1-antitrypsin constructed with the ATSP6UNI2 primer. Expression-PCR α_1-antitrypsins were not expressed as efficiently as α_1-antitrypsin transcripts from the SP6 vector. Band excision and scintillation counting revealed that 70% of expression-PCR M α_1-antitrypsin was secreted; identical to that obtained with vector transcribed M α_1-antitrypsin.

Another difference between expression-PCR transcribed RNA and RNA produced from the SP64TRCF vector is the poly(A) tail attached to 3'end of vector transcribed RNA. α_1-Antitrypsin template produced with the ATSP6UNI2 primer was *in vitro* transcribed, and then polyadenylated by addition of 1 μl Poly(A) polymerase (1 unit) (Gibco BRL) at 37°C for 15 minutes, prior to phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). Although polyadenylated mRNAs were translated *in vitro* as efficiently as non-adenylated mRNAs, polyadenylation did not

have any effect on the secretory properties of expression-PCR transcribed α_1-antitrypsin (data not shown).

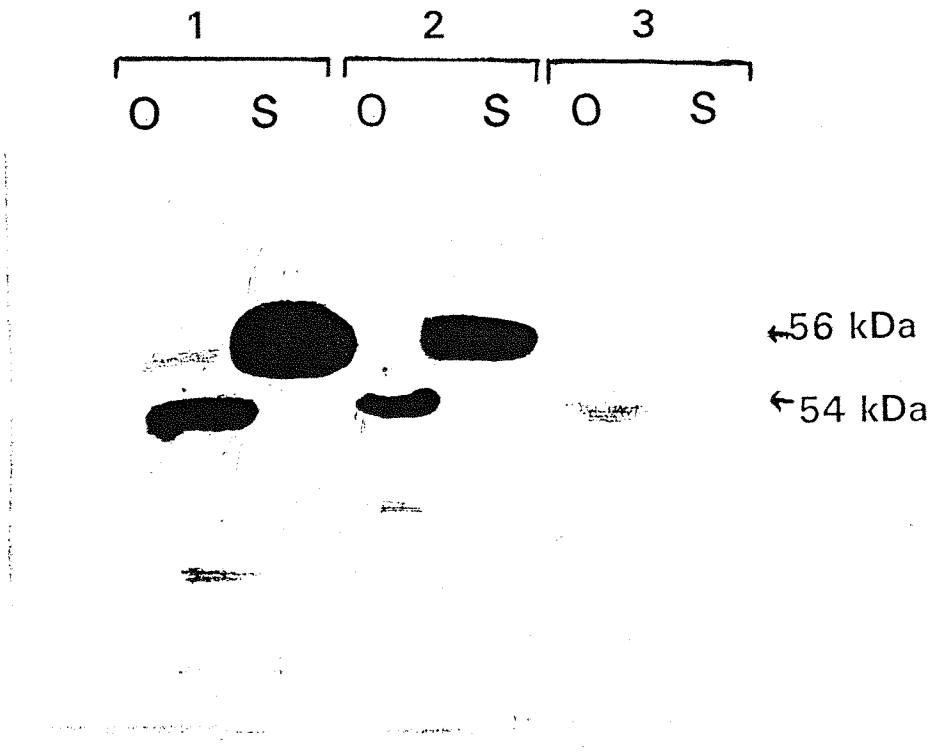


Figure 6.3 Synthesis of M and Z α_1-antitrypsins mRNAs produced by expression-PCR in *Xenopus* oocytes. Twenty oocytes were injected with messenger of a given α_1-antitrypsin variant and incubated in media containing ^{35}S methionine. Labelled protein was immunoprecipitated from cell extracts and incubation media, and separated by SDS-PAGE as described in *Methods*. O represents oocyte extract material; S represents material secreted into the surrounding medium. Lanes: 1, M α_1-antitrypsin transcribed from α_1-antitrypsin cloned into pSP645RCF; 2, M α_1-antitrypsin produced by expression-PCR; 3, Z α_1-antitrypsin produced by expression-PCR. The primer used in expression-PCR was ATSP6UNI2. Molecular mass was determineD by the co-migration of standard protein markers.

