α_1-ANTITRYPSIN MUTATIONS: AN INVESTIGATION OF THEIR EFFECTS ON PROTEIN PROCESSING AND SECRETION

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To The Future

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If you smell what The Rock™ is cooking!!!!

ABSTRACT

The mechanism of loop sheet polymerization has been shown to have an important role in the formation of aggregates of α1AT that occur in the liver and lungs of patients with α1AT deficiency. Crystallographic analysis and predictive molecular modelling have interpreted the effects of point mutations known to cause a clinical deficiency in terms of their ability to promote loop sheet insertion.

This study examined the relative secretion of a number of both existing deficiency variants (Z, Sii, S, F and I), and novel mutations (termed P5 V, Q & S and Tyr^{38}→Phe/Cys), compared to M α1AT, following injection of mRNA into *Xenopus* oocytes and protein analysis using immunoprecipitation and SDS-PAGE. The results show that the secretion of Z (16%), Sii (13%), F (53%), P5S (53%), I (26%) and S (49%), along with Tyr^{38}→Phe (48%), is reduced compared to M (63%). Secretion of the other 2 P5 mutants V (62%) & Q (60%) shows no sig. difference ($p>0.01$) to M. This demonstrates the importance of mutations affecting specific regions of the molecule – particularly the A β-sheet (Z, Sii), the reactive centre loop (F and P5S) and the shutter domain (I, S and Tyr^{38}→Phe), on the secretion of α1AT.

The form of α1AT within oocytes following mRNA injection was also examined and compared between Z, Sii, F & I variants and M α1AT. Oocyte secretory pathway was isolated and α1AT protein content analysed using Native-PAGE and immuno-blotting. Results indicated the presence of high MW forms of α1AT within the pathway of oocytes injected with Z, Sii and I mRNA, but not those injected with M and F mRNA. This suggests that the blockade in secretion occurring within *Xenopus* oocytes, similar to that seen in liver cells, is due to the formation of high MW aggregates of α1AT protein.

The effects of 3 similar RCL peptides on the polymerization of α1AT was also examined both *in vitro* and *in vivo*. Results showed that all 3 peptides blocked protein polymerization *in vitro* to varying extents, indicating that insertion of these peptides into the gap in β-sheet A may be occurring to block polymerization. The addition of peptide to the oocyte secretory system had no significant effect on protein secretion and this needs further examination to ensure peptide delivery to site of protein synthesis within these cells.

Further experiments involved the production of a novel system to closer examine the secretory pathway of α1AT. α1AT mRNA was translated in the presence of microsomal membranes to produce 49kDa protein sequestered in the microsomal vesicles. Microsomes were then injected into oocytes and protein secretion analysed by immunoprecipitation and SDS-PAGE. The results show secretion of M α1AT (37%) is significantly higher ($p>0.01$) than Z (17%) and Sii (9%) variants. This demonstrates that exogenous microsomes can integrate successfully with components of the oocyte secretory machinery and that the secretory phenotype for α1AT protein is retained. We could detect no high MW forms of protein within microsomes prior to injection, suggesting that if aggregation does occur, it must occur subsequently. This novel secretory system may be useful in examining the early post-translational events of α1AT biosynthesis and that of other proteins with aberrant handling in the ER.

AIMS OF THE RESEARCH

The high incidence and severity involved with α_1-antitrypsin deficiency has greatly increased the interest and research into this hereditary genetic disorder over the last 20 years. This study has used an *in vivo* surrogate secretory system – *Xenopus laevis* oocytes – to examine the effects of a number of existing deficiency variants, as well as novel variants, on protein secretion. By examining the relationship between the positions of these mutations on the protein structure and any relative blockade in secretion, an increased understanding into the mechanisms by which secretory blockade may occur may be deduced. This study also examines this secretory blockade in more detail by isolating and comparing the 'protein processing pathway' of both normal and variant protein using a variety of *in vitro* and *in vivo* techniques. For the first time the protein retained within the oocytes will also be closely examined to determine whether the similar pattern of α_1-antitrypsin secretion, compared to *in vivo* liver cells, produced by these cells is due to a similar mechanism of protein polymerisation and aggregation or occurs via an oocyte-mediated effect. The results from these experiments may act to further validate the role of *Xenopus* oocytes in the study of the effects of α_1-antitrypsin variants on protein secretion and may increase their use in the development of agents to reduce or abolish protein polymerization and allow normal levels of secretion of variant proteins.

CONTENTS

FIGURES

ABBREVIATIONS

α_1-AT	α_1-antitrypsin
BC11	reactive centre loop peptide of eleven residues
BSA	bovine serum albumin
CD	circular diochroism
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Endo H	endoglycosidase H
ER	endoplasmic reticulum
Hepes	N-2-hydroxyethylpiperazine-N$'$-2-ethanesulphonic acid
HPLC	high pressure liquid chromatography
K_{ass}	association rate constant
kb	kilobase pairs (1000 nucleotide bases)
kDa	kilodalton
LB	Luria broth
Met	methionine
mRNA	messenger RNA
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
Pi	proteinase inhibitor variant
Pipes	piperazine-N,N$'$-bis-2-ethanesulphonic acid
PMSF	phenylmethylsulphonyl fluoride
r.p.m	revolutions per minute
RER	rough endoplasmic reticulum
RNase	ribonuclease

S→R	serpin 'stressed' to 'relaxed' conformational change
SDS	sodium dodecyl sulphate
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of SDS
SEC	serpin enzyme complex
SOS	Standard Oocyte Saline
TCA	trichloroacetic acid
TEMED	N,N,N′N′,- tetramethylethylenediamine
Tris	tris(hydroxymethyl)-aminomethane
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 The Serpin Superfamily

The serpins are a widely distributed group of serine proteinase inhibitors found in viruses, plants, insects and animals but not prokaryotes (Carrell & Stein, 1996). Despite the great evolutionary divergence of these organisms, their serpins are highly conserved, both in sequence and structure. The majority of these have been isolated from plasma, have structures that are variously glycosylated and have molecular weights ranging from 45 to 100kDa (Schulze *et al.*, 1994).

The major proteinase inhibitor in human plasma was discovered in 1894, isolated in 1955 and later named α_1-antitrypsin. In 1980, Hunt and Dayhoff recognised a familial relationship between the serine proteinase inhibitors antithrombin III (ATIII) and α_1-antitrypsin on the basis of a comparison of the primary structure for these inhibitors as well as the non-inhibitory protein ovalbumin. In 1985, Carrell and Travis chose the term SERPINS (SERine Proteinase Inhibitors) to describe members of this highly homologous family of plasma proteins. Classical and DNA derived protein sequencing and more recently, X-ray structures show the similarities between members of this family extend to a tertiary structure level, with a core domain of about 350 amino acids being common to each member of the family.

The majority of more than 100 known members of the serpin superfamily are true inhibitors of serine proteinases, best exemplified by the archetypal serpin α_1-antitrypsin. Certain serpins, however, have lost this inhibitory function and are not known to be inhibitors of serine, or any other proteinase; for example, ovalbumin, which apparently functions as a storage protein in birds eggs, angiotensinogen which acts as a peptide hormone precursor and those serpins that serve as transport proteins for small ligands, such as thyroxine binding globulin (TBG) and corticosteroid binding globulin (Grasberger, 1999).

The serpins are mechanism-based, suicide substrate inhibitors that appear to form irreversible covalent complexes with the target proteinase through a branched

pathway mechanism that can lead either to stable complex formation or else to cleavage of the serpin as a substrate. This is in marked contrast to the low molecular weight protein inhibitors of serine proteinases, such as the Kunitz or Kazal inhibitors, which form a tight, stable and non-covalent complex with their target proteinases by a lock and key interaction (Laskowski & Kato, 1980). There is now considerable information on the different conformations that serpins can adopt, based on X-ray structure determinations. (Schulze *et al.*, 1994, Wilczynska *et al.*, 1997, Wright & Scarsdale, 1995, O'Malley *et al.*, 1997)

These structural studies have shown that, unlike almost all other proteins, serpins appear to fold in a kinetically trapped metastable conformation which, under appropriate conditions, can change to a more stable conformation.

One of the main reasons for interest in serpins is that a number of them play critical roles in the regulation of important physiological processes such as blood coagulation (antithrombin), fibrinolysis (PAI-1), complement activation (C1-I), and extracellular remodelling (α_1-antitrypsin) (Carrell & Travis, 1985). In relation to this wide range of functions, the study of serpins has been disease led and originated with the observation by Laurell and Eriksson in 1963 that a common genetic abnormality of α_1-antitrypsin was associated with a predisposition to chronic lung disease

1.1.1 Structural Studies

The understanding of the mechanisms underlying the disease states produced by abnormalities of α_1-antitrypsin, and other serpins, has been increased by the elucidation of the molecular structure of members of this proteinase inhibitor family. The crystallographic structures of several serpins have now been resolved but, in 1984, the first cleaved structure of the archetypal serpin, α_1-antitrypsin, was determined by Loebermann *et al.* and provided the first model for the structure of inhibitory proteins in the serpin superfamily. A RASMOL representation of this structure is presented in Fig 1.1.

This model shows a globular, elongated protein structure with 30% of the molecule in the form of nine α-helices (A-I) and 40% as three crossed β-sheets (A-C). The protein was shown to be glycosylated with three carbohydrate side chains attached to residues Asn^{46}, Asn^{83} and Asn^{247} on the outside surface of one half of the elongated structure (Loebermann *et al.*, 1984). Subsequent experiments examining recombinant non-glycosylated α_1-antitrypsin (Rubin *et al.*, 1990, Hopkins *et al.*, 1993, Lomas *et al.*, 1995 (b)), have shown that glycosylation is not required for functional activity and suggested that the role of these carbohydrate moieties in serpin function may be 3-fold: to aid secretion, as a tag for the removal of proteinase/inhibitor complexes from the circulation and in recognition by receptors for complex uptake.

As the representative model of α_1-antitrypsin in Fig 1.2 illustrates, the six stranded, mostly antiparallel, β-sheets A and B are the most extensive structures of the molecule. Three strands of β-sheet B, along with helix B, provide the hydrophobic core highly conserved among serpins (Schulze *et al.*, 1994). Sheet A has a large number of conserved hydrophobic interactions and several hydrogen bonds exist between strands 1-3 of sheet A and helix F. The strands of sheet A are orientated perpendicular to the β-strands of sheet B. Most of the rest of the molecule is folded into α-helices. Helix F partially covers sheet A, including

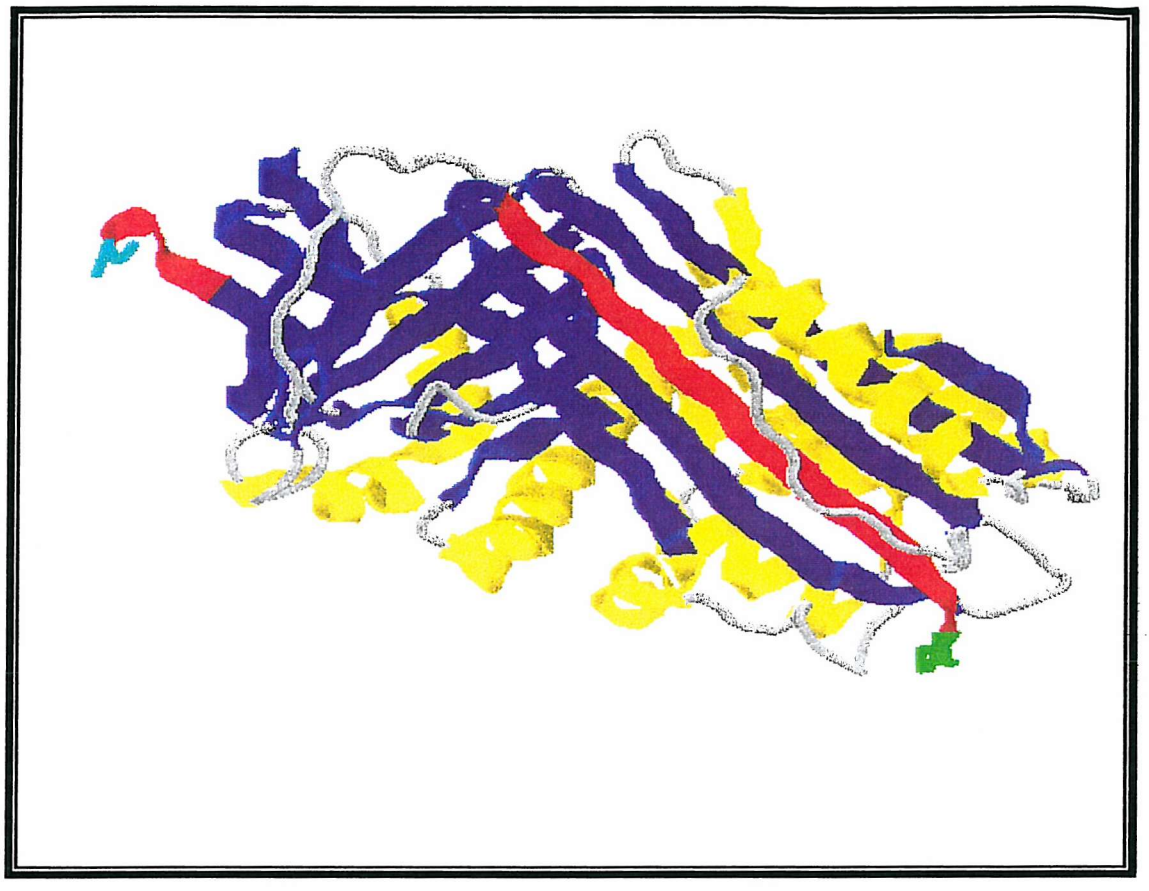


Fig 1.1 RASMOL diagram representing the crystallographic structure of reactive site cleaved α_1-antitrypsin as proposed by Loebermann *et al.*, (1984). The active site residues Met^{358} (green) – Ser^{359} (cyan), are separated on the molecule by 70Å as the RCL enters β-sheet A as strand 4.

strand s4A. Helix D, absent in the viral serpins, most probably forms the heparin binding site in antithrombin. Helices G and H are found under and behind β-sheet B covering the putative recognition site for the SEC receptor (Permutter, 1994), on strand s4B and, thus any reorganization of the molecule to expose the recognition site must involve the movement of these helices away from their hydrophobic packing against sheet B (Crystal *et al.*, 1989).

The crystallographic structure of cleaved α_1-antitrypsin defined by Loebermann *et al.* in 1984 provided extensive information about the structure of the inhibitory serine proteinases. It also identified the two most important areas of the serpin structure in relation to function: the five-stranded A β-sheet and a mobile reactive centre loop that contains the proteins inhibitory site for interaction with the target proteinase.

1.1.2 Reactive Centre Loop

The mobile reactive centre loop is a unique characteristic of the serpins and defines their specificity as it contains the active inhibitory site - the P1-P1' bond. As illustrated in Fig 1.3, the structure is highly conserved between different classes of serpins, despite the fact that the proteins themselves vary in overall structure (Mast *et al.*, 1992).

The 1984 structure presented α_1-antitrypsin in a 'cleaved' conformation with the reactive centre loop incorporated into the A sheet as strand s4A, to produce a six stranded β-sheet A (Loebermann *et al.*, 1984). In 1991, Baumann determined the crystal structure of another inhibitory Serpin - cleaved α_1-antichymotrypsin. The structure was shown to be very similar to cleaved α_1-antitrypsin including the presence of strand 4A which, when liberated by proteolysis, is inserted as the middle strand in β-sheet A. This structure acted to confirm the validity of the cleaved α_1-antitrypsin model determined by Loebermann *et al.* in 1984. These models both demonstrated the serpins structure following interaction with a

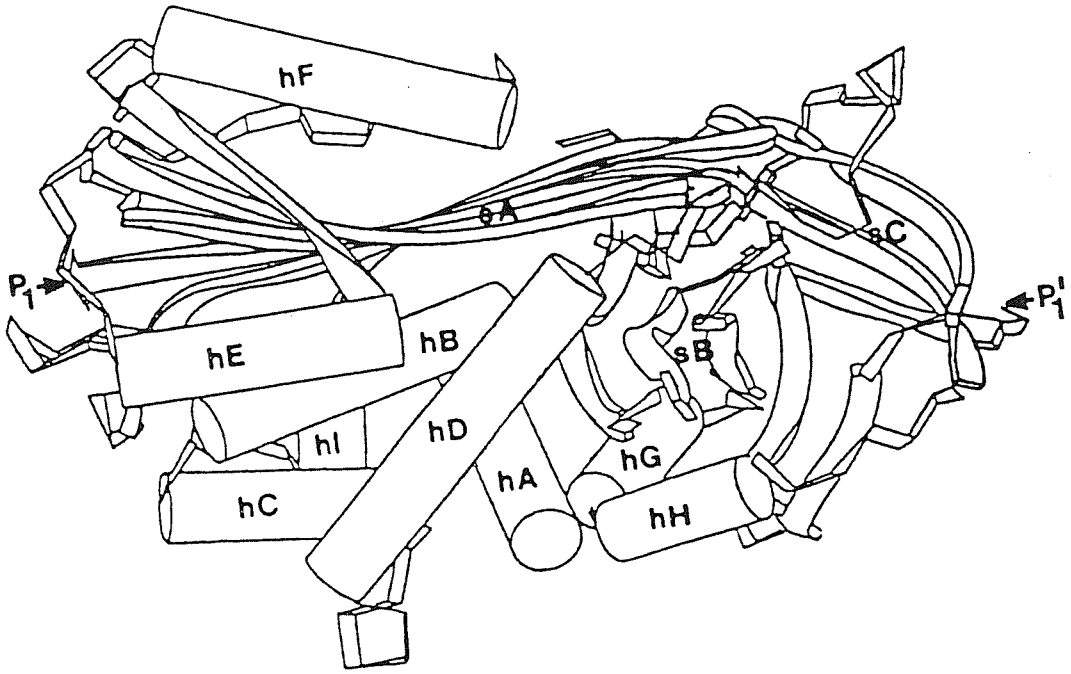


Fig 1.2 Cleaved structure of α_1-antitrypsin. hX represents helix X; sY represents sheet Y. Cleavage at P1-P1^1 bond (Met^{358}-Ser^{359}) of reactive centre loop results in a dramatic conformational change with the P1-P1^1 residues separated by 69Å (reprinted from Lomas *et al.*, 1992).

	Reactive Loop Sequence P15-P1′															
Residue Position	**15**	**14**	**13**	**12**	**11**	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**1′**
Amino Acid	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359
(Inhibitory)																
α_1-AT	G	T	E	A	A	G	A	M	F	L	E	A	I	P	M	S
Antithrombin	G	S	E	A	A	A	S	T	A	V	V	I	A	G	R	S
α_1-ACHY	G	T	E	A	S	A	A	T	A	V	K	I	T	L	L	S
PA1-I	G	T	V	A	S	S	S	T	A	V	I	V	S	A	R	M
C1-I	G	V	E	A	A	A	A	S	A	I	S	V	A	-	R	T
(Non-Inhibitory)																
Ovalbumin	G	R	E	V	V	G	S	A	E	A	G	V	D	A	A	S
Angiotensinogen	D	E	R	E	P	T	E	S	T	Q	Q	L	N	K	P	E

Fig 1.3 The Reactive Center Loop Sequence (P15-P1′) of selected Serpins. RCL sequences of various serpins aligned to α_1-antitrypsin (amino acid number refers to that of α_1-antitrypsin) show high homology, particularly between Inhibitory Serpins, indicating that amino acid selection is important for protein function. P1-P1′ denotes the reactive site bond.

proteinase and it was not until the work of Elliott *et al.*, in 1996 (a), that a 2.9Å structure of wild-type α_1-antitrypsin showed the intact molecule with no stabilizing mutations and an exposed reactive centre loop. A RASMOL interpretation of this model is illustrated in Fig 1.4 and shows, for the first time, the native structure of α_1-antitrypsin which allowed a definite picture of the mechanism of inhibition of the inhibitory serpins to be elucidated.

1.1.3 Inhibitory Loop Mechanism

In the intact model of α_1-antitrypsin (Elliott *et al.*, 1996 (a)), the mobile reactive centre was seen to form an exposed loop, hinged to the protein core at each end (P15 and P5′), and characterised by a defined mobility that allowed it to adopt an alternative secondary structure as an essential part of the inhibitory process (Sifers, 1995).

The loop contains the inhibitory site of the protein centred around two amino acids, unique to each inhibitor. For α_1-antitrypsin, this involves a Met-Ser bond at residues 358 (P1) and 359 (P1') (Sifers, 1995). The overall structure of the molecule stresses the active site loop such that the Met^{358}-Ser^{359} tip fits closely into the active pocket of the target proteinase where it is held with great affinity. Cleavage of the Met-Ser bond produces a conformational change in both the inhibitor and the enzyme as the reactive centre loop re-enters the central β-sheet of the molecule as strand 4A, in what is termed the Stressed→Relaxed, (S→R), transition. Fig 1.1 represents the crystallographic model of cleaved α_1-antitrypsin (Loebermann *et al.*, 1984), and demonstrates the insertion of the reactive centre loop as strand 4A. The active site Met^{358} and Ser^{359} are indicated and are separated by 70Å. Subsequent formation of a tight, equimolar complex between inhibitor and proteinase renders both molecules inactive (Sifers, 1995, Crystal *et al.*, 1989).

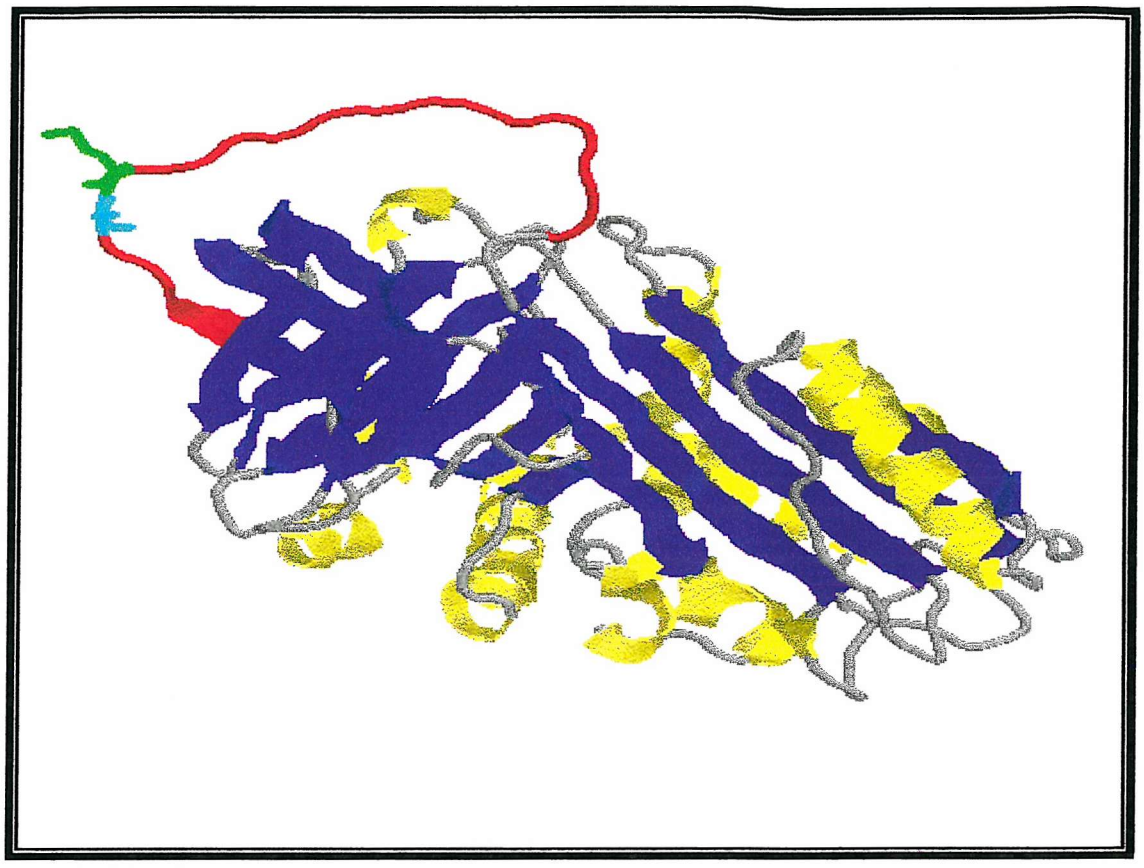


Fig 1.4 RASMOL diagram representing the crystallographic structure of intact α_1-antitrypsin as proposed by Elliott *et al.*, (1996). In this active form, the RCL of α_1-antitrypsin (red) is represented by a partial β-strand extended from β-sheets A and C.

1.1.4 Hinge Region and Shutter Domain

In order for the S→R transition that follows loop cleavage to occur, the loop must move from its exposed position away from the body of the molecule to form s4A of the A β-sheet. This movement is achieved by the insertion of about 7 amino acids (P9-P15), immediately proximal to the exposed portion of the loop (P8-P5′) into the body of the molecule and this region is referred to as the 'hinge' domain – highlighted in Fig 1.5 (Engh *et al.*, 1990).

The primary structure of this hinge region is strongly conserved in all inhibitory serpins and mutations in this region result in a disruption of normal inhibitory activity, indicating the regions importance in inhibitory function (Hopkins *et al.*, 1993, Potempa *et al.*, 1994, Huntington *et al.*, 1997). The P9-P12 residues are usually short chain amino acids, predominantly alanines; P13 is glutamic acid; P14 again usually has a short side chain, often serine or threonine; and P15 is almost always glycine.

In the intact crystal structures of ovalbumin (Stein *et al.*, 1991) and α_1-antitrypsin (Elliott *et al.*, 1996 (a)), it can clearly be seen that this hinge-region is where the reactive centre loop turns and joins the A β-sheet as strand 5A. The study of dysfunctional variants in the N-terminal region (P15-P10) provided evidence that mobility of the loop is necessary for inhibitory activity. These experiments revealed that when the small aliphatic side chains conserved in this hinge region are replaced with bulky or polar side chains, insertion of the N-terminal end of the loop into β-sheet A, necessary to achieve the optimal inhibitory conformation, is prevented (Hopkins *et al.*, 1993, Stein & Carrell, 1995, Hopkins & Stone, 1995). For example, Hopkins *et al.*, in 1993 determined that the substitution of the P10 glycine residue to proline converted the majority of the α_1-antitrypsin protein into a substrate for its target proteinase. These results provided evidence that the insertion of a portion of the loop, between P15 and P8, into the A β-sheet maintains the loop in a taut conformation, similar to that of the low molecular weight inhibitors, enabling the formation of a stable complex with the proteinase.