6.3 DISCUSSION

Conventional methods of protein expression from plasmid DNA entail a number of genetic manipulations that require a specialized expression vector which contains a RNA polymerase promoter upstream from a multiple cloning region (Krieg & Melton, 1984; Melton *et al.*, 1984). Browning (1989) demonstrated that PCR templates containing a suitable RNA polymerase promoter could be used directly for *in vitro* transcription of products downstream of the promoter without the need for cloning. This has been termed expression-PCR (Kain *et al.*, 1991) because it eliminates the need for specialized vectors, cloning, plasmid purification and restriction endonuclease digestion. 5′-primers that contained the bacteriophage SP6 promoter sequence (Table 6.1) were designed for α_1-antitrypsin and ovalbumin and together with a 3′-universal downstream primer used to amplify the Z variants (P_{17}Glu→Lys) of both proteins produced previously by PCR-based site directed mutagenesis was performed (Landt *et al.*, 1990) as described in *Methods*. Wild type template was also prepared in a single PCR reaction, with the 5′-SP6 primer and the downstream 3′-universal primer to amplify wild type template and so attach the bacteriophage SP6 promoter sequence. Expression-PCR templates were used for *in vitro* transcription, translated in reticulocyte lysates and the protein synthesized subjected to SDS-PAGE.

In vitro translated α_1-antitrypsin and ovalbumin proteins gave the correct molecular weight band on SDS-PAGE, but were not expressed in microinjected oocytes. *In vivo* translation can be problematic, and is often dependent on the characteristics of sequences 5′ to the initiation codon. The efficiency of translation is dependent on the distance of the 5′-end of the RNA from the AUG initiation codon (Struhl, 1987); if the distance is either too short or too long transcription may be hindered. The major difference with expression-PCR template and vector pSP64TRCF template is that flanking the cloning site of the vector is 5′ and 3′ untranslated regions from

Xenopus laevis ß-globin (Krieg & Melton, 1984; Foreman, 1987). These regions contain a 5′ capping site, ribosome binding site and 3′ poly(A) tail coding site. Thus, mRNA produced from the vector bears the hallmarks of a well expressed amphibian mRNA, making it suitable for translation in *Xenopus* oocytes. All transcripts injected into oocytes were capped with the cap analog mG(5′)ppp(5′)m by including this dinucleotide in the SP6 transcription reaction (as described in *Methods*). A 5′ cap structure on mRNA is essential to ensure mRNA stability and efficient translation in mRNA injected oocytes (Krieg & Melton, 1984; Drummond *et al.*, 1985). Subsequent experiments were conducted on α_1-antitrypsin to provide 5′ and 3′ untranslated sequences to increase translation efficiency.

It has been demonstrated that addition of an untranslated leader sequence (UTL) 5′ to the initiation codon increases the efficiency of translation *in vitro* (Struhl, 1989). Expression-PCR was performed on α_1-antitrypsin using a primer that contained the UTL from the coat protein of the alfalfa mosaic virus (AMV) between the bacteriophage SP6 promoter and the initiation codon. It has been previously shown that replacement of a gene's indigenous untranslated leader sequence with the UTL from AMV can increase the translational efficiency substantially (Jobling & Gehkre, 1987). This increase has been attributed to the reduced requirements of translational initiation factors by mRNA containing the AMV untranslated leader sequence (Browning *et al.*, 1988). Although some protein expression was achieved in *Xenopus* oocytes, it was of very low intensity. It may be necessary to replace the 5′-UTL with a UTL from an efficiently expressed *Xenopus* protein such as the ß-globin sequence. This sequence is cloned into the SP64TRCF vector, 5′ to the *Pst* I cloning site (Foreman, 1987).

The role a 3′-poly(A) tail in stabilizing mRNAs injected into oocytes remains controversial. Some studies have demonstrated that transcripts with a 3′OH poly(A) tail have a longer half-life than deadenylated (poly(A)$^-$) transcripts in oocytes (Marbaix *et al.*, 1975; Drummond *et al.*, 1985). Huez