The extent of the insertion is still a matter of debate, but biochemical evidence obtained from peptide annealing experiments and mutational analysis, suggests that insertion at least as far as P12 is required (Skriver *et al.*, 1991, Schulze *et al.*, 1992, Hopkins *et al.*, 1997).

Another important structural component of the molecule is the 'shutter region' – highlighted in Fig 1.5. This area of the molecule is so-called as its structure and conformation allows a shutter-like movement of the A-sheet to open the s4A position and allow insertion of the reactive centre loop. The sliding movement resulting in the opening of the sheet involves strands 1, 2 and 3 of the A-sheet and the overlying F helix. These 2 areas are covalently linked through the D helix to strand 2A and also at the end of strand 3A. Interactions with helix B also form part of this domain and the shift that creates the space for reactive centre loop insertion involves a sliding movement of strand 3A in the grooves of helix B (Stein and Chothia, 1991).

The P′ side of the reactive centre loop has been less extensively studied. In the crystal structure of cleaved α_1-antitrypsin, this region forms strand 1 of the C β-sheet from P5′ to P10′, and naturally occurring mutations of this region are known to interfere with normal inhibitory activity (Hofker *et al* 1987, Lane *et al.*, 1992, Eldering *et al.*, 1995). In uncleaved models it has been shown that strand 1C can detach from the C β-sheet upon insertion of the P-side of the reactive centre loop into the A β-sheet. Also, molecular modelling has shown that any insertion of the hinge region of the loop into the A β-sheet, to P12 or beyond, requires either cleavage of the P1-P1′ bond or the detaching of strand 1C from the C β-sheet, although this uncoupling is not necessary for inhibitory function of α_1-antitrypsin (Whisstock *et al.*, 1995, Hopkins *et al.*, 1997).

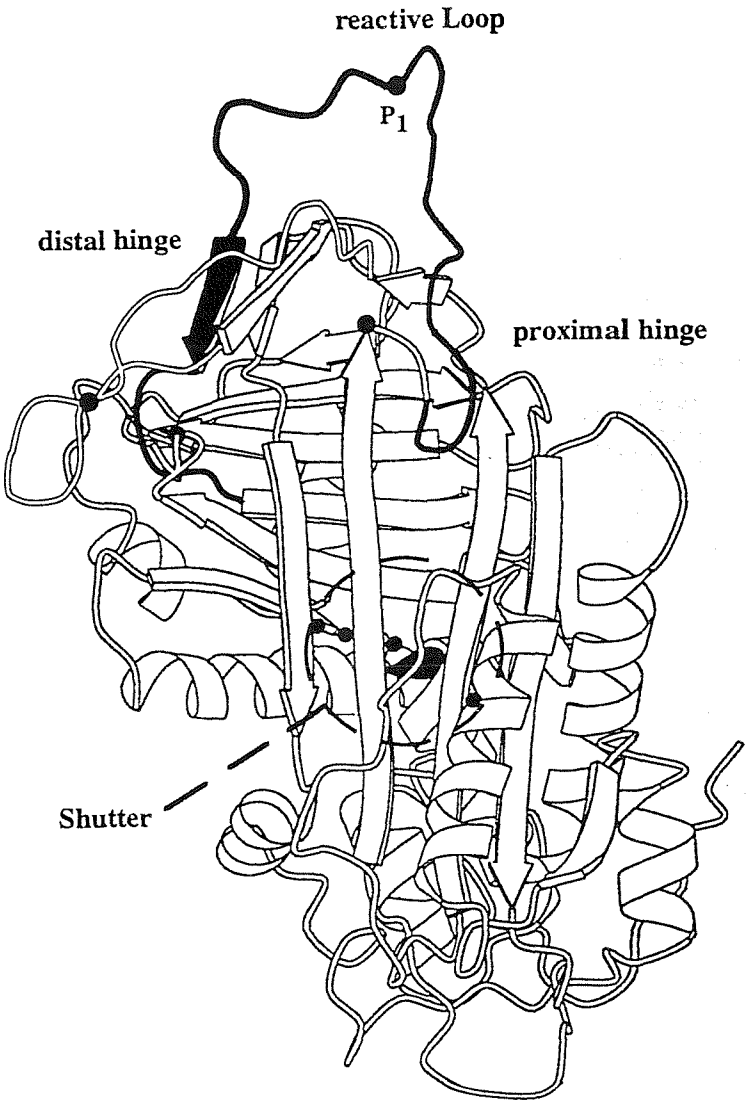


Fig 1.5 Template structure of an intact serpin (active antithrombin), illustrating the principal functional domains in which mutations frequently produce dysfunction and disease. The shutter domain (circled) is centred on the conserved Phe-Ser-Pro sequence at the commencement of helix B (black area) (reprinted from Carrell & Stein, 1996).

1.1.5 Native loop configuration

The actual conformation of the loop in the active inhibitor has been the subject of much interest. It was first thought to resemble that of Kazal type inhibitors when uncleaved (Bode & Huber, 1992). But the structures of inactive ovalbumin (Stein *et al.*, 1990), and a loop-swap variant of α_1-antichymotrypsin (Wei *et al.*, 1994), both presented loops as a three-turn helix, which is not competent for proteinase binding (Bode & Huber, 1991). More recent studies published structures of α_1-antitrypsin, stabilized by various point mutations. Song *et al.* in 1995 modelled the loop of an α_1-antitrypsin protein with 3 point-mutations also as a helical structure which may uncoil prior to or during proteinase attachment (Skiver *et al.*, 1991). However, Ryu *et al.* in 1996 demonstrated that the loop conformation of a similar model, but with 7 stabilizing point-mutations, was that of a β-strand. This β-strand conformation was also illustrated in 1996 by Elliott *et al.*, (a) who modelled the loop of an α_1-antitrypsin molecule with only 1 stabilizing mutation.

In 1998, Elliott *et al.*, (a) presented a 2.9Å structure, illustrated in Fig 1.4, of recombinant wild-type α_1-antitrypsin that presented the intact molecule for the first time with no stabilizing mutations. The structure revealed that the reactive centre loop is in a canonical conformation in the absence of significant insertion into the A β-sheet. The reactive centre loop of wild-type α_1-antitrypsin is exposed at the pole of the protein and adopts a canonical inhibitory structure between P3 and P3′ (Ile^{356} to Pro^{361}) with extension as a β-strand between P3 and P8 (Ile^{356} and Met^{351}). The loop is held in this configuration by a salt bridge between P5 glutamate, a ring of arginine residues at 196, 223, and 281, and the main chain amide of Met^{226} (Elliott *et al.*, 1996 (a)). The native structure is 'trapped' in a highly unstable, high free-energy state and it seems likely that the unfavourable configuration of these positively charged residues contributes energy to the "spring" toward the lowest free-energy state that drives loop insertion following reactive loop cleavage (Lawrence *et al.*, 1995). This dramatic structural rearrangement results in trapping of the proteinase-inhibitor complex as the rapid shift in the relative positions of the two molecules is thought to distort the active

site of the proteinase and prevent further peptide bond cleavage (Hopkins *et al.*, 1993). The model presented by Elliott *et al.*, in 1998 (a), illustrates this stabilization of the loop in the native structure by the P5 glutamate-Arg^{223} bond and this provides strong support that the structure represents the circulating form of α_1-antitrypsin *in vivo*.

1.1.6 S→R transition

Three serpin conformations have been described that involve partial to total insertion of the reactive centre loop into a gap between strands 3 and 5 in sheet A. The initial 'native' conformation of the inhibitor, as present in the circulating plasma prior to interaction with the proteinase, with fully exposed loop has been termed the 'quiescent' form by Mouray *et al.*, in 1993. On interaction with the proteinase, stabilization of the proteinase-inhibitor complex results in a structural transition to a stressed 'locking' conformation characterised by partial loop insertion into sheet A (Huber & Carrell, 1989).

Upon cleavage of the P1-P1′ bond on the reactive centre loop of inhibitory serpins, the proximal portion of this loop inserts into the A β-sheet to form a 6-stranded antiparallel β-sheet. This is accompanied by a dramatic increase in thermal stability of the protein as well as increased resistance against guanidine hydrochloride induced unfolding, indicating that the cleaved from of the protein is more stable than the uncleaved form. This information leads to the theory that the protein circulates in an unstable 'Stressed' (S) inhibitory conformation and, following cleavage, this stress is released, leading to the formation of a more stable 'Relaxed' (R) cleaved form. The difference in heat stability seen with this transition is likely to be attributable to a large number of weak hydrogen bonds in secondary structure elements that become stronger in the cleaved form, without any alteration in tertiary structure (Perkins et al., 1992). The occurrence of this S→R change takes place in all the known inhibitory serpins but not in the non-inhibitors, such as ovalbumin (Schulze *et al.*, 1994, Hopkins *et al.*, 1993).

Previous experiments have provided evidence for the importance of this transition for inhibitory activity. Lawrence *et al.*, in 1994, Schulze *et al.*, in 1991 and Hopkins & Stone in 1995 modified the amino acid sequence of the hinge region of α_1-antitrypsin, replacing the small amino acids that are highly conserved within this region of the reactive centre loop with larger, bulkier amino acids. The results demonstrated that, following such modification, strand insertion was affected and the proteins inhibitory activity was reduced, converting the inhibitor into a substrate. Therefore, the partial strand insertion required for movement of the active protein into the stressed conformation is essential for inhibitory activity. Alongside this 'intermediate' loop configuration seen in the active protein, two structural extremes of loop configuration have been determined by resolution of crystal structures of 2 non-functional serpins, ovalbumin and latent PAI-1. In the non-functional ovalbumin, S4 is not inserted at all into Sheet-A, and a pseudo-reactive site bond (P1-P1′) is exposed on the final turn of a mobile 3-turn helix protruding from the underlying protein scaffolding (Stein *et al.*, 1991). In the case of Plasminogen activator inhibitor-1 (PAI-1), the reactive centre loop is totally incorporated into Sheet-A. This is termed the 'latent' conformation and results in the reactive-site being inaccessible to proteinases (Mottonen *et al.*, 1992).

1.1.7 Ovalbumin

The 1.9Å structure of ovalbumin was the first intact native non-inhibitory serpin conformation to be determined. The reactive loop of ovalbumin in this model was shown as an extended three-turn α-helix extended on two peptide stalks from residue P17 in the A-sheet to P5′ in the C-sheet (Stein *et al.*, 1990). Comparison of the A β-sheet, in particular, demonstrates the structural difference between this non-inhibitory serpin and inhibitory α_1-antitrypsin. As illustrated in Fig 1.6, the structure differs from cleaved α_1-antitrypsin, primarily through the absence of β-strand s4A in sheet A. In ovalbumin, this sheet possesses a nearly ideal 5 stranded β-sheet geometry, and is exceptional among serpins as this five-stranded structure

is maintained in the cleaved ovalbumin molecule - plakalbumin (Carrell & Stein, 1996).

Sequence alignment based on the crystal structure of the cleaved form of the archetypal serpin, α_1-antitrypsin, indicated that the two serpins share a common, highly ordered structure. Ovalbumin is a well-conserved member of the serpin family showing sequence identity of 30% with α_1-antitrypsin, and a putative reactive centre at Ala^{358}-Ser^{359}. However, it shows no measurable inhibitory activity and does not undergo the typical conformational change on cleavage at this P1-P1′ site. The structure of plakalbumin suggests features that would prevent accommodation of the ovalbumin loop sequence, as in the cleaved α_1-antitrypsin structure, and may cause ovalbumin to be locked in the native conformation with a fully extended reactive centre loop (Stein *et al.*, 1990). Comparison of the hinge region demonstrated that residue 345 (P14) is an arginine in ovalbumin, but is otherwise conserved as threonine, serine, valine, or alanine among all other inhibitory serpins. This suggested that the presence of a small, preferably hydrophobic residue at this site must be a prerequisite for the insertion of stand s4A into sheet A and inhibitory activity. However, in 1994, a P14 arginine variant of α_1-antitrypsin was produced by Hood *et al.*, who demonstrated that this variant was still an inhibitor of trypsin and elastase. Donovan & Mapes in 1976 and Huntigton *et al.*, in 1995, have also demonstrated that partial insertion of the loop of ovalbumin does occur, but at a very slow rate and requiring high activation energy. Therefore, it is still not clear why ovalbumin is not an inhibitory serpin but Huntington *et al.*, in 1997 have suggested that the slow rate of loop insertion, due to the presence of the bulkier amino acids within the loop structure, may have a role in its lack of inhibitory activity.

In other serpins, it is likely that the complete insertion of exposed reactive centre loop residues into strand 4A, either spontaneously (latent PAI-1) or upon cleavage (α_1-antitrypsin), drives the conformational change underlying inactivation (Mottonen *et al.*, 1992).

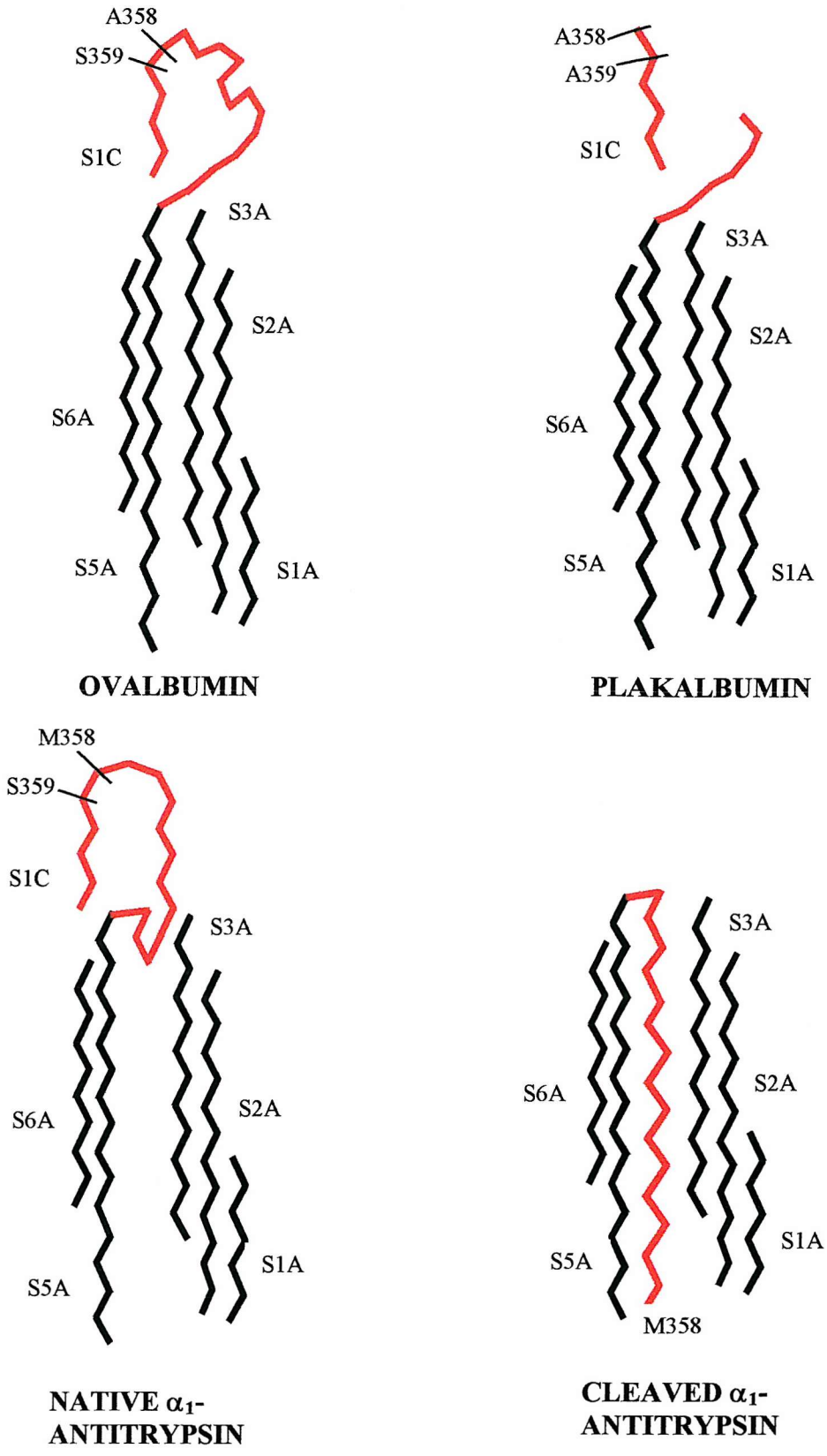


Fig 1.6 Schematic diagram of β-sheet A in various Serpins. The reactive centre loop (red), adopts a helical conformation in Ovalbumin, is partially absent in Plakalbumin and enters β-sheet A as strand 4 in cleaved α_1-antitrypsin. Experimental evidence (Schulze *et al.*, 1992), suggests that partial insertion of the RCL into β-sheet A at least as far as P12, is required for inhibitory activity of α_1-antitrypsin. (adapted from Schulze *et al.*, 1994).

1.1.8 Latent conformation

The other structural extreme related to the mobility of sheet A, strand 4A and sheet C was demonstrated with the structure of latent human PAI-1 (Mottonen *et al.*, 1992). The latent PAI-1 crystal structure is a hybrid of the uncleaved and cleaved inhibitor serpins. The uncleaved reactive centre loop of latent PAI-1 is fully inserted into strand A of its structure from the 'hinge' at P15 to residue 335 (P4), resembling the post-cleavage reactive loop strand of inhibitory serpins (a representation of cleaved α_1-antitrypsin is illustrated in Fig 1.6). In contrast to the cleaved inhibitory serpins, however, this insertion is accompanied by the removal of strand C on the carboxyl-side of the reactive centre loop. This provides the extension to bridge the intact, inserted reactive centre loop back into the unaltered structure of the rest of the molecule (Mottonen *et al.*, 1992).

This crystal model illustrated that the lack of inhibitory activity in PAI-1 is due to the premature sequestration of the entire reactive centre loop into strand A, removing it from exposure to proteinase. The formation of this stable, inactive state is termed 'latent' as inhibitory activity can be returned through denaturation and renaturation from 8M urea. In the circulation it is maintained in the active conformation by an interaction with another plasma protein – vitronectin (Carrel & Stein, 1996). The physiological advantage of this latent conformation is that it allows PAI-1 to be stored, or circulate in a non-functional form that is not vulnerable to proteolytic cleavage.

PAI-1 is the only serpin known to exist in a latent form under physiological conditions, but mild denaturation of other serpins can induce forms with similar properties and structures. Schreuder *et al.*, (1994) and Carrell *et al.*, (1994), have reported 3Å resolution structures of dimers of ATIII. In these structures the dimer is comprised of a latent, or possibly cleaved, molecule and an intact molecule. The latent (or cleaved) molecule has the same conformation as PAI-1, with the reactive centre loop fully inserted into sheet A and a strand from β-sheet C displaced to form a bridge. The structure of latent PAI-1 is similar to one formed

after cleavage in other serpins and the stability of both latent PAI-1 and the cleaved serpins may be derived from the same structural features (Mast *et al.*, 1992).

1.1.9 Interactions with Proteinases

The serpins form tight, stable complexes with proteinases. The association rate constants for Serpin-Proteinase interaction are high (e.g. $k_1 = 10^6$-$10^7 M^{-1}Seconds^{-1}$) and the ki values are low (e.g. ki = 10^{-10}M for the initial steps of plasmin inhibition by antiplasmin (Longstaff & Gaffney, 1991)). The serpins were first characterised as slow, tight-binding inhibitors because a slow step after the initial fast association precedes the formation of a stable complex. This slow step has rate constants of the order of $k_2 = 10^{-2}$-$10^{-3}Seconds^{-1}$ ($t½$= 78 mins for α_2-antiplasmin) and is virtually irreversible (Wright, 1996).

Serpins form enzymatically inactive, 1:1 complexes (denoted EI*) with their target proteinases that induces conformational change in both the enzyme and the inhibitor moieties (O'Malley *et al.*, 1997). The mechanism of EI* formation is incompletely understood and continues to be a source of controversy. Serpins are cleaved in their native complexes with cognate proteases suggesting that complex formation stops at a stage after reactive centre cleavage. In the case of PAI-1, Wilczynska *et al.*, (1995), demonstrated that complex formation and reactive centre cleavage are rapid and co-ordinated events. The complex is arrested as an acyl-enzyme intermediate or possibly, following addition of a H_2O molecule, as a tetrahedral intermediate. The cleavage of serpins by their target proteinases cannot be reversed and it is this characteristic that identifies serpins as suicide substrate inhibitors (Rubin *et al.*, 1990, Patston *et al.*, 1991).

Fig 1.7 demonstrates that after the initial formation of the acyl-enzyme itermediate (E*I*) the reaction may proceed along 2 divergent paths. One (k_4) leads to the formation of a stable enzyme-inhibitor complex (EI*), as a result of

loop insertion, and the other (k_3) to reactive centre cleaved serpin (I*) and active enzyme (E), (Potempa *et al.*, 1994, Hopkins & Stone, 1995).

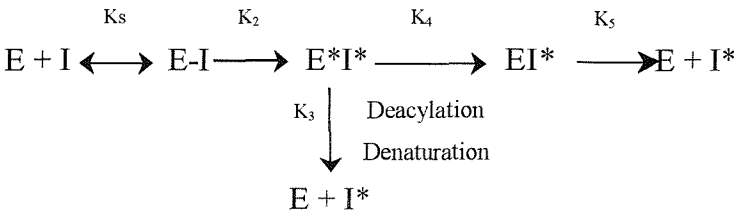


Fig 1.7 Mechanism for Serpin-Proteinase interaction, as proposed by Patston *et al.*, 1994. E (active enzyme), I (active inhibitor), * indicates conformationally changed species.

The relative amounts of E*I*, EI* and I* present depend on both the conditions for complex formation, individual rates of the various reactions, and the proteinase being complexed (Patston *et al.*, 1991). For some serpin-proteinase interactions, full inhibition of proteinase requires >1 eq of serpin. The ratio of moles of inhibitor required per mole of proteinase for 100% inhibition is defined as the stoichiometry of inhibition (SI). Proteinase serpin pairs can be placed on a scale of this ratio such that serpins without inhibitory activity, such as ovalbumin and angiotensinogen, pass exclusively through the substrate cleavage pathway and have a very high stoichiometry of inhibition (approaching infinity). The most efficient inhibitors such as α_1-antitrypsin and α_1-antichymotypsin, in comparison, have a low stoichiometry of inhibition, approaching 1.0, and form inhibited complex almost exclusively, giving rise to "suicide inhibitor"-type kinetics (Rubin, 1994, O'Malley *et al.*, 1997).

A characteristic feature of the serpin-proteinase complex is that it is stable to both heat and SDS treatment, implying covalent bond formation between enzyme and serpin. Results from O'Malley *et al.*, in 1997, provided strong evidence that the covalent bond within EI* occurs between the proteinase active site serine hydroxyl (Ser^{195} in α-chymotrypsin) and the P1 residue on the reactive centre loop

of the serpin. In intact inhibitor, this loop extends out from the rest of the molecule and is the primary interaction site between the inhibitor and the target proteinase. On interaction, the P1 residue of the inhibitor is positioned close to the catalytic centre of the serine proteinase, and the flanking residues interact with subsites on the proteinase (Hopkins *et al.*, 1993). The end effect is a tight complex of proteinase and inhibitor in which the active site is constrained in the catalytic inhibitor intermediate (Michaelis) form (Carrell & Evans, 1992).

These inhibitors may then be cleaved by the target proteinase following docking. Recent results, that EI* formation coincides with liberation of a new N-terminus at P1′, demonstrate that within EI*, I has been cleaved at the P1-P1′ bond within the reactive centre loop of the molecule, which in α_1-antitrypsin corresponds to positions 358-359 (O'Malley *et al.*, 1997). Cleavage of intact I to form I* is accompanied by a large decrease in free energy and a substantial gain in stability toward denaturing by either heating or specific denaturing agents. In I*, residues P1-P14 are inserted into β-sheet A, the dominant structural element in α_1-antitrypsin, as strand 4A. β-sheet C is also reinforced, with the result that the P1 and P1′ residues are separated by 70Å as illustrated by the cleaved model of α_1-antitrypsin determined by Loebermann *et al.* in 1984.

This rapid movement of the reactive centre loop into the body of the protein brings the inhibitor closer to its lowest free energy state. Data produced by Lawrence *et al.*, (1995), suggests a model for serpin action in which the drive toward the lowest free energy state results in trapping of the proteinase-inhibitor complex as an acyl-enzyme intermediate. Loop insertion leads to a large increase in thermal stability, presumably due to reorganisation of the 5-stranded β-sheet A from a mixed parallel-antiparallel arrangement to a 6-stranded, predominantly antiparallel, β-sheet. This dramatic stabilisation has led to the suggestion that, unlike almost all other proteins, native inhibitory serpins may be metastable structures, kinetically trapped in a state of higher free energy than their most stable thermodynamic state. (Elliott *et al.*, 1996 (a))

Lawrence *et al.*, (1995), also proposed that since the inserting reactive centre loop is covalently linked to the enzyme via the active-site Ser (S1), this transition from intact to cleaved inhibitor should also affect the proteinase and significantly change its position relative to the inhibitor. Such a rapid shift in the relative positions of the two molecules, centred at the enzymes active site, might sufficiently distort the active site geometry to prevent efficient deacylation and thus trap the complex. Alternatively, the new position of the acylated proteinases active site may exclude the H_2O necessary for deacylation from the contact surface between the 2 molecules (Hopkins *et al.*, 1993).

Stratikos & Gettins, (1998), have recently proposed a model of Serpin-proteinase interaction. In this study, they determined that the P1-P1′ bond must be exactly 14 residues away from the hinge point of the reactive centre loop to allow insertion into β-sheet A. This would make the loop region, upon complete insertion, just long enough to have residues P2 and P1 protrude from the end of the sheet in order to reach the proteinase active site. If the scissile bond were closer to the hinge point the acyl intermediate could not be trapped by such a full insertion mechanism. If the scissile bond were further away the resulting peptide that extends beyond β-sheet A would be so long that there could be no restraint imposed on the conformation of the acyl ester linkage, no disruption of the catalytic site of the proteinase, and hence no kinetic trap. These findings suggest a serpin-proteinase complex in which the reactive centre loop is fully inserted into the β-sheet, and the proteinase is at the far end of the serpin from its initial site of docking. This model is also consistent with previous results (Lawrence *et al.*, 1995), demonstrating that reactive centre loop insertion is not required for protease binding but is necessary for stable inhibition.