and colleagues have shown that deanylated globin mRNA has a half-life of of less than 12 hours following oocyte injection (Huez *et al.*, 1974), but restoration of the poly(A) restored the stability of the molecule to 3 days (Marbaix *et al.*, 1975). The poly(A) tract may stabilize mRNA perhaps by preventing binding of a 3′→5′ exonuclease. Nevertheless, others have shown that poly(A)$^-$ mRNAs have similar stability to polyadenylated mRNAs in oocytes (McCrae & Woodland, 1981; Krieg & Melton, 1984). Additionally, significant increases in mRNA translation have been reported following polyadenylation of injected transcripts (Huez *et al.*, 1977; Drummond *et al.*, 1985; Khorana *et al.*, 1988). However, little or no effect on translational efficiency was observed by other studies (Deshpande *et al.*, 1979; Soreq *et al.*, 1981). RNA transcripts were polyadenylated post-transcriptionally in a reaction catalyzed by poly(A) polymerase and microinjected into oocytes, in a view to increase *in vivo* stability and/or protein expression. Polyadenylation of expression-PCR transcribed RNA did not increase the translational efficiency *in ovo*. Gel electrophoresis of transcription reactions had revealed that injected transcripts consisted of a single species of RNA with an electrophoretic mobility consistent with the size predicted from the nucleotide sequence. In addition, protein of the predicted molecular weight was detected following translation and SDS-PAGE. Thus, the low expression in *Xenopus* oocytes may possibly be due to transcript instability. Additional experiments could be performed to determine *in vivo* stability of injected mRNAs. Such investigations would involve injection of radiolabelled mRNA (for example, with ^{32}P UTP) followed by RNA extraction and analysis by gel electrophoresis at various time periods (Drummond *et al.*, 1985). Other aspects of the 3′ terminal structure of the RNAs may also be involved in the reduced translational efficiency of the expression-PCR mRNAs.

7. CONCLUSION

A discussion of the experimental findings present in this thesis have been included at the end of individual chapters. At this point I will attempt to summarise these findings and draw some conclusions on their relevance to serpin conformational change and molecular pathology.

The *Xenopus* oocyte secretory system was shown to be a valid system for the study of α_1-antitrypsin deficiency variants, because the rare S_{iiyama} ($Ser^{53}\rightarrow Phe$) and M_{Malton} (Phe^{52} deleted) variants duplicated the secretory defect seen in hepatocytes that results in decreased plasma α_1-antitrypsin levels. Digestion with Endoglycosidase H localised the retained protein to a compartment prior to entry into the Golgi complex, suggesting a common site of accumulation of the non-secreted inhibitor.

The X-ray crystal structure of α_1-antitrypsin localises the position of the Z mutation at the junction of the head of the fifth strand of the A sheet and the base of the reactive centre loop while the S_{iiyama} and M_{Malton} mutations are located in the B-helix underlying the A sheet (Loebermann *et al.*, 1984) (Figure 3.12). The location of the mutations at well separated regions of the α_1-antitrypsin molecule does not support the hypothesis that the mechanism of accumulation results from the mutations exposing an endoplasmic reticulum retention sequence (McCracken *et al.*, 1989). The mechanism of loop-sheet polymerization, involving the insertion of the reactive centre loop of one molecule into a ß-pleated sheet of the next to give polymers, has been proposed for the formation of insoluble intracellular α_1-antitrypsin inclusions in hepatocytes (Lomas *et al.*, 1992; 1993a,b). Molecular modelling data suggests the B-helix that underlies the A ß-sheet, providing grooves along which the sliding movements involved in opening and closing of the A ß-sheet occur (Stein & Chothia, 1991). These mutations will perturb the structure of the A ß-sheet, and lock the conformation of the A sheet in an open form favouring the formation of loop-sheet polymers (Lomas *et al.*, 1993a,b).

I investigated whether loop-sheet polymerization was a cause or an effect of intracellular accumulation of the above-mentioned α_1-antitrypsin deficiency variants. One way to distinguish the importance of these events is to design mutations that prevent polymerization and determine the effect on the intracellular accumulation of α_1-antitrypsin. Two main approaches were taken that involved: (a) mutations in the hydrophobic core underlying the A sheet and (b) mutations in the loop which would restrict loop mobility and impede insertion into the A sheet, a prerequisite to polymerization and protein aggregation. The B-helix Phe^{51}→Leu mutation which stabilises M α_1-antitrypsin against polymerization (Kwon *et al.*, 1994), predictably by locking the A sheet in the closed position, completely abolished the intracellular accumulation of S_{iiyama} α_1-antitrypsin and reduced significantly the accumulation of Z α_1-antitrypsin. Replacement of the loop hinge alanines at positions P_{11} and P_{12} by valines reduced the secretory blockage of Z α_1-antitrypsin by increasing protein export two-fold. Thus a decrease in Z and S_{iiyama} α_1-antitrypsin accumulation was obtained with mutations designed to either close the A sheet or decrease loop mobility in the hinge region. The mutations provided good indirect evidence of α_1-antitrypsin linkage by loop-sheet polymerization as the cause of protein aggregation in hepatocytes.