Insertion of the hinge region appears to be the rate limiting processes in the formation of the stable complex (Hopkins *et al.*, 1997). Previous studies, (Loebermann *et al.*, 1984, Carrell *et al.*, 1994, Mottonen *et al.*, 1992), indicate that mutations in the hinge region of serpins reduce the rate at which the stable complex is formed from the intermediate E*I*, presumably by slowing down the

rate of insertion of the hinge region into the A β-sheet. Examination of the crystal structure of α_1-antitrypsin and other serpins indicates that substitution of larger or charged amino acids in the hinge region would hinder, or raise the energy barrier for strand insertion (Hopkins & Stone, 1995). If reactive centre loop insertion is blocked, or if deacylation occurs before reactive centre loop insertion, then the cleaved serpin is turned over like any other substrate and the active enzyme released (Wilczynska *et al.*, 1995). As discussed in section 1.1.7, this may provide the mechanism which results in the non-inhibitory activity of ovalbumin and suggests that it is the ratio of the rate of deacylation to the rate of loop insertion that determines whether a serpin is an inhibitor or a substrate. If deacylation (k_3) is faster, then the substrate reaction predominates. However, if reactive centre loop insertion and distortion of the active site (k_4) occurs before deacylation, then the complex is frozen in a covalent acyl-enzyme form (Lawrence, 1997).

1.1.10 Removal of enzyme-inhibitor complexes

A major function of serpins is to provide a tag for the removal of proteolytic enzymes from the circulation via cellular receptors, thought to occur when complexation with the serpin leads to conformational change which reveals a receptor recognition site. (Schulze *et al.*, 1994). Three types of receptor, involved in this process, have been described: (i) the α2-macroglobulin/low density lipoprotein (α2M/LDL) related protein receptor, (ii) a proposed serpin enzyme complex (SEC) receptor, and (iii) the urokinase receptor; the latter recognizes and binds urokinase independent of serpin binding.

The α2M/LDL receptor is implicated in hepatic uptake of proteinase-inhibitor complexes, as well as proteinase-inhibitor-receptor complexes, from the circulation and extracellular space. Evidence for its involvement in internalization and degradation of these complexes comes from experiments that show blockade of PAI-1 degradation by addition of anti-α2M receptor antibody in monocytes (Nykjaer *et al.*, 1992).

Permutter *et al.*, in 1988, observed that structural rearrangement of the α_1-antitrypsin and elastase molecules during the formation of an α_1-antitrypsin-elastase complex exposes a domain that can be recognized by specific cell surface receptor(s). This proposed interaction was then examined using synthetic peptides, analogous to this domain, to block any binding of serpin-enzyme complexes and these peptides were observed to bind specifically and saturably to neutrophils, monocytes and hepatoma derived hepatocytes (Permutter *et al.*, 1990). Peptide studies have also demonstrated that a specific pentapeptide sequence, amino acids 370-374, within this carboxy-terminal domain of α_1-antitrypsin, which is highly conserved among serpins, is sufficient for binding to this 'SEC' (Serpin-Enzyme Complex) receptor. Permutter *et al.*, in 1990, also determined that the SEC receptor is involved in the clearance/catabolism of serpin-enzyme complexes in Hep G2 cells and Joslin *et al.*, in 1993 then demonstrated that, not only does the SEC receptor mediate internalization and intracellular catabolism of the macromolecular complex, it also mediates a 'positive feedback' increase in the synthesis of α_1-antitrypsin.

1.2 α_1-antitrypsin

The main focus of this study is the archetypal member of the serpin family - α_1-antitrypsin. This name is actually a misnomer as, although it is an effective inhibitor of trypsin, its main physiological function *in vivo* is as an inhibitor of the elastase released by the principal inflammatory white cell, the neutrophil (Potempa *et al.*, 1994). α_1-antitrypsin is an also an inhibitor of numerous other serine proteinases, including chymotrypsin, cathepsin G, plasmin, thrombin, tissue kallikrein, factor Xa, and plasminogen. For this reason it is often referred to in more general terms as "α_1-antiproteinase" or "α_1-proteinase inhibitor". However, the association rate of normal α_1-antitrypsin with human neutrophil elastase is $\sim 10^7 M^{-1}s^{-1}$, a value 25-fold greater than for the interaction of α_1-antitrypsin with any other proteinase (Beatty *et al.*, 1980, Travis & Salvesen, 1983).

α_1-antitrypsin is the serpin present in the greatest concentration in the plasma. In normal humans, 34mg of α_1-antitrypsin are produced daily per kg of body weight, giving serum levels of 20 to 48μM (Jones *et al.*, 1978, Wewers *et al.*, 1987). With a molecular weight of 52kDa, the mature protein is capable of diffusion into most organs, but the molecular mass of the protein results in lower concentrations of α_1-antitrypsin within the organ. For example, the α_1-antitrypsin level on the epithelial surface of the lower respiratory tract of normal individuals is 2 to 5μM, approximately 10% that in the plasma (Hubbard & Crystal, 1988).

Under 'normal' physiological conditions α_1-antitrypsin alone provides >95% of the protective screen against the large proteolytic capacity of neutrophil elastase. This enzyme is a 220 residue, single chain protein with 2 carbohydrate side chains and a molecular weight of 29kDa (Baugh & Travis, 1976). Neutrophil elastase is a powerful proteinase capable of cleaving most protein components of the extracellular matrix, a variety of proteins of the coagulation and complement cascades, and E.coli cell wall components. It is also one of the few human enzymes capable of cleaving insoluble, cross-linked elastin, a rubber-like macromolecule that modulates the elastic recoil of tissues, including the alveolar walls of the lower respiratory tract (Janoff, 1985).

Like other serine proteinases, the proteolytic capacity of neutrophil elastase depends on a specific catalytic triad; His^{41}-Asp^{88}-Ser^{173}, centred in its reactive site pocket, as illustrated in Fig 1.8. When a substrate is presented to the reactive centre, a transfer of a proton within the triad allows the Ser^{173} to become a highly reactive nucleophile capable of attacking the peptide bond within the substrate. As discussed in section 1.1.9, α_1-antitrypsin rapidly inactivates neutrophil elastase by interacting tightly, and essentially irreversibly, with the neutrophil elastase active site pocket and binding is a suicide interaction for both molecules (Potempa *et al.*, 1994, Carrell & Stein, 1996).

Although α_1-antitrypsin is normally an excellent inhibitor of neutrophil elastase the Met^{358} at the active site can be easily oxidised and this leads to inactivation of

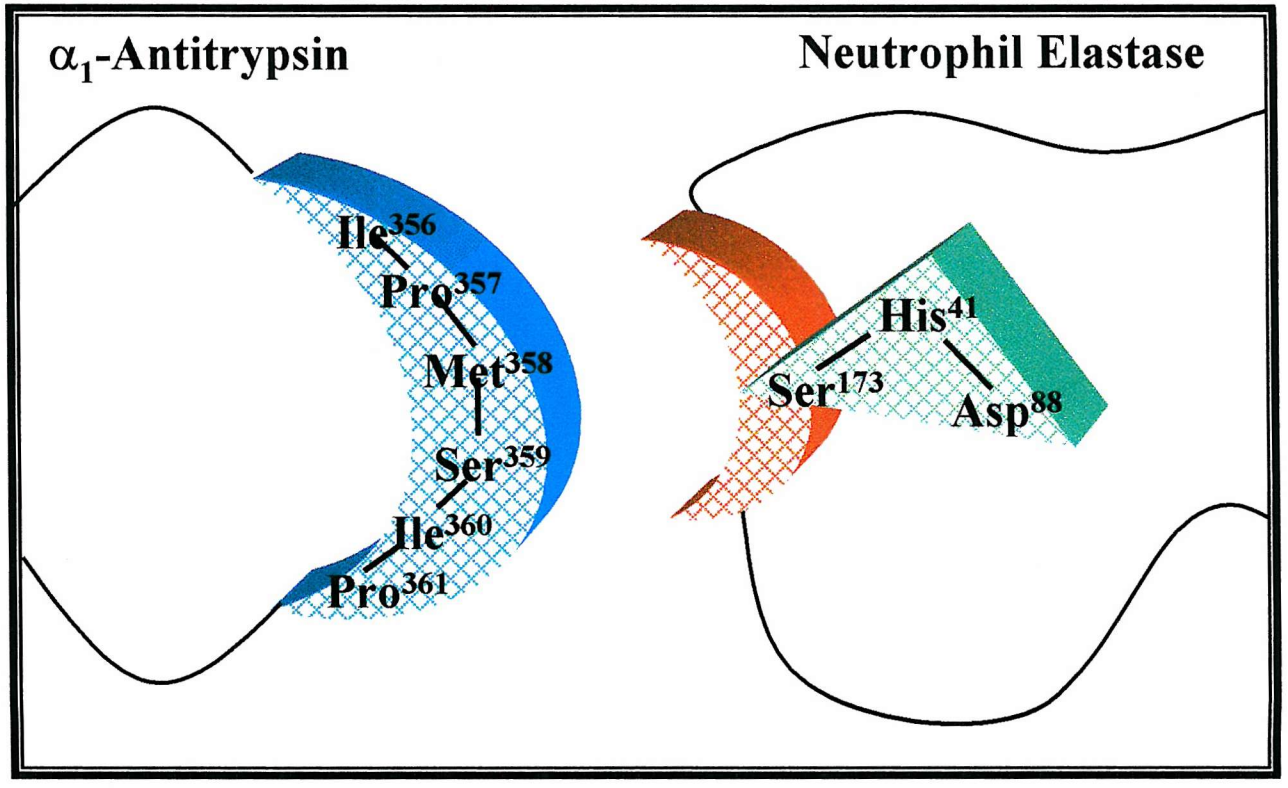


Fig 1.8 Active sites of α_1-antitrypsin and its natural substrate, Neutrophil Elastase. The inhibitory site of α_1-antitrypsin, Met^{358}-Ser^{359}, is exposed on a stressed loop (-Ile^{356} -Pro^{357} -Met^{358} -Ser^{359} -Ile^{360} -Pro^{361}-) (blue region), extended from the body of the molecule. The reactive centre of Neutrophil Elastase has a specificity pocket (Val^{168}, Phe^{170}, Ala^{187}, Val^{190}, Phe^{203}) (orange region), adjacent to the catalytic triad (Ser^{173} -His^{41} -Asp^{88}) (green region). Dissociation of the two molecules is rare and binding is a suicide interaction (adapted from Crystal *et al.*, 1989).

the protein. When this occurs, the association rate constant of the α_1-antitrypsin for neutrophil elastase is reduced 2000-fold (Carp *et al.*, 1981). Exposure to cigarette smoke and inflammatory cells in the lower respiratory tract are among possible factors that can oxidize the Met^{358}, and it is for this reason that cigarette smoking is seen to markedly accelerate the development of emphysema in individuals with exsiting α_1-antitrypsin deficiency (Larsson, 1978, Gadek *et al.*, 1979).

1.3 Genetics of α_1-antitrypsin

1.3.1. Gene Structure

The 12Kb α_1-antitrypsin gene, illustrated in Fig.1.9, is comprised of 7 exons and 6 introns and has been mapped to chromosome 14q31-2.3 (Purello *et al.*, 1987, Billingsley *et al.*, 1993, Crystal *et al.*, 1989). Exons II-V contain the protein coding region whereas exons 1A, 1B and 1C are "non-coding" i.e they contain sequences found in α_1-antitrypsin mRNA transcripts, but do not code for the α_1-antitrypsin protein. The carbohydrate attachment sites are coded in exons II and III and the active site is coded by exon V (Crystal *et al.*, 1989). The sequence coding for the α_1-antitrypsin protein translated by mRNA starts with the codon ATG in exon II (Purello *et al.*, 1987).

1.3.2 Gene Expression

The 2 major sites of α_1-antitrypsin gene expression are hepatocytes (Laurell & Jeppsson, 1975) and the Mononuclear Phagocyte (MP) cell family (Perlmutter *et al.*, 1985). The hepatocytes contain approximately 200-fold more α_1-antitrypsin mRNA transcripts per cell than do MP's (including monocytes and alveolar macrophages) and they represent the major site of α_1-antitrypsin biosynthesis (Fabbretti *et al.*, 1992). In the region just 5' of exon 1C there is at least one hepatocyte cis regulatory elements capable of interacting with specific nuclear

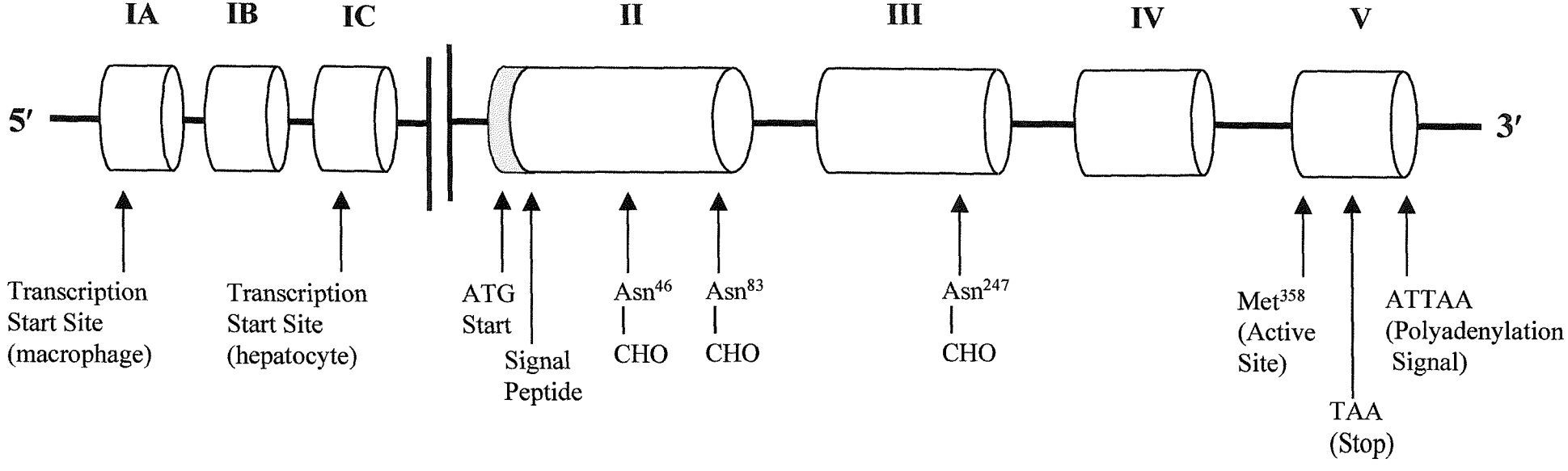


Fig 1.9 Organization of the normal α_1-antitrypsin (aat) gene. The boxes denote coding elements (exons) of the aat gene, the lines between the boxes are intervening sequences (introns). The gene is comprised of 7 exons spanning 12.2kb of chromosome 14 at q32.1. At the 5 end of the gene, exons IA, IB and IC are regulatory elements essential for normal aat expression. IA and IB are macrophage-specific regulatory elements and IC has both macrophage and hepatocyte-specific regulatory elements. These 3 non-protein coding exons are followed by 4 protein coding exons II-V. The start codon (ATG) for translation of aat mRNA, the signal peptide (shaded area) and 2 of the 3 CHO attachment sites (Asn^{46} & ^{83}) are in exon II. Exon III encodes the 3rd CHO attachment site (Asn^{247}). The active site (Met^{358}), the stop codon (TAA) and the polyadenylation signal (ATTAA) are coded for in exon V.

proteins, presumably to control expression of the gene. Most of the information relating to control of α_1-antitrypsin expression is related to hepatocyte expression and less is known about regulation of α_1-antitrypsin gene expression in MP's. Perlino *et al.*, in 1987, investigated the mechanism of expression of the human α_1-antitrypsin gene in macrophages and discovered a macrophage-specific promoter located ~2000bp upstream of the hepatocyte specific promoter. This suggested that α_1-antitrypsin gene transcription responds to two different cell-specific regulatory mechanisms and that transcription of the two α_1-antitrypsin promoters must be mutually exclusive, with the hepatocyte promoter silent in macrophages and vice versa.

1.4 Variant Serpins

A key contribution to our understanding of the molecular physiology of the serpins has come from the study of human variants associated with disease. These variants provided the earliest supporting evidence of the unique mobility of the reactive loop of the serpins and of the mechanisms and regions of the protein that allow and control this mobility (Carrell & Stein, 1996).

Presently, over a hundred variants of different serpins have been identified and the diseases they cause are characteristic for each serpin: mutants of antithrombin (Lane *et al.*, 1993) result in thrombotic disease, mutations of C1-I (Verpy *et al.*, 1995) result in angioedema and mutants of α_1-antitrypsin (Brantly *et al.*, 1988) predispose to emphysema and cirrhosis. As well as these common diseases, mutations of other serpins have been implicated in hypertension and in defects of hormone transport and in lung disease (Poller *et al.*, 1993).

1.4.1 Allelic Variants of α_1-antitrypsin

α_1-antitrypsin deficiency is a hereditary disorder characterised by low serum levels of α_1-antitrypsin, a high risk for the development of emphysema and a

possible risk for the development of liver disease, particularly in childhood. The disorder was discovered in 1963 by Laurell and Eriksson, when they noted a reduction of the α_1-globin band in approximately 1 in 500 serum protein electrophoresis patterns obtained from members of the Swedish population, chosen at random.

Since the 2 parental genes are codominantly expressed, the α_1-antitrypsin serum phenotype, referred to as Pi (Proteinase inhibitor), is determined by the independent expression of the 2 parental alleles (Fabbretti *et al.*, 1992). The α_1-antitrypsin gene, located on chromosome 14, is one of the most polymorphic loci in the human genome and at least 100 α_1-antitrypsin alleles have been identified (Blank & Brantly, 1994). These are conveniently categorised into 4 groups (Brantly *et al.*, 1988), based on the status of the α_1-antitrypsin protein in serum:

1) "**normal**" (associated with normal serum levels of α_1-antitrypsin with normal function);
2) "**deficient**" (associated with serum levels of α_1-antitrypsin <35% of normal, but available protein has normal function);
3) "**dysfunctional**" (the α_1-antitrypsin protein is present but does not function normally) and
4) "**null**" (no detectable α_1-antitrypsin protein in serum)

With the exception of the null alleles and a few rare alleles, the variants are classified alphabetically by the Pi nomenclature, relating to the position of the migration of the α_1-antitrypsin protein during isoelectric focusing (IEF) of plasma between pH 4.5 and 4.9. For example; PiBB is the homozygote for an anodal variant and PiZZ for the cathodal variant described by Laurell and Eriksson, with PiMM representing the homozygote for the normal allele (Hoffmann & Van Denbroek, 1977). When 2 alleles have an identical IEF pattern, and the sequence difference is known, the relevant residue is specifically indicated (for example, the 2 most common α_1-antitrypsin alleles, M1[Val^{213}] and M1[Ala^{213}], have IEF patterns of M1 but differ at residue 213 by the neutral amino acids Val and Ala

(Nukiwa *et al.*, 1987)). Some rare alleles are labelled by a letter indicating the IEF position together with the birth site of the allele (e.g. Mprociada). The null alleles are labelled "null" together with the place of origin (e.g. Pi $null_{Bellingham}$) (Satoh *et al.*, 1988).

Extensive epidemiological studies have carefully delineated the association of certain α_1-antitrypsin phenotypes and the risk for development of disease. While severe α_1-antitrypsin deficiency is the major factor leading to emphysema and related pulmonary disease, a small number of deficiency phenotypes have also been associated with the development of neonatal hepatitis and cirrhosis (Lam *et al.*, 1997). Genetic studies have shown that homozygous inheritance of the Z deficiency allele is associated with an increased risk for both emphysema and liver disease. In comparison, the homozygous inheritance of a null allele (e.g. Pi $null_{Bellingham}$) is associated with a much greater risk for the development of emphysema but no risk for liver disease (Fagerhol & Cox, 1981).

The α_1-antitrypsin variants that have been of particular interest are the common Z and S phenotypes, both of which are associated with severe plasma deficiency. The protein product of the Z allele is associated with plasma concentrations equivalent to only 15% of that of the normal M allele (Jeppsson *et al.*, 1976), and the S allele results in only 60% of normal protein levels (Gitlin & Gitlin, 1975). Mixed heterozygotes e.g. MZ, MS, SZ will give proportionally altered plasma levels. Genotypes that pose a risk to health have been established as PiZZ (15%), PiSZ (35%), and combinations with any rare null gene e.g. PiZ- (8%) (Carrell *et al.*, 1982).

1.4.2 Normal Category

Four alleles (M1[Ala^{213}], M1[Val^{213}], M3[M1[Val^{213}][Asp^{376}] and M2[M3[His^{101}]) represent >95% of the known α_1-antitrypsin variants associated with normal serum α_1-antitrypsin levels (Kneppers & Christopherson, 1978,

Brantly *et al.*, 1988). Among Caucasians, the only racial group for which there is extensive data, M1 (Val^{213}) is the most common. All of the normal M-family alleles, when inherited homozygously, are associated with serum levels of 20 to 48μM and all inhibit neutrophil elastase equally (Brantly *et al.*, 1988). At least 42 other "normal" α_1-antitrypsin variants have been identified. Except for M4 (alleic frequency ≈0.01-0.05) all are rare, with frequencies of less than 1% (Brantly *et al.*, 1988, Constans *et al.*, 1980).

1.4.3 Deficiency Category

At least 11 α_1-antitrypsin deficiency variants have been identified and a number of these are listed in Fig 1.10. The 'classic' deficiency allele is Z; it was this variant that was inherited in a homozygous fashion in the original patients described by Laurell and Eriksson in 1963.

Z is commonly found in Caucasians of N. European descent, representing 2 to 5% of all α_1-antitrypsin alleles in this population. It is rarely found among Blacks or Orientals. The serum levels of individuals homozygous for the Z gene are markedly reduced, and values are typically 2 to 5μM (Fagerhol & Cox., 1981). In α_1-antitrypsin synthesising cells of Z homozygotes, mRNA levels, mRNA translation and core glycosylation of the newly synthesised α_1-antitrypsin in the RER are all normal (Mornex *et al.*, 1986, Bathurst *et al.*, 1984, Verbanac & Heath, 1984). Two amino acid substitutions are the only identified structural differences between the inefficiently secreted Z variant and the efficiently secreted 'normal' M form of α_1-antitrypsin. The first is a G to A transition which alters the codon **G**AG (specifies Glu^{342}) to **A**AG (specifies Lys) (Long *et al.*, 1984, Lomas *et al.*, 1993). The second is a T to C transition which alters the codon G**T**G (specifies Val^{213}) to G**C**G (specifies Ala). As the substitution of Val for Ala^{213} is documented not to affect secretion of α_1-antitrypsin (Sifers *et al.*, 1987) the replacement of Glu^{342} for Lys is the likely cause for defective secretion and hepatocyte accumulation of the Z variant (McCracken *et al.*, 1991).

ALLELE	EXON*	MUTATION		CLINICAL DISORDER
F	III	R223C	(CGT→TGT)	Normal
Z	V	E342K	(GAG→AAG)	Emphysema
Siiyama	II	S53F	(TTC→TCC)	+ Liver Cirrhosis
Mmalton	II	F52	(TTC→delete)	
S	III	E264V	(GAA→GTA)	Emphysema
I	II	R39C	(CGC→TGC)	

Fig1.10 Deficient human α_1- antitrypsin variants. * indicates the exon on the α_1- antitrypsin gene where the mutation is located. Clinical disorder refers to the homozygous state (Crystal *et al.*, 1989).

The most common deficiency allele is Pi S. This allele accounts for 5-10% of all α_1-antitrypsin alleles of North European Caucasians (Owen *et al.*, 1976), and has an especially high prevalence of up to 28% in Southern Europeans (Fagerhol, 1976). The coding exons of the S gene differ from the M1 (Val^{213}) gene at a single base, resulting in the amino acids substitution $Glu^{264} \rightarrow Val$ (Owen *et al.*, 1976), caused by an A to T substitution in exon III. α_1-antitrypsin synthesising cells of S homozygotes, as seen with the Z variant, have α_1-antitrypsin mRNA transcripts of normal length and levels, but the cells secrete only 60% of normal protein levels secreted by comparable cells of M homozygotes (Gitlin & Gitlin, 1975). This decreased secretion results serum α_1-antitrypsin levels of 13 to 19μM in the homozygous state. These levels appear to be sufficient to protect the lung, as PiSS individuals show no risk for developing emphysema, despite the relative deficiency state. However, expression of the S allele in a heterozygous form with the Z allele, results in serum levels of 6 to 11μM, levels that are 'borderline protective', and some of these individuals do develop emphysema (Larsson *et al.*, 1976, Nukiwa *et al.*, 1986). Unlike the Z protein, the newly synthesised S protein does not appear to accumulate in the hepatocytes as any retained protein is rapidly degraded (Curiel *et al.*, 1989 (a)), and PiSS individuals are not at risk of liver disease (Gordon *et al.*, 1972). Conversely, expression of a mixed SZ heterozygote, can lead to the development of cirrhosis associated with the α_1-antitrypsin deficiency (Mahadeva *et al.*, 1999).

The α_1-antitrypsin variants Siiyama ($Ser^{53} \rightarrow Phe$ (Seyama *et al.*, 1991)) and Mmalton ($Phe^{52} \rightarrow del$ (Curiel *et al.*, 1989 (b)) are similar to the Z protein in that both result in a plasma deficiency related to hepatocellular accumulation of the protein (Lomas *et al.*, 1993, Carrell & Stein, 1996).

A number of other genetic variants of α_1-antitrypsin are now being discovered which produce the same deficiency state as the Z, Siiyama and Mmalton variants. For example: the F ($Arg^{223} \rightarrow Cys$) variant of α_1-antitrypsin described by Fagerhol *et al.* in 1965. The F allele of the α_1-antitrypsin gene shows a mild functional deficit and predisposes to the development of emphysema if it occurs with another

deficiency allele, in particular Z. This FZ phenotype is rare, occurring in about 1/17000 of the population (Kelly *et al.*, 1989). The I variant ($Arg^{39}\rightarrow Cys$) is another rare variant associated with a slight decrease in α_1-antitrypsin secretion (Fagerhol, 1967). As with the F variant, expression of the I allele in combination with Z, has been observed to increase the mild deficiency and increase the possibility of the development of emphysema and liver disease. Due to the position of the Arg^{39} mutation, it has also been suggested that the loss of a bond between this residue and Glu^{264} may contribute to the common S deficiency variant in which glutamic acid 264 is replaced by valine (Huber & Carrell, 1989). Hence, the disruption of the complementary side of this bond, as occurs with the loss of Arg^{39} in the I variant, may result in a deficiency state similar to the S variant, resulting in a relatively mild clinical disorder (Graham *et al.*, 1989).