Recent structural studies on a dimer of antithrombin (Carrell *et al.*, 1994; Schreuder *et al.*, 1994) (Figure 1.15; section 1.13.4), suggest a C sheet model of polymerization as an alternative to the A sheet polymerization model. The A sheet model requires the loop of one molecule to insert into the A sheet of the next molecule. In the C sheet model however, incorporation of the loop of one molecule into the A sheet of the same molecule is accompanied with the release of strand s1C from its C sheet, and it is this strand that is replaced by the reactive loop of a second molecule to allow sequential reactive loop-C sheet polymers. The mutants that I constructed affected the interaction between the A sheet and the mobile loop, the initial step common to both polymerization models. However, the extent of loop insertion may differ between the two models.

The A sheet model demands that the gap between strands s3A and s4A remain free for intermolecular association, whereas the C sheet model necessitates incorporation of distal regions of the loop so that strand s1C is displaced from the C sheet. The $P_{11/12}$Ala→Val mutation prevented the accumulation of Z α_1-antitrypsin presumably by preventing this distal region of the loop from insertion into the A sheet, and agrees with, but does not, confirm the C sheet model of serpin polymerization. The major problem that remains to be addressed is whether polymerization occurs by the A sheet or the C sheet model.

It is unfortunate that no physical and inhibitory properties were obtained from the mutants secreted by *Xenopus* oocyte. However, prospective studies include subcloning the mutant DNAs into a suitable expression vector (as in Hopkins *et al.*, 1993) and then characterization of the mutants by assessment of inhibitory activity, and association rate constants with proteinase, for example elastase or α-chymotrypsin (as in Lomas *et al.*, 1993a). Structural studies on these mutants will shed some light on understanding the mutational effects on α_1-antitrypsin and other serpins in general. In particular the structure of serpin-proteinase complexes have yet to be determined.

A construct of ovalbumin known as "Z" ovalbumin, containing the amino acid substitution present in Z α_1-antitrypsin, namely P_{17}Glu→Lys, was made by oligonucleotide directed mutagenesis. Although very little normal ovalbumin was secreted by *Xenopus* oocytes, a 50% reduction in secretion was observed with the "Z" ovalbumin. This accumulation may occur by the mechanism of loop-sheet polymerization, in a similar manner to Z α_1-antitrypsin. This was surprising because the loop of ovalbumin is not expected to insert into the A sheet because of the large loop residues at positions P_{14} and $P_{11/12}$. Differences in the A sheets of α_1-antitrypsin and ovalbumin, rather than reactive loop differences, may have implications on the mechanism of polymerization. The inability of ovalbumin (and another

non-inhibitory serpin, angiotensinogen) to undergo the S→R conformational change has been attributed to the large loop hinge residues that constrain the loop in an immobile conformation (Stein *et al.*, 1989). However the "Z" ovalbumin was not informative because secretion from oocytes was too low to obtain sufficient protein for analysis of inhibitory activity and ability to undergo the S→R transition. Other factors may explain the decrease in "Z" ovalbumin secretion such as mRNA instability, or abnormal folding, but these were not pursued.

Further work is necessary to investigate in detail the conformational changes in α_1-antitrypsin, and the relationship with other serpins, that result from mobility of the reactive centre loop and abnormalities in loop conformation that produce dysfunction and lung or liver disease. In normal serpins intramolecular loop insertion is important for adoption of an ideal inhibitory conformation and for subsequent complexation with a target proteinase. Abnormal proteins that form loop-sheet polymers do so by intermolecular loop insertion.

Investigations into *Xenopus* oocyte processing of the S_{iiyama} and M_{Malton} variants and mutations designed to impede loop-sheet polymerization have been submitted for publication (Lomas *et al.*, 1995; Sidhar *et al.*, 1995).

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