There are a large number of other deficiency variants whose mechanisms are not as yet fully understood and many other rare deficiency alleles exist but the mutations responsible for these are still unknown.

1.4.4 Null Category

The expression 'null' refers to an α_1-antitrypsin gene that does not code for α_1-antitrypsin protein identifiable in serum and, thus, individuals inheriting a null gene from both parents have α_1-antitrypsin levels of 0. This phenotype is extremely rare, (<0.1% in North Europeans), and is responsible for a very high risk for the development of emphysema but not liver disease (Brodbeck and Brown, 1992). Null variant genes are usually caused by single amino acid deletions, substitutions or insertions which lead to the formation of a premature stop codon and, thus, an incomplete polypeptide (Crystal *et al.*, 1989).

1.4.5 Dysfunctional Category

α_1-antitrypsin 'Pittsburgh' is the only known example of a natural dysfunctional variant and was discovered in an individual with a bleeding disorder (Lewis *et al.*, 1978). This form of α_1-antitrypsin was partially sequenced and was observed to have a single amino acid substitution at the α_1-antitrypsin active site ($Met^{358} \rightarrow Arg$). This gives the active site of the α_1-antitrypsin molecule a high homology with antithrombin III, the natural inhibitor of thrombin, making the α_1-antitrypsin an effective inhibitor of thrombin and a poor inhibitor of neutrophil elastase. This mutation resulted in an approximately 6000-fold increase in the second order association rate constant with human thrombin ($k_{ass} = 48M^{-1} \times s^{-1}$ for the normal protein compared to $3.1 \times 10^5 M^{-1} \times s^{-1}$ for the arginine mutant), and a 4000 increase in relative inhibitory activity (Owen *et al.*, 1983, Travis *et al.*, 1986). With no protection from proteolytic attack in the lower respiratory tract and an increased inhibition of thrombin, the individual eventually died from haemorrhage following trauma. This finding not only explained the bleeding disorder and established the position of the reactive centre in each molecule but it also showed the critical contribution of the P1 residue in determining inhibitory specificity of serpins.

1.4.6 Identification of Phenotypes

The identification of α_1-antitrypsin phenotypes can be achieved by a number of methods. As the α_1-antitrypsin allele dictates the serum protein level, the phenotype may be determined simply by measuring serum α_1-antitrypsin levels combined with familial studies. IEF analysis of serum from individuals provides a more accurate analysis as this method can be used to separate the majority of α_1-antitrypsin alleles that differ by charge. More recently, detection of possible α_1-antitrypsin phenotypes is achieved using PCR analysis. Genomic DNA is extracted from α_1-antitrypsin producing cells (Miller *et al.*, 1988), and analysed

by agarose gel electrophoresis following PCR amplification and *Taq*I restriction digestion. This method allows rapid screening of large numbers of DNA samples and easy detection of abnormal species, which can then be analysed separately to identify the mutant allele (Dry, 1991, Tazelaar *et al.*, 1992, Lam *et al.*, 1997).

1.5 Clinical Manifestations of α_1-antitrypsin Deficiency

The importance of serpins within physiological systems is reflected by the range of hereditary diseases resulting from serpin deficiency. Antithrombin (ATIII) deficiency is a cause of recurrent thromboembolic disease, and mutations of C1-inhibitor can cause angioedema. Hypertension is associated with the noninhibitory serpin angiotensinogen. Increased PAI-1 activity is associated with noninsulin-dependent diabetes mellitus and α_1-antichymotrypsin is closely associated with plaque formation in Alzheimer's disease (Engh *et al.*, 1995).
Abnormal phenotypes of α_1-antitrypsin have been associated with a number of diseases including renal disease, arthritis and malignancies. However, the 2 strongest associations are with liver and lung disease (Carrell *et al.*, 1982).

1.5.1 Lung Disease

Serum α_1-antitrypsin acts by diffusing into the alveolar space and irreversibly inhibiting neutrophil elastase that digests elastin and most tissue matrix proteins in the lower respiratory tract (Carrell *et al.*, 1982). Due to its main physiological function, it follows that severe genetic deficiency in α_1-antitrypsin (levels <50mg/dl) is frequently associated with a high incidence of development of early onset pulmonary emphysema (Eriksson, 1965).

The loss of lung elasticity, which occurs in severe α_1-antitrypsin deficiency, represents the summation of cumulative proteolytic damage and the end point of this loss of elasticity is the development of the irreversible lung disease, emphysema. This is a common disease and only 1-2% of cases are actually due to

a genetic deficiency of α_1-antitrypsin. The recognition of the relationship between active α_1-antitrypsin and neutrophil elastase has increased the understanding of the pathogenesis of emphysema as being due to an imbalance between proteinases and their naturally occurring inhibitors in the lung tissue (Carrell *et al.*, 1982, Hubbard *et al.*, 1991).

Inactivation of α_1-antitrypsin by environmental factors such as smoking or air pollution, can have the same effect as decreased plasma levels of α_1-antitrypsin and will also lead to the development of emphysema (Engh *et al.*, 1995). Several studies have shown that although the concentration of α_1-antitrypsin in the alveoli of heavy smokers is normal, its elastase-inhibitory activity is markedly reduced. This loss of activity in the α_1-antitrypsin of the smoker can be shown to coincide with the oxidation of two of its methionines, 351 and 358, to methionine sulphoxide. This illustrates the critical importance of the methionines and suggests that oxidation of methionine 358 leads to a decreased capacity for inhibition of elastase (Johnson & Travis, 1979, Owen *et al.*, 1983, Larsson, 1978, Gadek *et al.*, 1979).

Like most plasma proteins, α_1-antitrypsin is secreted from the liver into the serum. There are, however, situations in which a localised action of the protein is required. Following inflammation or infection there is accumulation of leucocytes at the site of injury, and a consequent release of high amounts of proteases. In such situations, the rapid release of α_1-antitrypsin by intervening local macrophages acts to prevent extensive tissue damage and also results in a positive-feedback mechanism that leads to the increased production of α_1-antitrypsin by the liver (Sharp *et al.*, 1979, Carrell *et al.*, 1982, Courtois *et al.*, 1987).

1.5.2 Liver Disease

Although the effects of α_1-antitrypsin deficiency in the lower respiratory tract are easily explained, the pathophysiology of the liver disease, that accompanies the expression of a number of α_1-antitrypsin deficiency variants, is not completely understood. It is almost certainly related to the accumulation of α_1-antitrypsin in the hepatocyte, as it only occurs in association with variants that produce inclusions and whose defect is assumed to be one of secretion e.g. the Z variant, and not those, such as the null variants, that give an isolated plasma deficiency (Carrell *et al.*, 1982).

The amino acid mutation that produces the Z phenotype is known to result in serum levels of α_1-antitrypsin that are only 15% of the M variant. The majority of the 85% of the α_1-antitrypsin that is not secreted is degraded, but a small percentage aggregates within the ER to form insoluble hepatic inclusions (Lomas *et al.*, 1992). The M variant can also accumulate in liver cells but only following increased synthesis and, unlike the Z variant, the accumulated M protein is completely soluble following cell lysis. This suggests that the insolubility of the accumulated Z variant may be the cause of liver damage (Sifers, 1992).

Studies of the Z protein that accumulates in the liver observed the same polypeptide structure as the circulating Z protein, but the mutant protein had immature carbohydrate side chains, with a high mannose content and no sialic acid (Hercz *et al.*, 1978). This is compatible with the Z protein deficiency being due to a partial block in processing of the polypeptide, with some of the protein produced being mature and secreted into the plasma as normal, whilst the remainder is stopped in its passage through the secretory pathway (Carrell & Owen, 1979). The protein accumulation observed with the Z variant takes the form of intracellular amorphous aggregations of α_1-antitrypsin that can be demonstrated microscopically, as illustrated in Fig 1.11, as occasional deposits in the PiMZ liver and as massive deposits in the ZZ liver (Carrell *et al.*, 1982). 14%

of individuals with homozygous ZZ deficiency will die of liver disease, which is most apparent in the ZZ newborn, where it occurs as hepatitis with a wide variation in severity (Lomas & Carrell, 1993).

The link between infancy and the increased rate of liver disease is related to the observation of the greatly increased rate of aggregaton, *in vitro*, of Z α_1-antitrypsin at 41^{O}C compared with 37^{O}C (Lomas *et al.*, 1992). Infections, particularly in infants, are accompanied by fever and an acute phase response, to give conditions favourable for this aggregation, i.e., increased α_1-antitrypsin concentration and temperatures of 40^{O}C or higher. Hence, an infant who suffers from incidental and often minor infections is particularly at risk of severe liver damage. It explains the observations that around 50% of ZZ infants have minor changes in liver function, some 7% develop a severe obstructive jaundice and a smaller proportion go on to develop a fatal juvenile cirrhosis (Sveger, 1976).

Although childhood cirrhosis is the most obvious form of α_1-antitrypsin-linked liver disease there is also an independent and substantial risk of developing cirrhosis in adult life. This increases with age to give cirrhosis levels near 20% in ZZ individuals above the age of 50 (Carrell *et al.*, 1982). In an analysis of life expectancy in patients with the condition, Larrson (1978), found that 12% of 246 adult Swedes had cirrhosis, but he did not report any age or sex-related risks. Cox & Smyth, in 1983, then reported on a case series of 115 adults with the PiZZ phenotype and found an increased level of cirrhosis in elderly men. The risk of primary liver cancer related to α_1-antitrypsin deficiency is still unknown. A definite relationship has not been established and no increased frequency of carriers of the PiZ gene have been found when patients with primary liver cancer have been studied (Eriksson *et al.*, 1986).

The fact that only 12-15% of ZZ homozygotes in the population present liver injury suggests a role for additional genetic or environmental factors in the development of this phenotype and, in 1994, Wu *et al.* reported evidence that

susceptibility to liver disease in these individuals is related to differences in the protein degradation machinery of the ER.

1.5.3 Treatment for α_1-antitrypsin Deficiency

Strategies to prevent the emphysema associated with α_1-antitrypsin deficiency have focused on augmenting the elastase screen of the lower respiratory tract by administering infusions of purified α_1-antitrypsin. In contrast, as the pathogenesis of the liver disease is not completely understood, no rational therapeutic strategies have been devised and liver transplantation is the only option for treatment (Crystal *et al*, 1989).

Both of these treatments are not the ideal, and the focus of the continuing research into the mechanisms behind α_1-antitrypsin deficiency are focused on the reasons such single amino acid mutations affect the structure of the protein in such a way that a deficiency ensues. Work has particularly focussed on the common Z and S mutations, the reasons for the decrease in secretion and aggregation of these proteins at their site of synthesis and how this is linked to the presence of particular changes in the structure of the protein.

1.6 Hepatic Aggregation

The physical mechanism by which α_1-antitrypsin may aggregate was first demonstrated in detail when, in 1990, Schulze *et al.* observed that when high concentrations of native M α_1-antitrypsin was heated at 60^{o}C for 2hr, the single molecules aggregated to form high-molecular mass chains or polymers. The reactive centre loops of these molecules were not seen to be cleaved and closer examination of the protein chains led to the suggestion that such polymers had arisen from the insertion of the reactive centre loop of one molecule into the A β-sheet of a second. Evans (1991), subsequently showed that α_1-antitrypsin and

other inhibitory serpins were also able to form these polymers, not just at increased temperature, but also following incubation in mild denaturing conditions (~1M guanidinium hydrochloride) at 37^{O}C. Recent research has focussed on the structure of such polymers and the crystallographic structure presented in Fig.1.12 is the first such illustration of a polymer of α_1-antitrypsin molecules (Elliott *et al.*, 1996 (a)).

These observations demonstrated that denaturation of the α_1-antitrypsin protein, either by heat or chemical means, altered the protein structure in such a way that the normal mechanism, involving insertion of the loop into sheet A following cleavage, had become evident as the mechanism by which the loop of one α_1-antitrypsin molecule inserted into the open A sheet of another. This abnormal intermolecular version of a normal intramolecular process has been termed "**Loop-sheet polymerization** " (Lomas *et al.*, 1992).

1.7 Loop-Sheet Polymerization

1.7.1 Mechanism

Two distinct models for the loop-sheet polymerization of α_1-antitrypsin, and other serpins, have been proposed and these have been termed the A-sheet and C-sheet mechanisms. The major distinguishing factor between these two models is that the A-sheet polymers require the reactive centre loop of the donor serpin to be fully expelled and strand 1C to be partially separated from the C β-sheet prior to loop insertion from the second molecule (Carrell & Stein, 1996). The C-sheet mechanism requires insertion of the reactive centre loop, of the donor serpin, into its own A β-sheet to remove strand 1C from the C β-sheet, leaving space for the loop of the second serpin (Carrell *et al.*, 1994). Recently, in 1999, both Huntington *et al.*, and Bottomley *et al.*, presented crystallographic structures of α_1-antitrypsin polymers at atomic resolution. The structure of such polymers had

Fig 1.11 Polymers of α_1-antitrypsin protein, within the hepatocytes of Z homozygotes, observed by electron microscopy (as observed by Lomas et al., 1992).

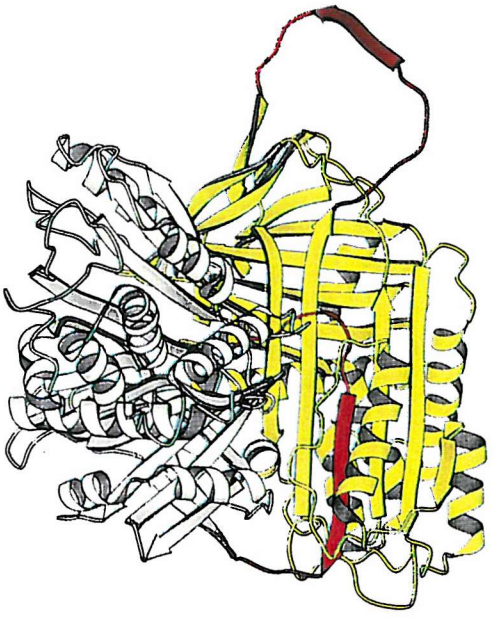


Fig 1.12 Polymerization of α_1-antitrypsin: Model for the interaction between adjacent molecules of α_1-antitrypsin in a fibrous polymer. The β-pleated A-sheet (in yellow), opens as observed in the cleaved structure, the reactive centre loop remains in the canonical β-strand conformation, but moves away from the body of the molecule to insert into the sheet of an adjacent molecule of α_1-antitrypsin (reprinted from Elliott *et al.*, 1996, with permission).

previously been suggested by Mast *et al.*, in 1992, and Elliott *et al.*, in 1996 (a), but no structural evidence was available. The results from both studies demonstrated polymers of cleaved α_1-antitrypsin, with β-strand linkage between molecules. In both structures, polymerization of the protein occurred as a section of the reactive centre loop (between P1 and P6) of one molecule inserted into a corresponding 'gap' at the bottom of the A β-sheet of another. These models provide evidence for a mechanism of polymerization that is intermediate between the A and C sheet mechanisms. The model determined by Bottomley *et al.*, illustrated that the P17-P6 portion of the reactive centre loop of the polymerized protein was inserted into its own A β-sheet (C-sheet mechanism), whereas the P5-P1 residues were inserted into the A β-sheet of a second molecule (A-sheet mechanism).

Fluorescence and molecular modelling studies have also demonstrated that, *in vitro*, α_1-antitrypsin is capable of undergoing both A-sheet and C-sheet linkage, depending on the buffer conditions. Results from Bottomley *et al.*, in 1998, observed that, under conditions of increased temperature, α_1-antitrypsin incubated in a Tris.HCl buffer will favour the formation of polymers via A-sheet linkage, whereas if a citrate buffer is used C-sheet linkage predominantly occurs. This ability to undergo polymerization via different mechanisms, or a combination of both, may also relate to the polymerisation seen with deficiency variants of α_1-antitrypsin. When α_1-antitrypsin polymerises, the conformation of the reactive centre loop may act as a factor to determine the form of linkage that occurs. If excess loop insertion into the A β-sheet has occurred then C-sheet linkage may predominate. If there is little, or no, insertion of the loop then A-sheet linkage will probably be seen. Mutations which affect the mobility of the loop, and the A-sheet will, therefore, have a significant role to play, not only in the process of polymerisation, but also in the type of linkage present between molecules (Lomas *et al.*, 1992, 1993, & 1995 (b)).

In 1999, Dafforn *et al.*, presented the possible kinetic mechanism for the polymerisation of α_1-antitrypsin:

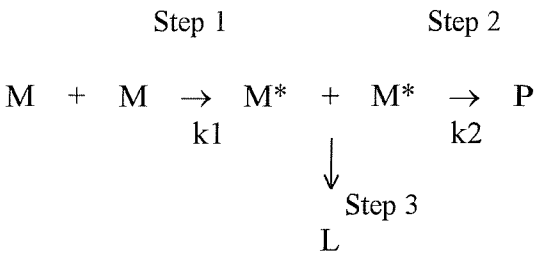


Fig 1.13 Possible mechanism for the polymerisation of serpins. Step 1 represents the conformational change of the serpin (M) to a polymerogenic form (M*), step 2 represents the formation of polymers (P) and step 3 a side pathway that may result in the formation of the latent conformation (L) (reproduced from Dafforn *et al.*, 1999).

This data suggests that polymerisation is a 2-step process with an initial fast conformational change within the protein producing a polymerogenic intermediate which may then undergo loop-sheet linkage. This mechanism for linkage leads to the theory that any factor that decreases protein stability will lead to an increase in the concentration of M* and increase the formation of polymers and/or the latent conformation. Deficiency mutations, such as Z and Siiyama, have been determined to have a role in the opening of the A β-sheet of the protein (Yu *et al.*, 1995, Stein & Carrell, 1993). This would increase the amount of M* and favour the formation of polymers. By the same mechanism, mutations that increase the stability of the serpins will reduce M*, reducing polymer formation (Lee *et al.*, 1996, Berkenpas *et al.*,1995).

1.7.2 Effects of α_1-antitrypsin variants on polymerization

The phenomenon of loop-sheet polymerization occurs spontaneously *in vivo* with several variants of α_1-antitrypsin and is thought to be the major mechanism involved in the accumulation of the protein in the hepatocyte (Lomas *et al.*, 1993). The most studied protein in this respect is the Z deficiency variant and the relationship of the position of the single amino acid substitution, Glu ^{342}→Lys, to

the resulting deficiency has increased the understanding of how and why such a blockade in secretion, of an otherwise functional protein, may occur (association rate for Z α_1-antitrypsin with neurophil elastase is only slightly reduced - $1.2x10^7M^{-1}S^{-1}$, compared to M - $5.3x10^7M^{-1}S^{-1}$ and Ki values are both low - <5pM, indicating tight complex formation (Lomas *et al.*, 1993)). Examination of the crystal structure of cleaved M α_1-antitrypsin revealed that the Glu^{342} residue lies on the 5th strand of the A-sheet at the hinge region of the reactive centre loop, and this led to a number of hypotheses as to why this particular mutation led to loop-sheet polymerization. Loebermann *et al.*, in 1984 suggested that this amino acid alteration disrupted a salt bridge between a positively charged lysine at position 290 and a negatively charged glutamic acid at position 342 which was important in the retention of α_1-antitrypsin within the hepatocyte. Foreman, in 1987, however, determined that severance of this salt bridge by mutating lysine 290 to glutamic acid (the reversal of the Z mutation) failed to inhibit protein synthesis or secretion. In subsequent experiments Sifers *et al.*, in 1989, and McCracken *et al.*, in 1989, also established that disruption of this salt bridge had little effect on the secretion of the Z protein. In 1990, Wu & Foreman then determined that it was actually the loss of positive charge at position 342 that results in the retention of α_1-antitrypsin within the endoplasmic reticulum. This was achieved by mutating the negatively charged glutamic acid to a variety of other amino acid residues. Only the insertion of a positive residue (lysine or arginine) in the 342 position resulted in the retention of α_1-antitrypsin within the secretory pathway. Thus, it was suggested that the Z mutation creates a positive charge at position 342 which may produce or unmask signal sequences which serve to retain the protein within the ER.

This work also led to the suggestion that the replacement of residue 342 could effect the molecular mechanisms involved in loop mobility. The position of the Z mutation, on the hinge region of the molecule, could either favour extension of the reactive centre loop to make the molecule a 'donor' for dimerization or increase the opening of the A-sheet to make the molecule a 'receptor' for dimerization (Lomas *et al.*, 1993). The circular dichroism spectrum in the near UV of Z α_1-

antitrypsin supported the second proposal and this information was also applied to explain the decreased secretion seen with 2 other, rarer, α_1-antitrypsin variants, Mmalton (Phe^{52}→del (Curiel *et al.*, 1989 (b)), and Siiyama (Ser^{53}→Phe (Seyama *et al.*, 1991)), that also polymerize to produce hepatocyte accumulation. Both of these variants have single amino acid mutations, at positions 52 and 53 respectively which are predicted to cause a physical displacement of the B helix that forms the base plate for the opening and closing of the A-sheet (Stein & Chothia, 1991). Hence, the molecular mechanism for loop-sheet polymerization is likely to involve fixation of the A-sheet in an open position, leading to an increased chance of dimer formation and subsequent polymerization (Lomas *et al.*, 1992).

To further assess the conditions under which polymerization takes place, both M and Z α_1-antitrypsin were purified from the plasma of homozygotes and then incubated at 37°C under physiological conditions. Changes in molecular mass were detected on a high-performance liquid chromatography (HPLC) gel filtration column. This was able to resolve native α_1-antitrypsin (52kDa) from high-molecular-mass polymers (>200kDa) and allowed the determination of percentage of polymerized material (Lomas *et al.*, 1992). Under these conditions Z, but not M, α_1-antitrypsin underwent spontaneous polymerization. The rate of polymer formation was accelerated by increasing the temperature to 41°C and was decreased by reducing protein concentration. M α_1-antitrypsin was similarly induced to form concentration-dependant polymers but only following incubation at higher, non-physiological temperatures (41-60°C). At this point the chaotropic effect of temperature actually drives the protein to polymerize irrespective of the Glu^{342}→Lys mutation (Lomas *et al.*, 1993, Carrell & Stein, 1996, Mast *et al.*, 1992).

Protein polymerization at high temperature and concentration was easily reproduced *in vitro* but to confirm that this α_1-antitrypsin polymerized *in vitro* was the same as that *in vivo*, hepatic inclusions were isolated from the liver of a Z homozygote undergoing orthotopic liver transplantation. Electron microscopy of

liver fractions revealed that *in vivo* polymerized α_1-antitrypsin was also formed from 'bead-like' chains of molecules (Lomas & Carrell, 1993). These 'chains' were seen to have a defined structure and the increase in molecular mass seen on incubating the protein under physiological conditions may result from the Z mutation causing a partial unfolding of the α_1-antitrypsin protein prior to aggregation.

To obtain more detail on the physical mechanism of loop-sheet polymerization and its effects on protein structure Lomas *et al.*, in 1992, compared the far UV circular dichroic (CD) spectra of monomeric plasma Z α_1-antitrypsin to that of Z loop-sheet polymers and denatured α_1-antitrypsin. This work allowed closer examination of the β-pleated sheets and α-helical packing and demonstrated that, unlike the denatured material, both the monomeric and polymeric forms of α_1-antitrypsin had a defined secondary structure. Furthermore, both had the profile that had been predicted for loop-sheet bonding, intermediate between that of intact (S) and cleaved (R) α_1-antitrypsin. Work by Cox *et al.*, in 1986, had demonstrated that Z and Mmalton α_1-antitrypsin in the plasma, like that in the liver, has a pronounced tendency to aggregate into a high-*Mr* form, while the M protein does not. These results indicated that aggregation occurs particularly, to a maximum of about 64%, in the presence of pH and salt concentrations typical of the hepatic intracellular fluid. The high-*Mr* complex consisted of a large range of *Mr* values, suggesting that aggregates of from two to many α_1-antitrypsin molecules are present, which cannot be disrupted by a reducing agent or by treatment with Triton X-100 detergent, but can be dissociated by boiling in 2% SDS. This shows that, although disulphide bonds are not involved in this complex formation, these bonds must be extremely strong. These results and those of Lomas *et al.*, in 1992, have determined that Z α_1-antitrypsin polymerized *in vitro* has identical properties and ultrastructure to the inclusions isolated from hepatocytes of a Z homozygote.

In 1998 Elliott *et al.*, (b) developed a novel method of characterizing the conformation of α_1-antitrypsin in bronchoalveolar lavage fluid (BAL) and show that, as well as in liver (Lomas & Carrell, 1993) and plasma (Cox *et al.*, 1986),

α_1-antitrypsin can also form polymers within the lungs of Z homozygotes. Spontaneous polymerization *in vivo* will further reduce the antiproteinase screen and may even exacerbate the lung damage as these polymers may be chemotactic for neutrophils. The polymers obtained by lung lavage were composed of approximately 2 to 7 α_1-antitrypsin molecules, and migrated further into the gel than did the M α_1-antitrypsin control heated at $60^{O}C$ for 3hr, which generated polymers of 15-20 α_1-antitrypsin molecules. The length of polymers identified in BAL was comparable to that of polymers obtained previously upon incubating isolated plasma Z α_1-antitrypsin under physiological conditions. Therefore, α_1-antitrypsin loop-sheet polymerization can be added to reactive-loop cleavage, enzyme-inhibitor complex formation and oxidation of the P1 Met as a mechanism of inactivating α_1-antitrypsin at its site of action.

The results from these experiments have indicated that the process of α_1-antitrypsin secretory blockade is not just a case of aggregation and denaturation of protein, but occurs by a precise and well-defined mechanism -loop-sheet polymerization (Lomas & Carrell, 1993). The *in vitro* experiments performed by Lomas, in 1992, determined the importance of temperature and concentration on polymer formation of protein. Therefore, it is most likely that the formation of liver aggregates is likely to also be dependent on temperature and concentration. α_1-antitrypsin is an acute phase protein and, as such, undergoes a manifold increase in production in association with temperature increases of up to $41^{O}C$ during bouts of inflammation. During these times of stress or inflammation, the formation of inclusions in the hepatocyte is also seen to increase dramatically. These stresses must contribute to the variable severity of hepatocellular damage associated with fever in α_1-antitrypsin deficient children. This temperature dependence of loop-sheet polymerization has implications for the management of the liver disease of the newborn ZZ homozygote (Lomas *et al.*, 1992).

In relation to the concentration dependent mechanism of loop-sheet intermolecular bonding, aggregation will occur in the ER as this is the point of greatest protein concentration in the hepatocyte. Furthermore, results from Lomas

in 1992 also observed that loop-sheet polymerization is an equilibrium process and, thus, there will always be some monomers as well as polymers. This accounts for the secretion of 15% of Z α_1-antitrypsin into the plasma as functional monomers, but this is a level that is insufficient to protect the lungs against the proteolytic damage that leads to emphysema.

The physiological significance of the mechanism of loop-sheet polymerization has been established with its possible relationship to a large number of other serpin-related diseases. Eldering *et al.*, (1995), identified spontaneous loop-sheet polymerization in variants of C1-inhibitor and a mutant antithrombin that produces pyrexially-induced thrombotic disease. Protein folding defects have also been implicated in a few other inherited diseases such as cystic fibrosis (Thomas *et al.*, 1992), and possibly scrapie (Cohen *et al.*, 1994).

1.8 Protein Processing

The relationship between mutation and loop-sheet polymerization that results in aggregation has been extensively studied and the discovery of new variants, as well as the creation of novel mutations in specific regions of the proteins structure, have increased the understanding of this relationship further. The process of aggregation occurs at the main site of synthesis, the ER of hepatocytes, and this is the main site of interest for the determination of methods to prevent polymer formation. Although the relationship between mutation and loop-sheet polymerization is being increasingly understood, the actual mechanism by which loop-sheet polymerization leads to a decrease in protein secretion is still not clear.

The folding and assembly of secretory proteins, such as α_1-antitrypsin, has been extensively studied and the folding pathway has become the primary target for studying the polymerisation process. Synthesis occurs on the cytoplasmic face of the rough ER and nascent proteins are then translocated into the cisternal space. Here the signal peptide is cleaved; initial co-translational folding involving secondary structure and some native tertiary structure occurs; addition and initial processing of high mannose N-linked oligosaccharides takes place, as well as the

formation of disulphide bonds (Le *et al.*, 1990). During these early stages of the pathway, molecular "decisions" are made to determine the fate of each newly synthesized protein. The proteins are then translocated to the Golgi apparatus where they are packaged into vesicles for secretion (Ruddon & Bedows, 1997).

Human α_1-antitrypsin is synthesized as a single polypeptide chain that is modified by the covalent addition of three N-linked oligosaccharides during its transit through the ER. One function of N-linked glycans is to facilitate protein folding and conformational maturation. When N-linked chains are eliminated, many ER-synthesised glycoproteins misfold, aggregate and are degraded within the ER (Le *et al.*, 1990).

The most studied α_1-antitrypsin variant is the Z mutation but the reason that the Z type protein fails to fully secrete is still unclear. Fig 1.14 illustrates the relative processing and secretion of M and Z α_1-antitrypsin. The accumulation of Z α_1-antitrypsin within the microsomal vesicles of *Xenopus* oocyte cells, as shown by Verbanac & Heath in 1986, suggested that α_1-antitrypsin deficiency was the result of an alteration in the transport of the protein through this normal secretory pathway. McCracken *et al.*, (1991), then established that apparently normal levels of Z α_1-antitrypsin are produced and the preprotein is transported normally into the lumen of the ER. Here it is processed and core-glycosylated in the same manner as the M variant, but, compared to M, the rate of movement of Z from ER to Golgi is dramatically reduced. Therefore, a large portion of the newly synthesized protein was seen to accumulate or be degraded within the ER, instead of being secreted.

The results of Yu *et al.*, in 1995, then demonstrated that, *in vivo*, the mutation at residue 342 causes a kinetic defect that leads to accumulation of a folding intermediate with a high tendency to aggregate. These results, and those of Kang *et al.*, in 1997, suggest that the defective step lies at the last stage of folding from a compact intermediate to the native protein. The accumulated folding intermediate is in a state in which the A β-sheet is not completely closed and these

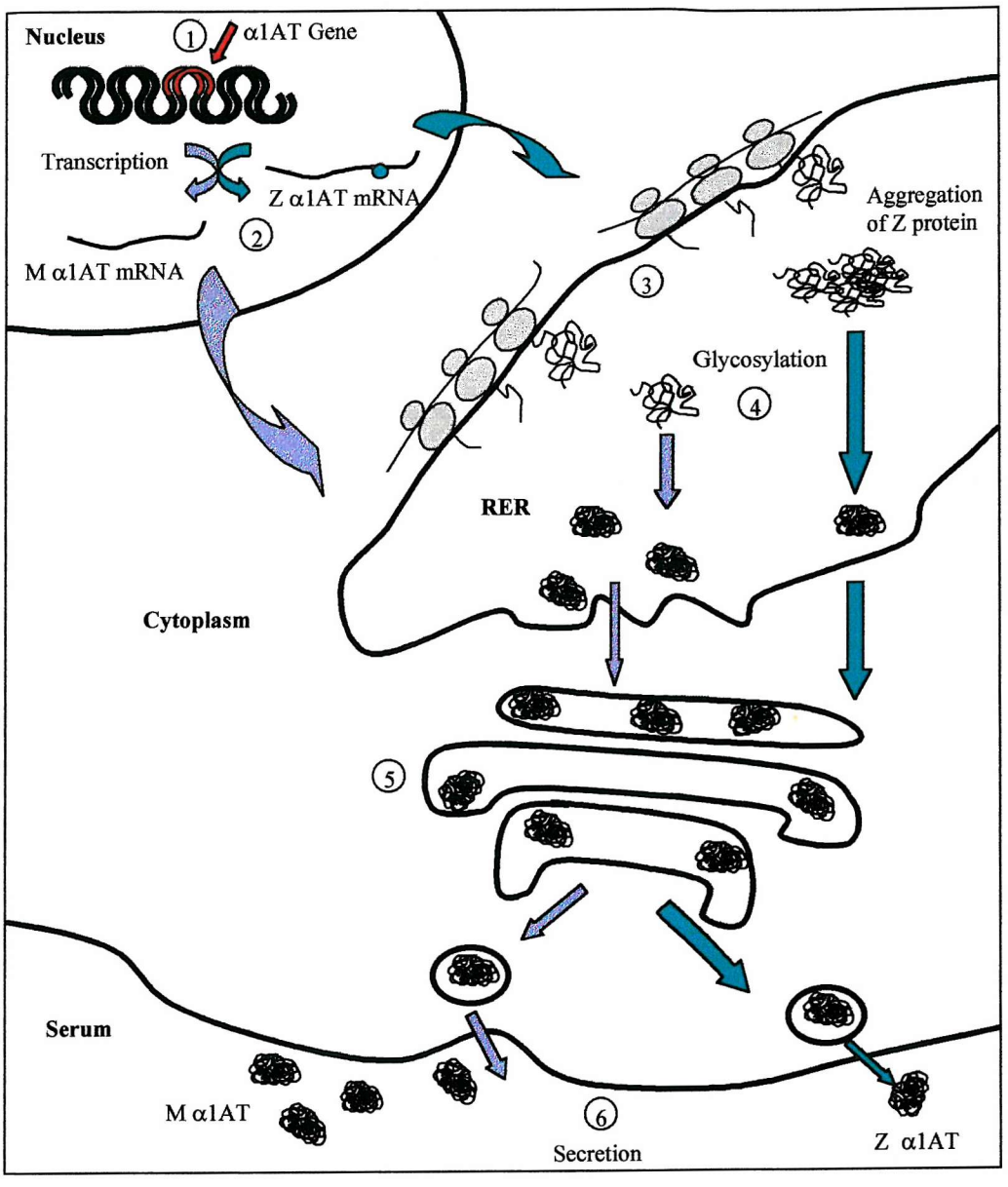


Fig 1.14 Diagram representing the intracellular processing of normal (M) and abnormal (Z) α_1-antitrypsin from gene to protein. (1) Transcription of gene to produce mRNA. (2) Transfer of mRNA from nucleus to cytoplasm. (3) Translation of mRNA on Rough ER to produce protein. (4) Glycosylation of protein and transport to the Golgi. (5) Carbohydrate side-chains added and sialic acid residues added. (6) Glycoprotein is secreted into the serum. The green dot denotes the Z mutation on the Z α1AT mRNA. Processing of Z α1AT differs from stage 3 when the single amino acid change affects protein folding resulting in polymerization, aggregation and degradation of the Z variant protein which greatly reduces secretion compared to the normal M protein (based on Blank & Brantly, Review Article, 1994).

intermediates are, therefore, likely to be a precursor for the formation of loop-sheet polymers. In 1997, Kang *et al.*, also observed that the majority of the secreted Z protein retains inhibitory activity, indicating that the primary cause of the Z type retention in the ER is likely to be due to the folding retardation. In contrast, the retention of another deficiency variant - Siiyama (Ser^{53}→Phe) - appears to be due to a stability defect within the protein. Being at the protein surface, the mutation of Glu^{342}→Lys, does not appear to induce a substantial loss of stability. In contrast, the conformational stability of Siiyama, located at the hydrophobic core, appears to be drastically affected. Results from this study and those of Sidhar *et al.*, in 1995, have also indicated that a successfully secreted Siiyama α_1-antitrypsin protein carrying additional stablizing mutations is not active and, therefore, even the stabilisation of this deficiency variant did not result in inhibitory activity. These observations indicate that retention of the two variants occurs due to different events, with retention of the Z variant related to a trapped folding intermediate and that of the Siiyama variant related to a denatured protein structure.

Although the retention of these two variants may be due to different primary causes, the end result for both is aggregation and degradation of the proteins at their site of synthesis. The cause of aggregation has been established as loop-sheet polymerization, but the reason why not all of the aggregated protein is degraded is still unclear.

1.9 Retention and Degradation of variant α_1-antitrypsin

Only 10-15% of individuals with the ZZ genotype develop sufficient protein aggregation to cause liver disease. Therefore, mechanisms in addition to those simply involved in loop-sheet polymerization, such as inherited trait(s) or environmental factor(s), must participate in the intracellular retention of the Z variant (Sifers, 1995).

The export of many newly synthesized soluble and integral membrane proteins from the ER requires that they must first fold or assemble into their native conformation. Failure to fulfil this requirement will result in either degradation of the misfolded protein within the cell or protein retention until the native structure is produced and secreted (Le *et al.*, 1994). Intracellular degradation of transport-impaired secretory proteins is initialized 30-45 min following their synthesis and translocation into the ER. The inability of a variety of inhibitors of ER-to-Golgi protein vesicular transport, e.g. the carboxylic ionophore monensin, to hinder the degradative process suggests that degradation of the Z variant occurs prior to its delivery to the Golgi complex. Attachment of the ER-recycling signal KDEL to the carboxyl terminus of the recombinant protein also prevented its intracellular degradation (Le *et al.*, 1990).

It has been established that N-linked oligosaccharides of glycoproteins in the ER exhibit sensitivity to digestion by Endoglycosidase H (Endo H). The action of glycolytic enzymes present in the medial stacks of the Golgi complex alter the structure of these oligosaccharides such that they no longer serve as substrates for Endo H digestion. Studies by Le *et al.*, (1990), demonstrated that α_1-antitrypsin mutants did not exhibit oligosaccharide moieties that were resistant to digestion by this enzyme, another indication that degradation of the PiZ variant must occur before export to the Golgi.

Interestingly, the overall rate of degradation in the retained mutant protein is significantly accelerated when all the subcellular compartments are buffered to pH6. In contrast, degradation is virtually abolished when intravesicular compartments are buffered at pH 8. However, despite the pH sensitivity neither lysosomotrophic amines, leupeptin, brefeldin A (promotes redistribution of Golgi enzymes to the ER), or leucine methyl ester (a potent irreversible inhibitor of lysosymal proteolytic activity in hepatocytes), have an apparent effect on the intracellular removal of the Z variant, suggesting that degradation is independent of lysosomal proteases (Le *et al.*, 1990). Degradation of Z α_1-antitrypsin was also prevented by adding the non-specific metabolic poison cyclohexamide

(Novorovdovskya *et al.*, 1998). This evidence, and the fact that degradation of Z protein occurs at a pre-Golgi stage, suggested another mechanism for the retention and degradation of variant protein that involved interaction of the slowly folding protein with other components of the cell.

1.9.1 Molecular Chaperones

Many polypeptides can reform native structure easily by themselves *in vitro* (usually small single domain proteins) while others (more complex, multidomain, or oligomeric proteins) fold and assemble efficiently only when facilitated by physical interaction with additional proteins that are not constituents of the final native protein itself. These additional proteins have been called "molecular chaperones", a term applied by Ellis and Hemmingsen (1989), to the expanding families of proteins of bacterial and eukaryotic components involved in protein folding, assembly and translocation.

Glycosylated folding intermediates of secretory proteins, including α_1-antitrypsin, have been noted to transiently associate with the molecular chaperone Calnexin (Yu *et al.*, 1995). Also designated p88 or IP90, Calnexin is a calcium-binding molecular chaperone of the ER membrane, whose association with such proteins is transient and noncovalent (Wada *et al.*, 1991). An important feature of Calnexin is that it exhibits an affinity for monoglycosylated oligosaccharides, which are intermediates formed during cotranslational trimming by ER α-glucosidases (Kornfeld & Kornfeld, 1989).

It has been suggested that the folding of glycoproteins determines the length of their association with calnexin, which in turn determines their retention time in the ER. Incompletely folded proteins often exhibit a persistent physical association with one or more molecular chaperones and are retained in the ER prior to intracellular disposal. This mechanism has been termed "quality control" and apparently functions to ensure transport of only correctly folded proteins beyond the ER (Hurtley & Helenius, 1989). This quality control machinery is

thought to participate in the molecular pathogenesis of plasma α_1-antitrypsin deficiency as the defective intracellular transport of the aberrantly folded protein through the compartments of the secretory pathway decreases circulating levels of the inhibitor (Sifers *et al.*, 1994).

Chaperones appear to act sequentially in protein folding pathways by binding to folding intermediates and passing them on to the next chaperone or chaperone complex in the cascade, eventually releasing a competent native protein. Binding does not involve specific amino acid residues and usually involves interaction of chaperones with hydrophobic residues on the surface of unfolded proteins. This was demonstrated by Le *et al.*, (1994), who reported that dissociation of the calnexin-null$_{\text{Hong Kong}}$ variant complex with deoxycholate suggests that their interaction might result from the exposure of hydrophobic regions at the surface of the misfolded variant polypeptide. These hydrophobic sequences would normally be on the inside of native proteins, and this provides a way for the chaperones to discriminate between folded and unfolded proteins (Ruddon & Bedows, 1997).

The observed folding retardation of Z α_1-antitrypsin fits well with this model to explain the ER retention of the mutant protein. However, although the interaction of the Z variant protein with calnexin may be involved in the degradation of the Z polypeptides, the involvement of calnexin in the aggregation of Z protein has not been shown (Yu *et al.*, 1995). Work by Qu *et al.*, in 1996, demonstrated that, once in the lumen of the ER, the Z protein binds to the luminal domain of calnexin, while its cytoplasmic side becomes ubiquinated and degraded by proteasomes. This provides evidence that the proteasome, from its cytoplasmic location, induces the degradation of the luminal α_1-antitrypsin Z molecule by attacking the cytoplasmic tail of calnexin molecules that are associated with the variant molecule. Results from Novoradovskaya *et al.*, in 1998 then indicated a role for proteasomes in the intracellular degradation of the Z protein. These experiments observed that lactacystin (a specific, irreversible inhibitor of proteasomes), prevented Z degradation in CHO cells and transfected human fibroblasts, resulting

in retrieval of the protein via vesicular trafficking. The observation that inhibition of intracellular degradation of Z allows apparently normal vesicular trafficking supports the theory of Lee *et al.*, (1998), that very little Z α_1-antitrypsin exists in an insoluble form within the ER. Therefore, the bulk of intracellular Z protein remains available in a soluble form for either degradation or transport to another intracellular compartment.

The relative location of this ubiquitin-proteasomal system, and the protein retained within the ER degradation pathway, has led the suggestion of several mechanisms for by which the proteasome may gain 'access' to the protein. Retrograde translocation, by which substrates are transported from the ER lumen or membrane to the cytosol, is the most recognised of these suggestions. These studies (Biederer *et al.*, 1997, Bordallo *et al.*, 1998, De Virgilio *et al.*, 1998), suggest that the activity of the ubiquitin-dependant proteasomal system is a prerequisite for the retrograde translocation of substrates of the ER degradation pathway. As explained above, this may be particularly relevant to degradation of Z α_1-antitrypsin as ER degradation of this substrate appears to involve polyubiquitination on the cytoplasmic tail of calnexin only when it has bound Z α_1-antitrypsin at the luminal surface of the ER membrane (Teckman *et al.*, 2000).

Establishing the pathways and mechanisms by which degradation of Z α_1-antitrypsin occurs, enabled an increased understanding of why not all of the variant protein retained within the ER of the cell is degraded. Recently, in 1994, Wu *et al.*, subdivided α_1-antitrypsin-deficient individuals into 2 groups. Most were protected from liver injury because of a relatively efficient degradation of α_1-antitrypsin Z in the ER ("protected hosts"), whereas those affected by severe liver disease ("susceptible hosts"), showed a lag in ER degradation, resulting in greater net retention of potentially hepatotoxic α_1-antitrypsin Z within the ER. In one susceptible host, the lag in ER degradation was associated with a failure to interact with calnexin, raising the possibility that this interaction is necessary for entry into the degradative pathway. In several other susceptible hosts, the retained Z interacts well with calnexin, but is only slowly degraded. These hosts may have

a defect in calnexin that prevents its ubiquitination or may have a defect in ubiquitination or proteasomal activity. The interaction of Z with calnexin may not be unique and it is possible that Z interacts with other transmembrane proteins of the ER and that such, as yet unidentified, interactions can account for part of its degradation. It is not yet known how the entire α_1-antitrypsin Z-calnexin complex is degraded and the proteasome may initiate a process that is completed by other enzymes within the ER membrane or ER lumen. Since a significant proportion of Z (40-50%) is also degraded in an energy-independent fashion, ubiquitination-proteasome-mediated proteolysis is probably not the sole pathway for intracellular Z degradation (Qu *et al.*, 1996, Teckman *et al.*, 2000).

These studies all provide evidence that a defect in the folding of variant protein, rather than in the properties of the fully folded protein, may be the underlying cause of both lung and liver disease phenotypes associated with Z, and possibly other secretion deficient, variants of α_1-antitrypsin. The results also suggest that susceptibility to liver disease in α_1-antitrypsin deficiency may be caused by a relatively small increase in the burden of Z protein that accumulates in the ER. Certain individuals have been described as "susceptible" to these slight increases due to a relatively inefficient degradation system within the liver cells and, according to the accumulation theory suggested by Carrell in 1986, liver injury results from the hepatotoxic effect of retained Z α_1-antitrypsin (Carlson *et al.*, 1989). It is a possibility that altering the intracellular fate of a mutant protein may be an option in the treatment of diseases associated with misfolded but potentially functional proteins (Sifers, 1995, Burrows *et al.*, 2000, Teckman *et al.*, 2000).

1.10 Polymerization prevention

1.10.1 Peptide annealing experiments

The first of these novel studies was performed by Schulze *et al.*, in 1990 and involved the complexation of α_1-antitrypsin with a synthetic tetradecameric peptide mimicking strand s4A. This induced a state in which the protein no longer had inhibitory properties, but underwent substrate-like cleavage at the reactive site

on interaction with trypsin. The complex showed CD spectra and denaturation stability similar to that of the cleaved form, evidence that the peptide may bind in the position that the reactive centre loop occupies (strand s4A) in the X-ray structure of cleaved α_1-antitrypsin described by Loebermann *et al.*, in 1984. Fig 1.15 a) illustrates a crystallographic model of intact α_1-antitrypsin with the addition of a synthetic peptide mimicking s4A. The schematic representation (b) shows the peptide inserted as β-sheet 4A, causing blockade of loop insertion.

Similar experiments using a variation of the peptide used by Schulze *et al.*, in 1990, were performed by Mast *et al.*, in 1992. These experiments involved the insertion of a 16-mer peptide, again homologous to strand 4A of the A β-sheet of α_1-antitrypsin. The results from these experiments were consistent with those from Schulze *et al.*, in that the inhibitory activity of α_1-antitrypsin was lost, there was a change in the electrophoretic mobility and an increase in denaturation stability, similar to that seen with the cleaved form of α_1-antitrypsin. This indicated that the peptide had most probably inserted as s4A of β-sheet A. Heat-induced polymerization was also blocked by addition of the 16-mer peptide.

In the same year, Lomas *et al.*, produced similar results and observed that the mechanism of polymerisation was prevented by the addition of an excess of another synthetic peptide, BC13. This peptide differs from those used in the experiments by Schulze and Mast as it is was a 13-residue peptide derived from the reactive centre loop of antithrombin which formed binary complexes as readily with α_1-antitrypsin as antithrombin (k_{obs} = $8x10^{-2}M^{-1}s^{-1}$). The BC13 peptide is homologous to, and only 1 amino acid shorter than, the same sequence, as the peptide used in experiments by Schulze *et al.*, in 1990. These peptides, and the 16-mer peptide designed by Mast *et al.*, in 1992, are homologous to the same region of the molecule, strand 4A, which is reinserted into the A-sheet under conditions of loop cleavage. These results also indicated that this binary complexed Z α_1-antitrypsin was unable to undergo loop-sheet polymerization as the open A-sheet was already occupied by the peptide.

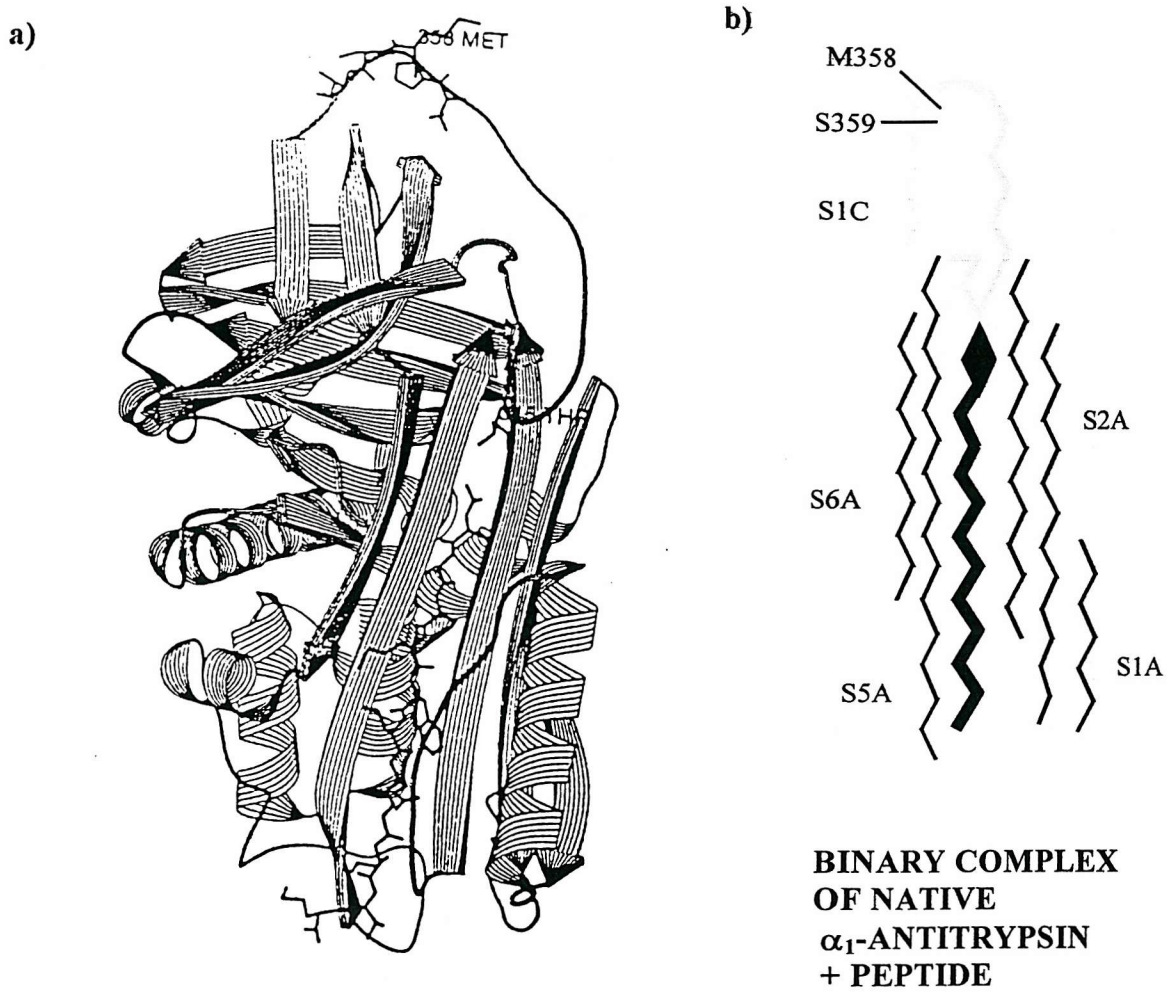


Fig 1.15 Model proposed by Schulze *et al.*, (1994), for an inhibitory complex of α_1-antitrypsin and a unadecamer peptide.

a) Crystallographic model of α_1-antitrypsin molecule showing binding site of inserted synthetic peptide as strand 4A.

b) Schematic diagram of isolated β-sheet A illustrating the peptide binding site.

In this conformation, a completely inserted synthetic peptide would allow only partial insertion of the RCL, including P14 (Thr^{345}) and possibly P13 (Glu^{346}). Insertion at least as far as P12 (Schulze *et al*, 1992), is required for inhibitory activity.

Recent experiments by Bottomley & Chang (1999), have also shown the effect of peptide insertion on polymerization. These experiments involved the incubation of a 12-mer peptide, representing the P14-P3 sequence of antithrombin first designed by Chang *et al.*, in 1996, with a range of chimeric (α_1-antitrypsin/α_1-antichymotrypsin/SERP1), serpins and resulted in the formation of binary complexes. This demonstrated that the A β-sheet of the chimeric proteins were able to accept reactive centre loop residues, increasing the possibility of reactive centre loop insertion and, consequently, polymerization.

The extent of loop insertion into β-sheet A, in relation to inhibitory activity and secretory blockade, has also been determined using similar peptide annealing experiments. Beginning with the full length tetradecamer peptide, first described by Schulze *et al.*, in 1990, these novel experiments were expanded to identify and examine the effects of smaller peptides on the polymerization of α_1-antitrypsin. The original 13-mer peptide was altered to a series of shorter peptides by progressively shortening the N-terminus by 1 amino acid per peptide, each new peptide was then complexed with intact α_1-antitrypsin. The resulting complexes were tested for inhibitory activity, with the hypothesis that inhibition should occur when the complexed peptide is short enough to still allow the necessary degree of insertion of the proteins own loop. These experiments revealed that insertion of Thr^{345} and part of residue 346 is required for inhibitor function (Schulze *et al.*, 1992). Comparison of the 3D structures of cleaved α_1-antitrypsin (Loebermann, 1984), plakalbumin (Wright *et al.*, 1990), and, recently, native α_1-antitrypsin (Elliott *et al.*, 1996(a)), supports this model in which α_1-antitrypsin requires the insertion of Thr^{345} into sheet A for activity. This model for the extent of loop insertion is also supported by the work of Mast *et al.*, in 1992, who determined that, following cleavage of the reactive centre loop of intact α_1-antitrypsin at P9 or P10, the protein undergoes spontaneous polymerization. Therefore, insertion of the P1- P8 region of the reactive loop of one molecule into the A-sheet of another is required for polymerization to occur (Mast *et al.*, 1992).

The information obtained from these novel experiments may prove useful in the prevention of polymerization at the proteins site of synthesis, the ER of hepatocytes. It is possible that specific peptides could be produced and introduced directly to the liver cells, thus preventing polymerization of protein *in vivo,* and the build up of hepatotoxic protein within these cells.

1.10.2 Other Targets

Results from Kim *et al.,* in 1995, strongly suggest that the hydrophobic core of α_1-antitrypsin regulates the opening and closing of the A-sheet and certain genetic variants that cause opening of the A-sheet can be corrected by inserting an additional stable point mutation into the hydrophobic core. The most significant of the stabilizing mutations studied are $Phe^{51}\rightarrow$Leu and $Met^{374}\rightarrow$Ile which lie in the shutter domain that controls mobility of the A β-sheet. The $Phe^{51}\rightarrow$Leu mutation is in a region predicted to be the acceptor site of the exogenous loop in the formation of loop-sheet polymers. This mutation did reduce polymerization of Z α_1-antitrypsin *in vitro* (Kim *et al.*, 1995), and *in ovo* (Sidhar *et al.*, 1995), supporting the assertion that it impedes opening of the A β-sheet. Comparison between the structures of wild-type and $Phe^{51}\rightarrow$Leu α_1-antitrypsin confirms that this mutation improves packing below the A sheet and allows Phe^{384} in s4B to move closer to Leu^{51}. The small structural differences have little effect on the conformation of the loop or the strands of the overlying A β-sheet, but the resulting enhancement of internal stability must hamper their parting to accept other reactive loops during polymerization.

Crystallographic modelling by Elliott *et al.*, in 1998(a), demonstrated the presence of a hydrophobic pocket lined by helices D, E and strand s2A, illustrated in Fig 1.16, which is filled in the cleaved structure of α_1-antitrypsin by the sideways expansion of strand s2A and is also likely to be filled during polymer formation. This pocket provides a potential target for rational drug design as agents that prevent A sheet mobility would hamper polymerization *in vivo,* while they would

not anneal to the A sheet which is known to inactivate the protein as an inhibitor. Computer generated models demonstrate that the pocket can be filled with tri-peptides containing a phenylalanine or tryptophan residue, but have yet to determine whether binding will be hampered by the carbohydrate side-chains not present in the recombinant α_1-antitrypsin structure.

Recent experiments have provided a number of potential targets that can be used to prevent polymerization. The study of the processes involved in aggregation is continuing to provide a number of potential therapeutic targets, but the understanding of why polymerisation leads to a blockade in secretion is still not fully understood, and it is this area of research which may provide the major route for prevention, rather than cure, of this protein related disease.

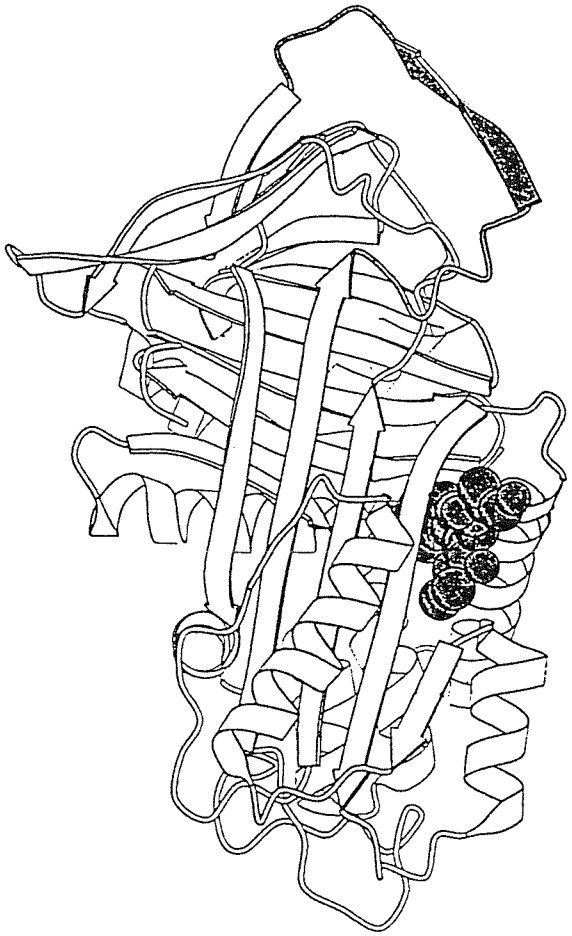


Fig 1.16 Model of the structure of wild-type α_1-antitrypsin, illustrating a hydrophobic 'pocket' domain on the proteins surface, which may provide an ideal target for rational drug design to prevent protein polymerisation, by blocking the movement of strand 2A (reprinted from Elliott *et al.*, 1998, with permission).

CHAPTER 2.

MATERIALS AND METHODS

2.1. Chemicals and Reagents

DNA and RNA modifying enzymes were purchased from Promega (UK), New England Biolabs (NEB) or Boehringher Mannheim (Germany) and were used as recommended by the manufacturers. L-[^{35}S]methionine (specific activity >1000 Ci.mmol^{-1}) for metabolic labelling of proteins and [^{33}P]ddNTP's for DNA sequencing were supplied by Amersham (UK). Sequencing of DNA, to ensure the desired mutation was in place, was performed by the dideoxy-chain termination technique using the Thermosequenase kit from Amersham (UK) and the 'SEQUAGEL' sequencing system supplied by National Diagnostics or using the 'Big Dye' cycle sequencing kit (Perkin Elmer) followed by automated sequence analysis using the ABI377 sequencer. cDNA's were transcribed *in vitro* using the Promega Ribomax sp6 Transcription System. Resulting mRNA was translated *in vitro* using the Promega Nuclease-treated Rabbit Reticulocyte Lysate system according to manufacturers instructions. Anti-human α_1-antitrypsin was purchased from DAKO and horseradish peroxidase-conjugated anti-α_1-antitrypsin from Amersham (UK). All other reagents were analytical grade or better and purchased from Sigma (UK).

2.2. Standard Techniques

Basic microbiological and molecular methods were performed according to Sambrook *et al.*, (1989).

2.3. Construction of α_1-antitrypsin Variants

2.3.1. Introduction

Two methods are described here for the construction of mutagenised α_1-antitrypsin cDNA. For α_1-antitrypsin variants F, I and P5 S, V and Q, described in Chapter 3, cDNA was supplied by Dr D. Lomas at the University of Cambridge.

cDNA of α_1-antitrypsin variants S and Tyr^{38}→Cys/Phe, described in Chapter 4, was created in this laboratory using the method of site-directed mutagenesis.

2.3.2. Method 1: Construction of F, I and P5 S, V and Q α_1-antitrypsin Variants

Variants were supplied by Dr D. Lomas as α_1-antitrypsin DNA insert containing the desired mutation within translation vector pWombat. The α_1-antitrypsin DNA transcript produced using this vector is unsuitable for expression of the protein using the *Xenopus* oocyte expression system. The expression of protein from this system requires the use of a modified version of the Sp64T vector described by Krieg and Melton in 1984. This modified vector includes the addition of a 5′ flanking region extracted from *Xenopus laevis* β-globin mRNA in the region of the sp6 primer recognition site in the vector. This region produces capping and ribosome binding sites and allows the efficient processing of readily translatable α_1-antitrypsin mRNA in *Xenopus* oocytes. The modified vector also contains a PstI site downstream from the 5′ flanking region to allow correct insertion and alignment of the α_1-antitrypsin cDNA in relation to the untranslated flanking regions (Foreman, 1987).

In order to transfer the α_1-antitrypsin DNA insert containing the mutation from the pWombat vector to the modified Sp64T vector, specific restriction enzymes were used, according to the methods detailed below and illustrated in Fig 2.1.

2.3.2.1. Restriction Enzyme Analysis

pWombat vector was treated with SpH1 (90min @ 37°C) & EcoRV (90min @ 37°C) enzymes (variants P5 S, Q and V) or EcoRV (90min @ 37°C) & BamHI (90min @ 37°C) enzymes (variants F and I), to remove α_1-antitrypsin insert containing the desired mutation. Sp64T vector was treated with SmaI (90min @ 25°C) & EcoRV (90min @ 37°C) enzymes (variants P5 S, Q and V) or EcoRV (90min @ 37°C) & BamHI (90min @ 37°C) enzymes (variants F and I), to remove the equivalent section from this vector.

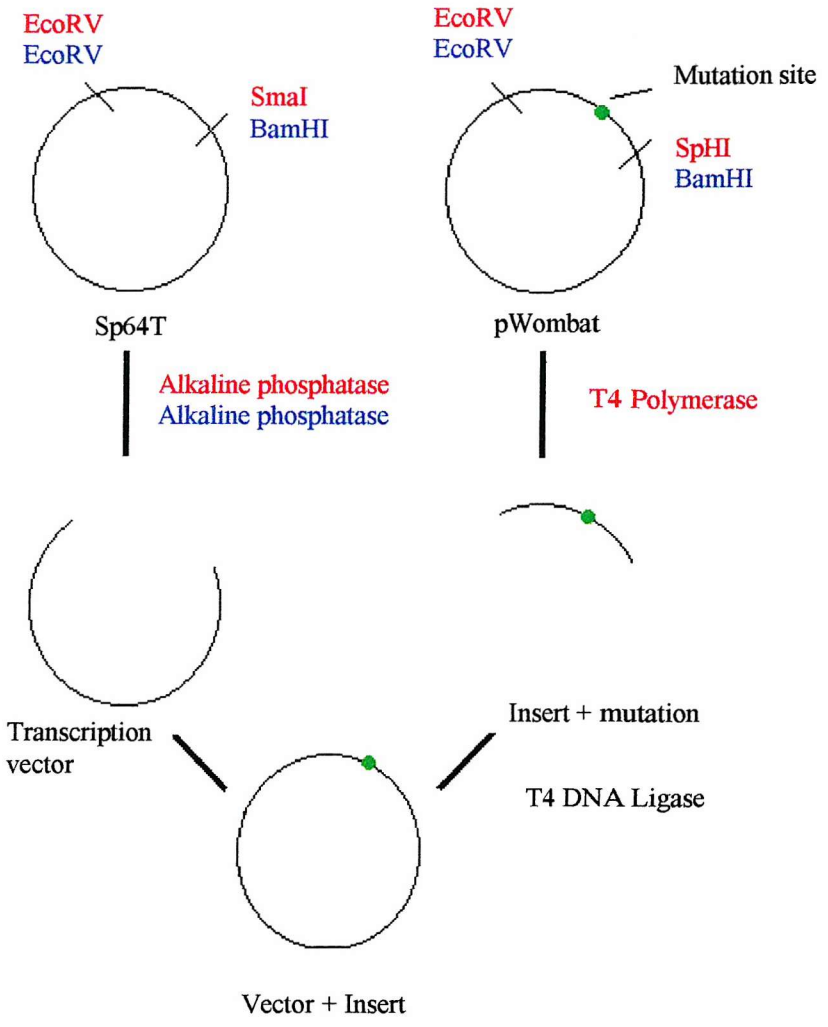


Fig 2.1 Construction of α_1-antitrypsin P5, F and I mutants. α_1-antitrypsin cDNA inserts were spliced from transcription vectors Sp64T and pWombat using appropriate restriction enzymes. Specific restriction enzyme sites and any additional procedures are highlighted in red for P5 variants and in blue for F and I variants. Transcription vector Sp64T was treated with calf alkaline phosphatase to prevent recircularisation during ligation. pWombat α_1-antitrypsin insert containing the desired mutation was incubated for 20min in the presence of T4 Polymerase to fill in 3' recessed termini created by restriction enzyme Sph1 and create a blunt end for successful ligation with the corresponding Sma1 restriction site of the receiving vector (P5 variants only). Vector and insert were ligated, using T4 DNA ligase (Promega), @ 16^{O}C overnight and transformed into competent TG2 *E.Coli* cells.

Pwombat insert and Sp64T vector DNA were then isolated using agarose gel electrophoresis.

2.3.2.2. Agarose Gel Electrophoresis

Agarose was dissolved by heating in 25ml 1xTBE buffer (89mM Tris, 89mM Boric Acid, 1.8mM EDTA), to achieve a 1% agarose solution. Upon cooling, 1.3µl of 1x working solution of ethidium bromide was added to the gel solution. The gel solution was poured in a H2 Horizontal Electrophoresis unit (Anachem) and allowed to polymerize at room temperature for 20 min.

The total amount of α_1-antitrypsin DNA/Vector digested using restriction enzyme analysis was loaded into the wells following addition of 2µl of 1x tracking dye. pWombat sample was separated from Sp64T sample by two wells to ensure no contamination of samples occurred. Agarose gels were run at 50 volts for ~ 90min in 1xTBE running buffer. The integrity and size of DNA samples was verified by co-migration of 1Kb DNA ladder (Promega). Records of gels were kept by transillumination of the gel using a UV light box and photography onto Polaroid 667 film through an orange filter.

Separation of vector and insert sections of both pWombat and Sp64T samples using agarose gel electrophoresis allowed the 'vector' section of the Sp64T and the 'insert' section of the pWombat vector to be removed from the gel. The presence of bands at ~ 3Kb and ~ 1Kb, represented the 'vector' and 'insert' sections of each sample respectively.

2.3.2.3. Isolation of DNA

'Vector' and 'insert' sections were isolated and reprecipitated from the agarose gel using DEAE membrane. The gel was visualized using a UV light box and slits cut in the gel 5mm in front of the sample band required. Following activation by treatment with 10mM EDTA (pH 8.0) for 5 min, 0.5M NaOH for 5min and 6

rinses in distilled H_2O, a piece of DEAE membrane, of equal size to the band required, was inserted into the slit. The gel was then replaced in the tank and run for a further 30min, or until all the sample was visualized on the membrane.
The membrane was then removed from the gel, rinsed in low salt buffer (50mM Tris.HCl (pH 8.0), 0.15M NaCl, 10mM EDTA) and heated for 45min @ 65°C in high salt buffer (50mM Tris HCl (pH 8.0), 1M MaCl, 10mM EDTA). The resulting 400µls solution was then purified by treatment with phenol/chloroform to remove buffers and agarose impurities. Vector and insert were then stored at –20°C until required.

2.3.2.4. Purification of DNA

DNA was purified from agarose impurities and reaction buffers using ice cold phenol:chloroform:isoamylalcohol (25:24:1).
An equal volume of H_2O saturated phenol was added to the 400µl DNA sample, extracted from agarose gel electrophoresis. The mix was then vortexed for 1min to ensure thorough mixing and centrifuged at 10Krpm for 3min at room temperature. The upper (aqueous) phase was removed and the above step repeated with 400µl phenol added to the lower phase (containing the DNA). The sample was vortexed and centrifuged as above and the upper phase discarded. The step was repeated once more, but with the addition of chloroform:isoamylalcohol (24:1). The upper layer was again removed following centrifugation and 0.3M sodium acetate pH5.2 and 2.5vols of 100% ice-cold sterile filtered ethanol added to precipitate the DNA. The samples were incubated at -20°C overnight or, alternatively at -70°C for 1 hour.

2.3.3. Method 2: Construction of S and Tyr^{38}→Cys/Phe α_1-antitrypsin Variants

2.3.3.1. Introduction

S and Tyr^{38}→Cys/Phe variants were constructed in this laboratory via the method of PCR site-directed Mutagenesis. Single amino acid mutations were introduced

into α_1-antitrypsin DNA insert in Sp64T vector using site-directed mutagenesis and samples were then cycle sequenced to allow determination of correct mutational insertion.

2.3.3.2. Introduction of Mutations to α_1-antitrypsin DNA insert

Sp64T vector containing 'normal' M α_1-antitrypsin DNA insert was incubated @37°C for 90min in the presence of restriction enzymes XbaI and HindIII to splice α_1-antitrypsin insert from Sp64T vector.

Following electrophoresis, alongside a standard 1Kb ladder, the presence of a 3Kb vector and 1Kb insert confirmed successful enzyme digestion. α_1-antitrypsin insert was isolated from agarose gel using DEAE membranes (section 2.3.2.3), and DNA purified from reaction buffers and agarose contaminents using QIAGEN PCR clean-up kit according to manufacturers instructions.

2.3.4. PCR site-directed mutagenesis

2.3.4.1. PCR Amplification

New primer pairs were used for all PCR reactions, therefore, each underwent a complete optimisation using the positive control DNA. This included varying primer concentrations, annealing temperature and PCR cycle parameters. $MgCl_2$ concentration is also important for PCR conditions but, as Pfu ploymerase was used during this study, the $MgCl_2$ concentration was already optimised within the supplied enzyme buffer.

2.3.4.2. Annealing Temperature (Ta)

This is the temperature at which the primers anneal to their complimentary sequences on the target DNA. This depends on, and varies with, the melting temperature (Tm) and the base sequence of the primers used:

$$Ta = Tm - 5°C = 2(A\&T) \& 4\ (G\&C) - 5°C$$

Therefore, to assess the effect of changing annealing temperature on the specificity and efficiency of the PCR amplification, annealing temperatures ranging from 55°C to 65°C were analysed for each primer pair.

2.3.4.3. PCR Cycle Parameters

The number of PCR cycles plays a critical role and is dependent on the efficient optimisation of the other parameters. One PCR cycle consists of the initial heat denaturation of the double stranded DNA template into 2 single strands. This is followed by cooling the reaction mixture to a temperature which will allow the primer to anneal to its target sequence. Finally, the temperature is increased to allow the optimal extension of the primer by the DNA polymerase. These 3 steps are repeated leading to an exponential increase in the number of copies of DNA generated. Therefore, for optimal PCR it is essential that DNA be examined in the exponential phase of amplification. For this, PCR was performed using a range of 25-35 PCR cycles.

2.3.4.4. Primer Concentration

The efficiency of amplification can be compromised if the concentration of primer is too low. Therefore, PCR was optimised with primer concentrations from 10-30pmol.

2.3.4.5. PCR Controls

To ensure accuracy and efficiency of PCR, the following controls were used:

-PCR amplification in the absence of cDNA

-PCR amplification in the absence of Pfu polymerase

Fig 2.2 shows the method of site-directed mutagenesis used to produce S and Tyr^{38}→Phe/Cys mutations.

2.3.5. S Variant

The S mutation involves a GAA→GTA substitution at position 264. A 19 base oligonucleotide containing this mutation was produced by OSWEL Laboratories at Southampton University:

*
3′ - TCGTGGACCATTTACTTGA - 5′ * indicates position of mutation

5′ and 3′ Universal primers were also constructed to allow sequencing of double stranded DNA. These primers were designed to contain the PstI restriction enzyme binding site that is engineered into the Sp64T vector at either end of the α_1-antitrypsin DNA insert to allow re-introduction of mutated DNA insert into vector.

	Direction
5′ Uni – CGTGCTGCA$^{\curlyvee}$GACAGTGAATCGACAA$_{(1)}$TG -	→
3′ Uni – GATCCTGCA$_{\curlywedge}$GTCCAGCTCAACCCTTC -	←

$^{\curlyvee}_{\curlywedge}$ indicates PstI restriction enzyme sites.

Step 1: Reaction Conditions

Reagent	Volume (per 50µl total mix)
10X Buffer	5µl
dNTP Mix	1µl
5′ Uni Primer	9µl (17.1pmol)
Primer + S mutation	8µl (17.9pmol)
DNA template (α_1-antitrypsin insert)	0.2µl (~200ng)
Pfu Polymerase	0.5µl (1.5unit)
H_2O	26.3µl

PCR - Based Site - Directed Mutagenesis

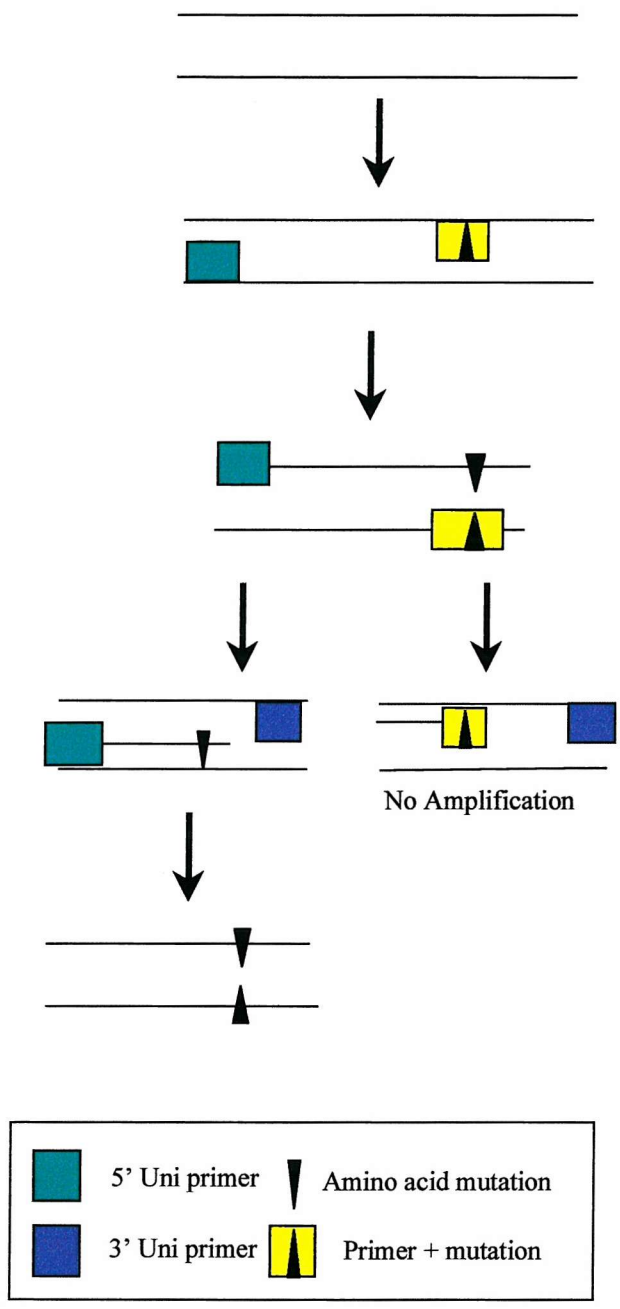


Fig 2.2 Diagrammatic representation of the method of PCR site-directed mutagenesis, as adapted for this study.

Cycling conditions were optimized to; denaturation of template @94°C for 1min, annealing of primer pair @55°C for 1min and elongation @72°C for 90sec. This 1° 5′ insert was then used as a primer to amplify the complimentary 3′ section of the DNA insert.

Step 2: Reaction Conditions

Reagent	Volume (per 50µl total mix)
10X Buffer	5µl
dNTP Mix	1µl
5′ Uni Primer	8µl (19.3pmol)
S mut. Primer from Step 1	3µl
DNA template (α_1-antitrypsin insert)	0.2µl (~200ng)
Pfu Polymerase	0.5µl (1.5unit)
H_2O	32.3µl

Cycling conditions were optimized to denaturation of template @94°C for 1min, annealing of primer pair @55°C for 1min and elongation @72°C for 90sec. Complete, amplified DNA inserts containing mutation were then purified using QIAGEN PCR Clean up Kit according to manufacturer instructions. Insert was then cut using PstI restriction enzyme @37°C for 90min to create restriction sites for re-ligation to PstI cut Sp64T vector.

2.3.6. Tyr38→Phe/Cys Variants

The Tyr38→Phe mutation involves a TAC→TTC substitution at position 38. A 19 base oligonucleotide containing this mutation was produced by OSWEL Laboratories at Southampton University:

```
      *
3′ - AGTCGGATAAGGCGGTCGA - 5′     * indicates position of mutation
```

The Tyr38→Cys mutation involves a TAC→TGC substitution at position 38. A 19 base oligonucleotide containing this mutation was produced by OSWEL Laboratories at Southampton University:

```
      *
3' - AGTCGGATACGGCGGTCGA - 5'     *indicates position of mutation
```

3′ and 5′ Universal primers were used as for the S variant.

Step 1: Reaction Conditions

	Volume (per 50µl total mix)	
Reagent	Cys mutation	Phe mutation
10X Buffer	5µl	5µl
dNTP mix	1µl	1µl
5′ Uni Primer	9µl (17.1pmol)	9µl (17.1pmol)
Primer + Mutation	6µl (18.3pmol)	9µl (16.3pmol)
DNA template	0.2µl	0.2µl (~200ng)
Pfu Polymerase	0.5µl (1.5unit)	0.5µl (1.5 unit)
H_2O	28.3µl	24.7µl

Cycling conditions were as described for the S variant. This 1° 5′ insert was then used as a primer to amplify the complimentary 3′ section of the DNA insert.

Step 2: Reaction Conditions

	Volume (per 50µl total mix)	
Reagent	Cys mutation	Phe mutation
10X Buffer	5µl	5µl
dNTP mix	1µl	1µl
3′ Uni Primer	8µl (19.3pmol)	8µl (19.3pmol)
Primer from Step 1	3µl	3µl
DNA template	0.2µl	0.2µl (~200ng)
Pfu Polymerase	0.5µl (1.5unit)	0.5µl (1.5 unit)
H_2O	32.3µl	32.3µl

Cycling conditions used were as described for the S variant.

Complete, amplified, DNA inserts containing mutation were then purified using QIAGEN PCR Clean up Kit according to manufacturers instructions. Insert was then cut using PstI restriction enzyme @37°C for 90min to create restriction sites for re-ligation to PstI cut Sp64T vector.

2.4. Ligation of Vector and Insert (all variants)

Ligation of Sp64T vector and insert containing mutation was performed using T4 DNA Ligase (Promega).

Prior to ligation, the Sp64T vector was treated with 0.01u/µl calf alkaline phosphatase and incubated @ 37°C for 15min, followed by incubation @55°C for 45min, to remove 5′ phosphate groups and prevent recirculization during ligation.

In the case of P5 V, Q & S variants, pWombat insert DNA containing the mutation was treated with T4 polymerase, (3 units @ 12°C for 40min), to fill in the recessed 3′ terminal created by digestion with SphI restriction enzyme and create a blunt end for successful ligation with the blunt end created by SmaI enzyme on the Sp64T vector (Sambrook *et al.*, 1989).

This step was not required for F and I variants due to the different restriction enzymes used to remove the insert, or for the S and Tyr^{38}→Phe/Cys variants as these were constructed using the Sp64T vector α_1-antitrypsin DNA template.
Ligation was performed at 16°C overnight using 3 units of T4 DNA ligase and varying ratios of vector and insert concentrations. 1:1, 1:2, 1:10 and 1:20 ratios of vector: insert were used to increase the possibility of single insert-vector ligation. Following ligation, complete plasmids were transformed into competent cells to select plasmids that contained insert for mutational analysis.

2.5. Preparation of Competant Cells

Escherichia Coli bacterial strain TG2 was used for the production of competent cells used in this study.
Bacterial cells were cultured in standard LB (Luria-Bertani) medium:

Reagent	g/l
Bacto-tryptone	10
Bacto-yeast extract	5
Sodium Chloride	10

LB medium was adjusted to pH 7 with 5M NaOH and sterilized.
Competent cells were prepared according to the method of Sambrook *et al.*, 1989.

Under sterile conditions, 50µl of TG2 *E.Coli* stock was incubated overnight (~16hrs) in 5ml LB medium. 0.5ml of this culture was then removed and added to 50mls pre-warmed LB. The culture was then incubated @ 37°C with constant shaking and aeration for 1hour or until the optical density of the culture had reached 0.5-0.6 OD units. Total culture was then centrifuged @4°C for 10min @ 4,000rpm to pellet the cells. Supernatant was removed and pellet resuspended in 10mls ice-cold $CaCl_2$ (0.1M). Cells were re-centrifuged under the same conditions

and resuspended in 2mls $CaCl_2$ (0.1M). Cell suspension was aliquoted into sterile ice-cold epindorffs – 200µl per tube – and incubated @4°C overnight.

Following the storage of competent cells @4°C for 24hrs, 10µl of the total 20µl ligation mix of plasmid DNA was added to a 200µl aliquot of competent cells and incubated on ice for 30min. Cells were heat shocked at precisely 42°C for 90sec, followed by incubation on ice for 2min. The Sp64T vector carries the gene for ampicillin resistance. 0.75ml of LB medium was added and the mix incubated for 45min at 37°C to allow time for expression of the ampicillin resistance gene.
To determine cells carrying the required plasmid, 50µl and 200µl aliquots of the competent cell mix were spread onto LB Ampicillin Agar plates. These plates were prepared by the addition of 15g bacto-agar per litre of LB medium prior to sterilization. Following cooling to below 50°C, to prevent loss of antibiotic function, ampicillin was added at a concentration of 50µg/ml, to allow only bacterial cells expressing the Sp64T vector to culture.
Plates were then cultured overnight @37°C and individual colonies then picked and transferred to 5ml LB medium containing ampicillin (LB Amp). These colonies were then incubated overnight at 37°C whilst shaking to optimize colony growth.

2.5.1. Negative and Positive Controls

In order to determine the efficiency and growth of the competent cells, cells were also transformed in the presence of H_2O and commercially produced Sp64T plasmid. The H_2O mix was spread on both LB only and LB Amp plates to ensure the ampicillin added was effective.

2.6. Isolation of Plasmid DNA from Bacterial Cells

DNA was isolated from bacterial cells using the QIAPrep Spin Miniprep Kit (QIAGEN), according to the manufacturers instructions.

1ml of bacterial culture was centrifuged @ 13Krpm to isolate the bacterial cells. The supernatant was then aspirated and the bacterial cells resuspended. Cells were then lysed using NaOH/SDS buffer and this buffer was then neutralised in high salt buffer to precipitate any denatured proteins, chromosomal DNA, cellular debris and SDS. Resulting lysate was centrifuged @ 13Krpm for 10min, leaving DNA in the supernatant which was pipetted onto a 1ml spin column. Supernatant was spun through the column silica gel membrane for 1min @ 13Krpm into a collecting tube, leaving the DNA bound to the column membrane. This membrane was then treated with alkali buffer to wash through any salts and ensure only DNA was bound to the membrane and DNA was eluted from the column using RNAse-free H_2O and centrifugation @ 13Krpm for 1min. This method produced 50µl of DNA per 1ml of bacterial cell culture.

The quality and integrity of isolated DNA was verified by 1% agarose gel electrophoresis with ethidium bromide staining. 1µl of the DNA obtained from the plasmid mini-prep was added to 7µl distilled H_2O and 2µl of 1x tracking dye and loaded into the wells. Agarose gels were run at 80 volts for ~30min in 1xTBE running buffer. Size of DNA samples was verified by co-migration of 1Kb DNA ladder. Records of gels were kept by transillumination of the gel using a UV light box and photography onto Polaroid 667 film through an orange filter.

2.7. Determination of Presence of Mutation

Following ligation, the presence of α_1-antitrypsin DNA insert, with the desired mutation, within the vector was determined by specific restriction enzyme analysis.

DNA was incubated in the presence of PstI enzyme for 90min @37°C and a small quantity of the sample – 1µl from 10µl mix, was added to 7µl H_2O and 2µl of 1x tracking dye and loaded on a 1% agarose gel. Following electrophoresis alongside a standard 1Kb ladder, the presence of a ~ 1Kb sized fragment indicated the presence of the α_1-antitrypsin insert within the vector.

To ensure correct orientation of the insert within the vector, F, I, and P5 variants V, Q and S, DNA was incubated in the presence of EcoRV (90min @ 37°C), and SmaI (90min @ 25°C), enzymes. Following electrophoresis alongside a standard 1Kb ladder, the presence of an 800bp fragment indicated the correct orientation of the insert within the vector. S and Tyr^{38}→Cys/Phe variant DNA was incubated in the presence of BamHI @ 37°C for 90min. The presence of a separate insert band following electrophoresis indicated the correct orientation of insert within the vector.

At least two individual clones for each variant were then sequenced to ensure the desired mutation was in place. Again, two methods were utilized depending on the mutation present. F, I and P5 S, V and Q variants were sequenced via PCR (Polymerase Chain Reaction) using the 'Thermosequenase' system (Amersham) and an automated thermocycler, followed by manual sequence analysis using audioradiography of sequencing gels (Method 1).

S and Tyr^{38}→Cys/Phe variants were also sequenced via PCR using an automated thermocycler, followed by automated sequence analysis using the ABI377 automated sequencer and ABI Prism analysis program for the Macintosh (Method 2).

2.7.1. Method 1: Sequencing of F, I and P5 S, V and Q Variants

PCR was performed using the 'Thermosequenase' system (Amersham) according to manufacturers instructions. 2µl of cDNA template was added a final concentration of 18pmol of each upstream or downstream primer (sequencing primers were provided by Dr D Lomas at Cambridge University). For each individual clone, 4 PCR reactions were performed using [^{33}P] labelled ddGTP/ATP/CTP/UTP termination nucleotides. 1unit of Pfu DNA polymerase was added to each mix and made to a final volume of 10µl.

The initial cycling conditions consisted of 1min denaturation of template DNA @ 94°C, primer annealing of 1min @ 55°C and 2min @ 72°C to allow extension of the primers from the 3′ end by the thermostable action of DNA polymerase, for a total of 30 cycles.

To prevent evaporation, an overlay of sterile mineral oil was included in each tube. All PCR reactions were performed on an automated thermocycler (HYBAID).

2.7.1.1. Confirmation of Variant α_1-antitrypsin PCR product by manual sequencing

PCR products were examined via sequencing gel analysis. G, A, T and C samples for each individual clone were added directly to adjacent wells on a polyacrylamide sequencing gel which was then ran at a constant current of 40mA for various time periods depending on relation of sequencing primer to mutation. Gels were then fixed (10% Methanol, 10% Acetic Acid), dried and autoradiographed @ -70°C overnight using GRI X-ray film.

2.7.1.2. Analysis of Sequencing Results

Gels were developed using an X-OMAT film developer and DNA sequence determined by reading GATC sequence from autoradiograph.

2.7.2. Method 2: Sequencing of S and $Tyr^{38} \rightarrow$Cys/Phe Variants

PCR of samples for sequencing was performed according to instructions for 'Bigdye cycle sequencing' (Perkin Elmer), used with the ABI377 automated sequencer:

Reagent	Volume (per 10µl total mix)
DNA	~ 200ng (0.2µl)
Terminator Ready Reaction mix	4µl
Primer	1.6pmol
Deionized H2O	9.5µl

Primer used for S variant sequencing was produced by OSWEL Laboratories at Southampton University:

S Variant sequencing primer:

5' – AAGAAGCTGTCCAGCTGG –

Primer used for sequencing of Tyr38→Phe/Cys variants was the same primer used for sequencing the I variant and provided by Dr D Lomas at Cambridge University.

Samples were then spun briefly and incubated under the following cycle sequencing conditions using an automated thermocycler: denaturation @ 96°C for 10sec, primer annealing @ 50°C for 5 sec and elongation @ 72°C for 4min, for a total of 25 cycles.

2.7.2.1.Confirmation of Variant α_1-antitrypsin PCR product by direct sequencing

Direct sequencing of PCR product was performed by James Willson and Dr. Mark Dixon at the University of Southampton. Alignment of normal and variant PCR product allowed the correct insertion of mutation to be identified.

2.7.2.2. Purification of PCR Product DNA

PCR product DNA was purified using ethanol/sodium acetate precipitation. 2.0µl of sodium acetate (pH4.6) and 50µl of 95% ethanol were added to each sequencing reaction. Samples were then vortexed briefly and incubated at room temperature for 30min to precipitate the extension products. Samples were then spun @13Krpm for 20min and the supernatants removed. Pellets were rinsed with 250µl of 95% ethanol, vortexed and spun @13Krpm for 5min. Supernatants were removed and the pellets dried by heating @90°C for 1min. Sample pellets were stored @ -20°C until required for sequencing.

2.7.2.3. Sequencing of Purified DNA Fragment

Sequencing was performed by James Willson and Dr Mark Dixon at the University of Southampton using a ABI377 automated sequencer.

2.7.2.4. Analysis of Sequencing Results

Data was obtained from the sequence report from the ABI377 sequencer generated using forward primers, and analysis of each sample followed using ABI prism software for the Macintosh. This allows the visualisation of each peak and the corresponding base assigned for this peak.

At least 2 distinct clones for each mutation were identified following comparison with wild-type M α_1-antitrypsin. DNA was stored at -20°C until required for transcription.

2.8. Preparation of Variant DNA for Transcription

DNA was linearized by incubation for 90min @ 37°C in the presence of EcoR1 restriction enzyme. This enzyme cuts the template just 5′ of the α_1-antitrypsin DNA insert.

2.8.1. Purification of DNA

Linearized DNA was cleaned for transcription using phenol:chloroform:isoamylalcohol (25:24:1), to remove any impurities, reaction buffers etc which may interfere with the transcription process.

The 50µl DNA sample, following linearisation, was diluted to 100µl by the addition of RNase-free H_2O. 100µl of H_2O saturated phenol was added, vortexed for 1min to ensure thorough mixing and then centrifuged at 10Krpm for 3min at room temperature. The upper (aqueous) phase was removed and the above step repeated with 100µl phenol added to the lower phase (containing the DNA). The sample was vortexed and centrifuged as above and the upper phase discarded. The step was repeated once more, but with the addition of chloroform:isoamylalcohol (24:1). The upper layer was again removed following centrifugation and 0.3M sodium acetate pH5.2 and 2.5vols of 100% ice-cold sterile filtered ethanol added to precipitate the DNA. The samples were incubated at -20°C overnight or, alternatively at -70°C for 1 hour.

Following precipitation, the suspension was centrifuged at 10,000g av for 10min @ 4°C forming a visible pellet. The supernatant was discarded and the pellet resuspended in 200µl of 70% ethanol. The sample was recentrifuged at 10,000g av @ 4°C and the supernatant discarded. The resulting DNA pellet was freeze dried for 3 minutes and redissolved in 10µl of RNase-free H_2O. The DNA was stored at –20°C until required for transcription.

2.9. Transcription and Translation of Variant Proteins

2.9.1. *In Vitro* Transcription System

50µl transcription reactions were performed on ~2µg EcoRI linearised DNA in the presence of sp6 polymerase, using the Promega Ribomax Transcription System, according to manufacturers instructions.

DNA was added to 5µl sp6 polymerase (as supplied), 0.57mM GTP, 4mM UTP, 4mM ATP and 4mM CTP. 3mM $m^7G(5')ppp(5')G$ cap structure (New England Biolabs), was also included to produce capped RNA suitable for expression in *Xenopus* oocytes. The mix was the incubated @ 30°C for 3 hours. 8units of DNase was subsequently added to disintegrate the DNA template and the sample incubated for a further 15min @ 37°C.

RNA was extracted using the QIAGEN RNeasy Total RNA kit. Transcription mix was treated with lysis buffer and then centrifuged through a spin column membrane. The membrane was then washed with sterile ethanol to alter binding conditions and RNA was eluted using 70µl RNase-free H_2O and centrifugation @ 13Krpm for 1min. RNA samples were stored in sterile, screw top cryogenic vials in liquid nitrogen until required for translation *in vitro* or injection into *Xenopus* oocytes.

2.9.2. *In Vitro* Translation System

To ensure that transcribed RNA was readily translatable to a correctly sized α_1-antitrypsin protein, RNA was translated *in vitro* in a cell-free Nuclease-treated Rabbit Reticulocyte Lysate System (Promega) according to manufacturers instructions. 1µl RNA was incubated in a translation mix composed of 70% reticulocyte lysate and 1µl RNase inhibitor. 0.2µl amino acid mix per sample was added and substituted with 6µCi ^{35}[S] methionine (per sample) to allow

radiolabelling and identification of translated protein. Translation mix was incubated @ 30°C for 90min and reaction stopped by incubation on ice.

2.10. Analysis of Translated Protein

Translation products were assayed by TCA precipitation to determine % amino acid incorporation. 2µl of translation mix was added to 98µl of 1M NaOH/2%H_2O_2 and incubated @ 37°C for 10min. 900µl of 25% TCA/ 2% Casamino acids was then added and sample left on ice for 30min to precipitate the translation product. The entire mix was applied to a Whatman 3mm filter attached to a Buchner Vacuum flask and the filter washed x2 with ice-cold 5% TCA to remove any smaller protein products and incubation buffers from the membrane. Filter discs were then dried and scintillation counted using OPTIPHASE scintillant (LKB) and a Beckman Scintillation counter, to determine % amino acid incorporation and, therefore, quantity of protein produced.

Protein products were also analysed by SDS-PAGE (PolyAcrylamide Gel Electrophoresis – method developed by Laemelli *et al.*, 1970), to determine the size of the protein product compared to molecular weight markers and a known sample of translated α_1-antitrypsin protein.

2.10.1. Preparation of SDS-PAGE gels.

A 3% polyacrylamide stacking gel and 10% polyacrylamide separating gel was prepared and cast in a H2 Horizontal electrophoresis system (Anachem). The 10% separating gel was composed of:

Reagent	Volume per Gel
Distilled H_2O	10.45ml
30% (w/v) bis-acrylamide	6.7ml
3M Tris.HCl (pH8.9)	2.5ml
10% (w/v) SDS	200µl
10% (w/v) AMPS	135µl
TEMED	25µl

TEMED and Ammonium Persulphate (AMPS), were added last and the gel left to polymerize at room temperature for 30min. An overlay of H_2O saturated butanol was added to the separating gel during polymerization to prevent oxidation of the gel.

The 3% stacking gel was composed of:

Reagent	Volume per Gel
Distilled H_2O	7.6ml
30% (w/v) bis-acrylamide	1.0ml
0.5M Tris.HCl (pH6.7)	1.25ml
10% (w/v) SDS	100µl
10% (w/v) AMPS	65µl
TEMED	12µl

Again, the TEMED and AMPS were added last, a 10 well comb inserted and the gel left to polymerize at room temperature for 30min.

Translated protein was diluted 1 in 5 in 10µl distilled H_2O and Maizel sample buffer (200µl 2-βMercaptoethanol, 0.2g SDS, 4ml Glycerol in 10mls in H_2O). A 1 in 5 dilution of molecular weight standards (Amersham), was prepared with distilled H_2O and Maizel sample buffer. Samples were heated for 2min @ 90°C to ensure protein degradation and solubilization, and then loaded into the wells. Gels

were electrophoresed at room temperature at a constant current of 28mA for ~90min, or until the tracking dye front reached the end of the gel.

2.10.2. Detection of Protein

Following separation, gels were fixed in 25% Acetic Acid, 10% Isopropanol in distilled H_2O for ~ 30min, followed by immersion in 'Amplify' (Amersham) fluorography reagent for a further 30min. Gels were then dried for 90min on a GRI Rapidry gel drying system. Gels were developed using the X-OMAT film developer following 4 hour exposure at -70°C using GRI X-ray film.

2.11. *In Vivo* Analysis of Variant α_1-antitrypsin Protein

2.11.1. Introduction

Following confirmation via TCA precipitation and SDS-PAGE analysis that translation of variant α_1-antitrypsin mRNA *in vitro* resulted in the formation of the expected sized protein, mRNA was injected into *Xenopus* oocytes to study the effects of these mutations of α_1-antitrypsin on its processing and secretion using an *in vivo* expression system.

2.11.2. Biosynthesis and Secretion of α_1-antitrypsin in *Xenopus* Oocytes

The large size (>1.2mm) and ample store of molecular components have made the amphibian oocyte a popular choice for the assay of biological materials after microinjection. Fig 2.3 shows healthy, stage V-VI oocytes used for microinjection. The *Xenopus* oocyte has clear advantages over cell-free systems in certain circumstances. Unlike the cell-free systems, the oocyte can correctly carry out the post-translational modification of many foreign proteins such as precursor processing, phosphorylation and glycosylation. The oocyte has, thus, provided an excellent vehicle for investigation of mRNA transcripts, DNA transcription, RNA splicing and protein segregation and secretion. (Wu & Foreman, 1990, Verbanac & Heath, 1986, Seyama *et al.*, 1991, Mahadeva *et al.*, 1998). Injected mRNAs, as

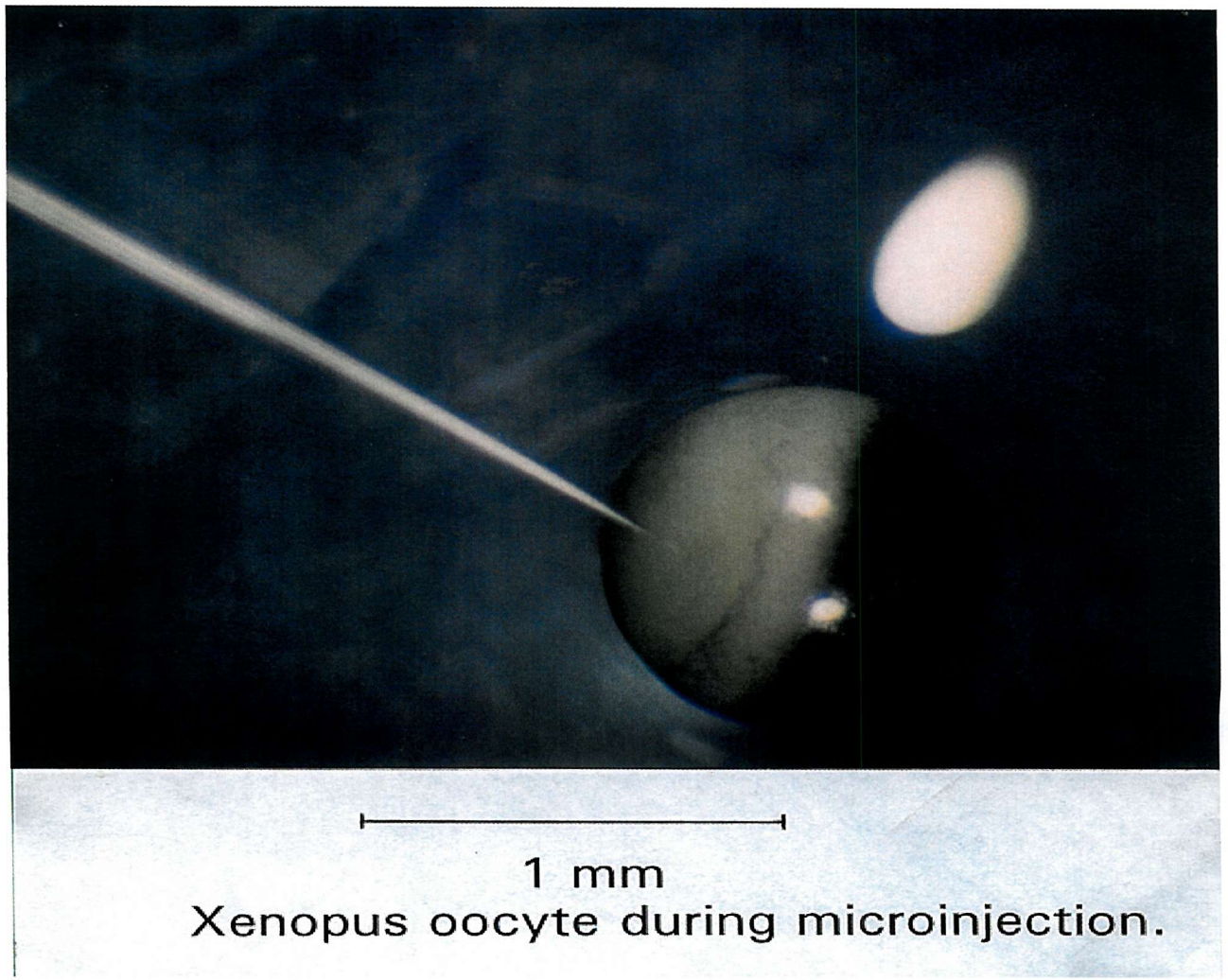


Figure 2.3: A healthy stage 5-6 *Xenopus leavis* oocyte during microinjection of RNA.

used in this study, translate very efficiently in *Xenopus* oocytes, a technique first demonstrated by John Gurdon and colleagues when rabbit globins were synthesised after the microinjection of rabbit reticulocyte 9S RNA (Gurdon *et al*, 1971).

The use of *Xenopus* oocytes to study and compare the secretion of wild-type and secretory deficient variants of α_1-antitrypsin has shown that the protein is not secreted as efficiently as from other cell systems including transfected mammalian cells, (e.g. the human murine embryonic fibroblast cell line NIH313 - Wu & Foreman, 1990), COS cells (McCracken *et al.*, 1989) and yeast, (e.g. *Saccharomyces cerevisiae* - Kang *et al.*, 1997). However, while absolute amounts of the protein secreted from these cell types may differ, it is now apparent that the qualitative effect of the various point mutations on the secretory properties of the resultant α_1-antitrypsin protein produced is constant regardless of the cell type used for expression.

2.11.3. Preparation and microinjection of Xenopus oocytes

A female *Xenopus laevis* toad was anaesthetized by submersion in a 0.1% Tricaine (ethyl-*m*-aminobenzoate) solution for approximately 45min. When anaesthesia was confirmed the toad was placed ventral side up on a dissecting board and the ovary was exposed by a 1cm incision on the posterior ventral side through the skin and abdominal wall. Lobes of oocytes were removed using forceps and immersed in Standard Oocyte Saline (SOS – 100mM NaCl, 2mM KCl, 1.7mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES – pH7.6 with NaOH). The incision was sutured and the toad allowed to recover in distilled H_2O. Single oocytes were stripped from the lobes and healthy, mature stage V oocytes (diameter $\approx$1.2mm) were selected for microinjection. The microinjection of *Xenopus* oocytes was as described by Coleman in 1984.

2.11.4. Secretion from *Xenopus* Oocytes

Oocytes were injected with ≈20nl mRNA into the cytoplasmic (vegetal) pole to avoid possible damage to cell components contained within the animal pole. Control oocytes were either non-injected or injected with RNase-free H_2O, with identical results. Oocytes were incubated overnight (~16hours), at 17-20^{O}C in SOS + antibiotics (0.1μM penicillin and streptamycin). The oocytes that were viable after this period (~85%) were then incubated for 7hrs in SOS supplemented with 200μCi.ml^{-1} L-[^{35}S] methionine. 96 well cell culture plates were used for incubation and 3 oocytes were placed in each well to limit the toxic effects of cell contents from burst oocytes on healthy oocytes. 25μl of ^{35}[S] methionine supplemented SOS was added to each well to keep media volume to a minimum. At the end of this period the rabiolabelling medium was replaced with an equal volume of unradiolabelled L-methionine (25mM) and the incubation continued overnight prior to immunoprecipitation.

2.11.5. Immunoprecipitation Procedure

Fig 2.4 shows the immunoprecipitation procedure for the analysis of all variant α_1-antitrypsin protein expressed within, and secreted by, *Xenopus* oocytes as described by Coleman in 1984.

Incubation media was removed from wells containing healthy oocytes by pipetting. Media from each injected variant was pooled and the sample left on ice until required. Oocytes from the relevant wells were removed and homogenised in buffer containing 0.1M NaCl, 1% Triton X-100, 1mM PMSF, 20mM Tris.HCl (pH7.6), using a 1ml glass Dounce homogenizer. Typically, 21 oocytes were homogenized per variant in 420μl buffer (20μl per oocyte). The protein from both incubation media (s) and homogenized oocyte extract (oo) was then immunoprecipitated by addition of 2μl of 10mg/ml rabbit anti-human α_1-antitrypsin (DAKO) in immunoprecipitation buffer (0.1M KCL, 5mM $MgCl_2$, 1%

Triton X-100, 0.5% SDS, 1% sodium deoxycholate, 1mM PMSF, 0.1M Tris.HCl (pH8.2)). Samples were then incubated for 90min @ 4°C.

Antibody bound α_1-antitrypsin protein was then conjugated to PANSORBIN bacterial cells (Calbiochem). 25µl (10% w/v suspention) of cells were added to each sample and incubated @ 4°C for 2 hours with constant shaking. The conjugated protein was then pelleted by centrifugation for 2 min at 13Krpm @ 4°C and the pellet washed using immunoprecipitation buffer to remove any unbound protein and antibody. The protein was extracted from the antibody and cells by resuspension of the pellet in Maizel sample buffer followed by incubation for 1 min @ 95°C. Samples were then centrifuged, the supernatant containing protein was removed and prepared for SDS-PAGE analysis and the pellet discarded. 2µl of bromophenol blue dye was added and each sample (~30µl) loaded directly onto a 10% SDS-PAGE gel (as described in section 2.10.1), and run at a constant current of 28mA. Gels were fixed, treated with 'Amplify', dried and autoradiographed to expose protein bands. The co-migration of molecular weight markers allowed size determination of the protein products from both the oocyte incubation media (secreted protein) and oocyte extract (retained protein). Quantitation of radiolabelled proteins was performed by scintillation counting of excised gel bands resuspended in OPTIPHASE scintillant (LKB) and counts were expressed as a percentage of the total immunoprecipitated protein material.

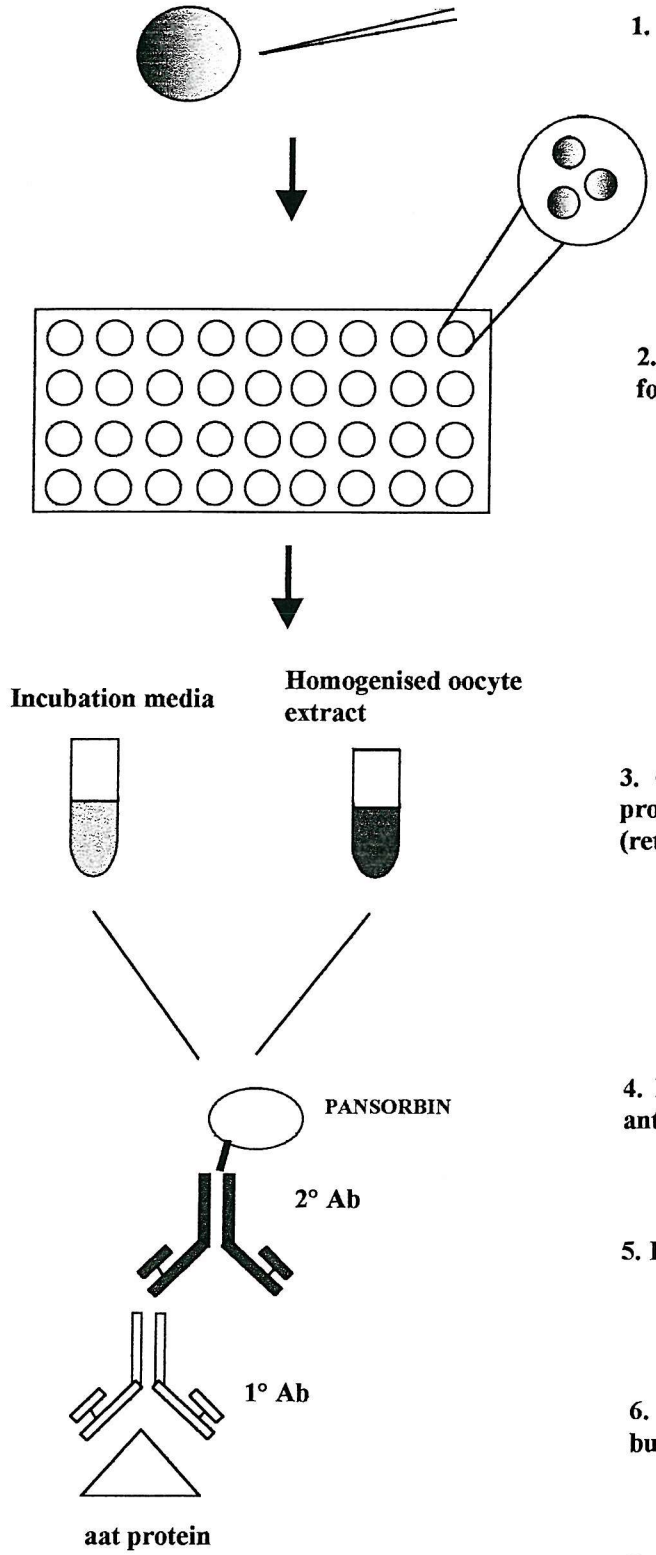


Fig 2.4 Immunoprecipitation procedure for *Xenopus* oocytes following injection with α_1-antitrypsin mRNA.

2.12. Microsomal Membrane Preparations

2.12.1. Introduction

The *in vivo Xenopus* oocyte system is a useful tool for studying the effects of mutations of α_1-antitrypsin on its secretion. In this laboratory the further study of the processing of the α_1-antitrypsin protein, *in vitro* and *in ovo*, was achieved using microsomal membranes. Microsomes are small vesicles of ER used in this study to achieve a higher level of processing of the α_1-antitrypsin protein than produced using an *in vitro* cell-free system.

2.12.2. Isolation and Preparation of canine microsomal membranes

Canine pancreas is a major source of actively translocating microsomes (Walter & Blobel, 1983). As the pancreas actively secretes digestive enzymes the procedure of microsome isolation was carried out as fast as possible and all the steps carried out at $4^{O}C$ to minimize degradation. Canine microsomal membranes (cmm) were prepared as described by Walter and Blobel (1983). Tissue was removed and immediately rinsed and immersed in ice cold Buffer A (250mM sucrose, 50mM TEA, 50mM KOAc, 6mM $Mg(OAc)_2$, 1mM EDTA, 1mM DTT, 0.5mM PMSF). The gland was then minced, tissue was weighed and 4 millilitres of Buffer A were added per gram of tissue. The tissue was extensively homogenised in a motor driven Potter-Elvehiem homgenizer and the homogenate was then centrifiged for 10min @ 1000g av. Floating fatty material was removed by aspiration and the supernatant was recentrifuged for 10 min @ 10,000g av. The supernatant was decanted from the pellet and rough microsomes were collected by centrifugation of supernatant for 2.5hr @ 140,000g av. using a Beckman SW41Ti rotor, through a 1.3M sucrose in Buffer A cushion. The resulting pellets were resuspended by manual homogenization in a glass Dounce homogenizer in Buffer B (250mM sucrose, 50mM TEA, 1mM DTT) to a concentration of ~$50A_{260}$ units/ml (determined in a 1% SDS solution). All concentrations of microsomes used in this study are related to the original crude rough microsome preparation, which has an

OD measurement of 541A_{260} units/ml. This study refers to 1µl of this suspension as 1 unit of cmm. Microsomal extract was stored at -70°C in 15µl aliquots to avoid freeze-thaw cycles which may disrupt microsomal integrity.

2.12.3. *In Vitro* Translocation

2µl (2 units) of microsomal membranes was added to a reticulocyte lysate translation mix in the presence of α_1-antitrypsin mRNA as described in section 2.12.3. The concentration of the lysate in the mix was reduced from 70% to 60% to allow for microsomal incorporation, but this did not affect translational activity. Samples were incubated @ 30°C for 2 hours.

Processed protein product was then analyzed using SDS-PAGE. Samples were prepared and loaded onto gel as described in section 2.10.1. PAGE gel was run at a constant current of 28mA for 90min or until the dye front reached the bottom of the gel. Gels were fixed, 'Amplified', dried and developed, following 4 hour exposure @ -70°C, using GRI X-ray film.

2.13. Injection of α_1-antitrypsin protein-loaded microsomes into *Xenopus* oocytes

2.13.1. Introduction

Translocation of microsomes in an *in vitro* Reticulocyte system resulted in the production of a partially processed α_1-antitrypsin protein. The protein produced in the presence of microsomes was identified and assessed and this protein then injected into *Xenopus* oocytes using the same method as mRNA injection. This method allows more detailed study of α_1-antitrypsin protein using both *in vitro* and *in vivo* systems.

2.13.2. Preparation of α_1-antitrypsin loaded microsomes for oocyte injection

α_1-antitrypsin mRNA was translated in the presence of microsomal membranes as detailed in section 2.12.3. Membrane bound ribosomes were then stripped from the microsomes by incubation for 15min on ice in the presence of 25mM EDTA. The purpose of this is to decrease endogenous mRNA activity by removing the bulk of membrane-bound ribosomes and mRNA as well as many adsorbed proteins or peripheral membrane proteins from rough microsomes (Walter & Blobel, 1983). The microsomal fraction, containing partially processed protein, was isolated from the other components of the translation system via ultracentrifugation through a 0.5M sucrose cushion in buffer B at 34Krpm for 1hour 45min.

The supernatant was then removed by aspiration and the pellet containing the microsomes resuspended in 4µl buffer B and stored on ice prior to oocyte injection.

2.13.3. Proteinase K Treatment of microsomal membranes

Membranes were treated with either 100µg/ml Proteinase K or Proteinase K + 1% Triton X-100 @ 4°C for 1 hour following *in vitro* transcription, to determine whether the glycosylated protein translocated within this system was sequestered within the vesicles.

2.13.4. Effects of EDTA-treatment on α_1-antitrypsin processing *in vitro*

To ensure the EDTA treatment of microsomal membranes did not decrease the processing efficiency of microsomal membranes *in vitro*, translation reactions were performed as described in section 2.12.3, with the addition of 25mM EDTA to the reaction mix. Samples were analyzed via SDS-PAGE to determine the effects of EDTA treatment on α_1-antitrypsin processing in the presence of microsomal membranes.

2.13.5. Nuclease-treatment of microsomal membranes

Rough microsomes can also be treated with staphylococcal nuclease to deplete them of endogenous mRNA activity (Pelham & Jackson, 1976). 20µl of 100mM $CaCl_2$ was added to 1ml microsomal membranes and incubated at 23°C for 10min. The reaction was stopped by addition of 40µl of 100mM EGTA (pH7.5). Membranes were then pelleted at 100,000g av. for 30min @4°C and the supernatant removed. Pellets were then re-suspended in 1ml buffer B and stored at -70°C until required for oocyte injection.

2.13.6. Gradient Native PAGE analysis of microsomal membranes.

To examine the contents of the microsomal membranes containing partially processed α_1-antitrypsin protein, microsomes, following retrieval via sucrose gradient centrifugation from translation mix, were loaded directly onto a Gradient Native PAGE gel (method adapted from Fischer and Lerman, 1979) followed by Western blotting.

2.14. Western Blotting for the Detection of α_1-antitrypsin Protein

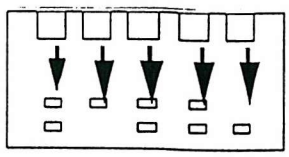
2.14.1. Introduction

Immunoblotting is used in the detection of proteins by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins by polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. Fig 2.5 illustrates the principle of Western blot analysis.

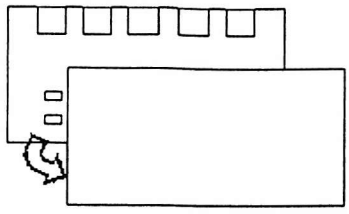
2.14.2. Gradient Native PAGE

Gradient (7-15% acrylamide) Native slab gels were used to allow refined separation of proteins. The separating gel was composed of a 7-15% acrylamide

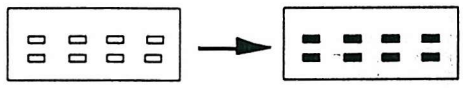
1. Seperate protein samples by SDS-PAGE electrophoresis



2. Transfer proteins to membrane



3. Block non-specific sites



4. Incubate the blocked membrane in primary antibody specific for antigen of interest

5. Wash membrane

6. Incubate the membrane in HRP conjugated secondary antibody specific for primary antibody

7. Wash the membrane

8. Incubate the membrane in chemiluminscent substrate

9. Expose the membrane to x-ray film

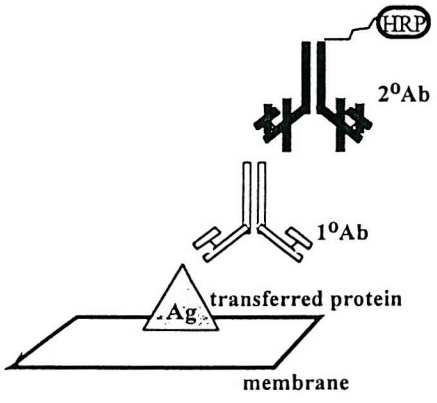


Fig 2.5 Diagrammatic illustration of the principle of Western Blotting

gradient created using a dual solution gradient gel maker. The 7% and 15% gel solutions were composed as follows:

Reagent	7%	15%
Distilled H_2O	3.5mls	1.75mls
30% (w/v) bis-acrylamide	1.75mls	3.5mls
1.5M Tris.HCl (pH8.9)	3.5mls	1.75mls
TWEEN 20	3.5µl	3.5µl
Sucrose	-	0.7g
TEMED	5.25µl	5.25µl
10% (w/v) AMPS	26.25µl	26.25µl

The high and low % acrylamide solutions were then added to the gradient gel maker (Fig 2.6):

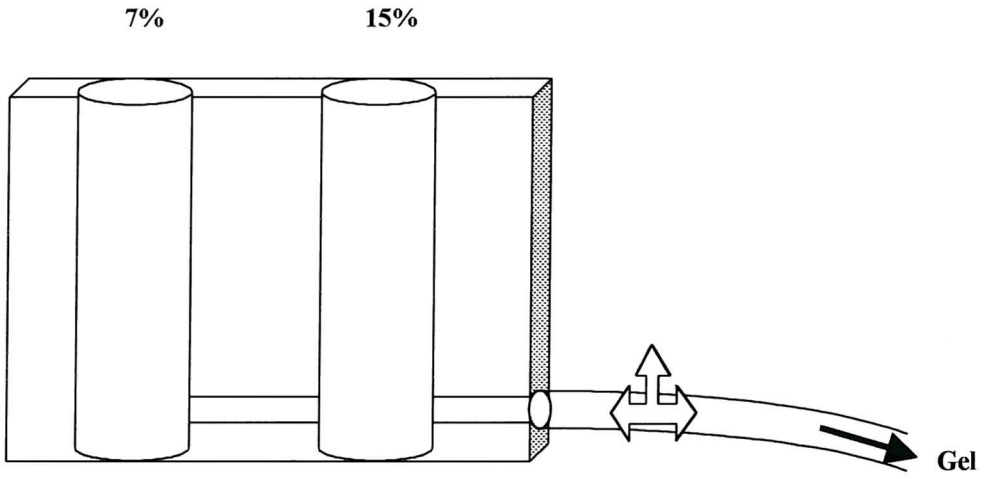


Fig 2.6 Gradient maker used for the production of 15→7% Native PAGE gels. Solutions were gradually mixed together to achieve an even gradient for increased separation of proteins.

AMPS and TEMED were added to both solutions and continuous mixing resulted in a gel with a 15→7% gradient. The decrease in acrylamide concentration and, thus, the accuracy of the gradient maker, was verified by adding blue dye to the 15% solution. Mixing resulted in a gel of decreasing intensity of dye relating to decreasing intensity of acrylamide.

The gel was left to polymerize at room temperature for 45min. A H_2O saturated butanol layer covered the gel during polymerization to prevent oxidation of the gel.

The 3% stacking gel was composed of:

Reagent	Volume per gel
Distilled H_2O	6.4ml
30% (w/v) bis-acrylamide	1.6ml
0.47M Tris/H_3PO_4 (pH6.9)	1.0ml
TWEEN 20	10µl
TEMED	15µl
10% AMPS	100µl

AMPS and TEMED were added last, a comb inserted and the gel left to polymerize at room temperature for 30 min.

Each sample was diluted 1:1 with Native Sample buffer (2ml Glycerol, 1% TWEEN 20, diluted to 10ml final volume in 1.5M Tris), made up to a final volume of 40µl and loaded directly onto the gel. 10µl of biotinylated molecular weight markers (New England Biolabs), were loaded onto the gel to determine molecular weight of protein samples. Gels were run at a constant current of 28mA for 90min or until the tracking dye front reached the bottom of the gel.

2.14.3. Blotting Transfer of Protein

Following electrophoresis, gels were rinsed in Towbins buffer (20% Methanol, 77mM Glycine, 100mM Tris) for 20 min to shrink the gel. The proteins were then transferred to nitrocellulose membrane using the method described by Towbin *et al.*, in 1979.

Nitrocellulose membrane and Whatman filter paper was cut to the approxiamate size of the gel and pre-soaked in Towbins buffer.
The blotting 'sandwich' was assembled in the following order (Fig 2.7):

- 1 piece filter paper
- Nitrocellulose membrane
- Gel
- 1 piece filter paper

and enclosed within a sponge lined plastic cage to allow the 'sandwich' to be fixed within the blotting apparatus. Air bubbles were removed and the 'sandwich' inserted into the blotting apparatus filled with Towbins buffer. Blotting transfer was performed at a constant current of 400mA for 2 hours.

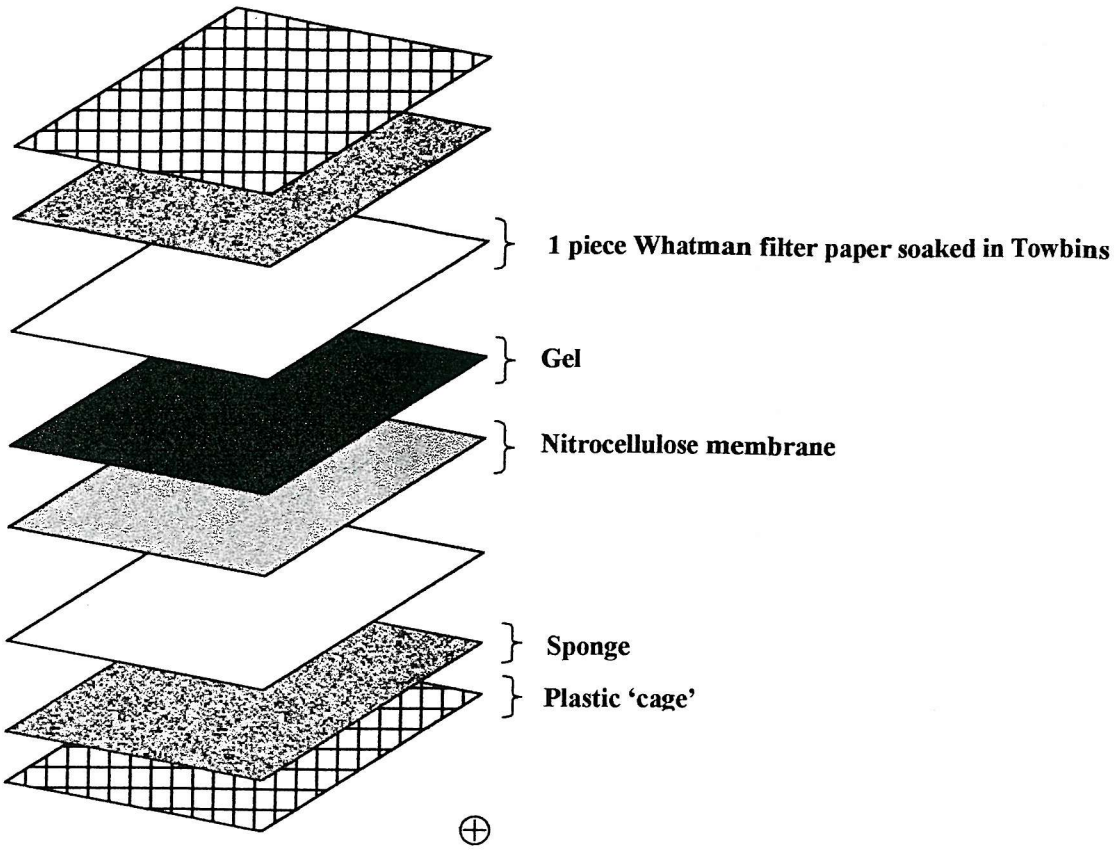


Fig 2.7 Diagram illustrating the order for assembly of filter paper, gel and membrane for Western Blotting transfer of proteins as used in this study (based on the method of Towbin *et al.*, 1979). Proteins were transferred from PAGE gel to nitrocellulose membrane by application of an electrical current across the blotting 'sandwich' shown above.

2.14.4. Detection of Protein

Following protein transfer, the membrane was blocked in 5% milk in 0.1% TWEEN/PBS (136mM NaCl, 2.7mM KCl, 1.4mM KH_2PO_4, 10mM Na_2HPO_4, pH7), overnight @ 4°C. The membrane was then treated with monoclonal rabbit anti-human α_1-antitrypsin antibody (DAKO), added at a concentration of 5µg/ml in 0.1% TWEEN/PBS, (antibody stock concentration – 10mg/ml, final concentration equivalent to 1:2000 dilution), and the membrane was incubated for 2 hours, shaking at room temperature. The membrane was then washed 5 times in 0.1% TWEEN/PBS for 10min and the membrane treated with anti-rabbit IgG

linked to HRP (Amersham) at a concentration of 5.5µg/ml, (stock concentration 11mg/ml, final concentration equivalent to 1:2000), for 2 hours, shaking at room temperature.

The membrane was washed as before and developed using the ECL system (Amersham) and developed on GRI X-ray film after exposure for 5sec-1min, dependent on protein concentration and band intensity.

2.14.5. Western Blotting Controls

To ensure the accuracy and efficiency of Western Blotting, a number of controls were utilized, including the incubation of 2° antibody in the absence of 1° antibody. In addition, by including molecular weight markers, the size of the band detected could be estimated and compared with the published size of each α_1-antitrypsin variant protein.

2.15. Microinjection of Microsomal Fraction into *Xenopus* Oocytes

Oocytes were obtained and prepared for injection as described in section. 2.11 and injected with the retrieved microsomal fraction from the *in vitro* translation system. Following injection of ~50nl of microsomal extract, oocytes were incubated overnight (~16 hours) @ 17-20°C in SOS containing antibiotics. Incubation media was then removed and oocytes homogenized. Both fractions were then immunoprecipitated and protein analyzed using SDS-PAGE as described in section 2.11.5.

2.15.1. RNase treatment of Microsomal Membranes

To ensure that protein isolated from the oocytes injected with microsomes was produced within the microsomes pre-injection and not from unprocessed mRNA present on the external surface of the microsomes, the membranes were incubated

with RNase A (1μg/ml) at room temperature for 10min pre-injection. Oocytes were then injected and protein analyzed as described in section 2.11.

2.16. Isolation of microsomal membranes from *Xenopus* Ooctyes Injected with Variant α_1-antitrypsin mRNA

2.16.1. Introduction

In order to examine the retained fraction of α_1-antitrypsin protein within the oocytes, the secretory pathway was isolated following mRNA injection and the protein contents analyzed using NATIVE-PAGE.

2.16.2. Microinjection of *Xenopus* Oocytes

Oocytes were obtained and prepared for injection as described in section 2.11, and injected with ~20nl α_1-antitrypsin mRNA per oocyte. Oocytes were then incubated in fresh SOS containing antibiotics overnight (~16 hours), at 17-20°C and oocytes viable after this period were transferred to 96 well cell culture plates, 3 oocytes per well to limit the toxic effects of oocyte contents from burst cells on healthy cells. 25μl of SOS was added to each well and incubation continued for 8 hours. After this time point, media was replaced and incubation continued overnight (~16 hours).

2.16.3. Isolation of Oocyte Secretory Fraction

The secretory system of *Xenopus* oocytes was isolated from normal and variant RNA injected oocytes by the process of sucrose density gradient centrifugation (In *Transcription and Translation: A practical approach.* Hames. B.D., and Higgins. J., IRL Press, 1984).

Following incubation, media from wells containing healthy oocytes, 39 oocytes were used in total, was removed and stored on ice until required. The oocytes from these wells were then homogenised in T buffer (50mM NaCl, 10mM MgAc,

20mM Tris.HCl (pH7.6)), containing 10% sucrose. The homogenate was then layered onto a step gradient consisting of equal volumes (6mls) of 20% (w/v) sucrose in T buffer above 50% (w/v) sucrose in T buffer. Samples were then centrifuged at 11Krpm in a Beckman SW41Ti swing out rotor. Following centrifugation, the layer at the 20%-50% interface (containing the secretory membraneous elements of the oocytes), was carefully removed using a glass pasteur pipette and the contents re-centrifuged at 10Krpm for 10min @ 4°C to pellet the membranes.

The supernatant was removed by pipetting and the pellet resuspended in Native-PAGE sample buffer. The protein contents of the membranes were then analysed by Native Gradient PAGE and Western Blotting as described in section. 2.14, to specifically identify any α_1-antitrypsin protein.

2.17. Peptide Analysis

2.17.1. Introduction

The effects of temperature and addition of peptide on the polymerisation of α_1-antitrypsin was examined in this study. Loop-sheet polymerization has been shown to be a temperature and concentration dependent process *in vitro* and the effects of the addition of synthetic loop peptides have also been studied previously (Lomas *et al.*, 1992, Schulze *et al.*, 1990, Mast *et al.*, 1992). In this study the effects of increasing temperature and increasing incubation time on the polymerisation of α_1-antitrypsin were examined as well as the effects of the addition of three synthetic reactive centre loop analogues *in vitro* and *in vivo*. Synthetic loop peptide BC11 was supplied by Dr D Lomas at Cambridge University, novel peptides REV1 and SEA1 were designed by Dr R. Foreman and synthesised by Prof R Sharma at Southampton University.

2.17.2. *In Vitro* Polymerisation of α_1-antitrypsin

6.18µg of α_1-antitrypsin protein (supplied by Dr D Lomas at Cambridge University), was incubated at a range of temperatures between 20 and 75°C (20, 37, 45, 60, and 75°C), for 60mins. The samples were then incubated on ice to prevent further aggregation and prepared for Gradient Native PAGE analysis. Each sample was diluted 1:1 with Native sample buffer, 2µl Bromophenol Blue dye added, and sample loaded directly onto the gel. Gels were run at a constant current of 28mA for 90min or until the tracking dye reached the bottom of the gel. Gels were then stained using Coomassie blue and destained using gel fixer solution (10% Isopropanol, 25% Acetic Acid).

2.17.2.1. Effects of reactive centre loop peptides on α_1-antitrypsin polymerization *in vitro*

6.18µg of α_1-antitrypsin protein was incubated @ 37°C for 18hrs, to allow optimal peptide insertion conditions, in the presence of various concentrations of BC11 peptide (3mg, 6mg and 12mg). Incubation then continued @ 65°C for 60min to reproduce the same polymerisation conditions shown in the absence of peptide. An identical sample incubated in the absence of BC11 peptide was used as a control to allow the effects of the peptide to be determined. The samples were then incubated on ice to prevent any further aggregation and analysed using Gradient Native PAGE and Coomassie staining.

Identical experiments were then performed using REV1 (2.28mg, 1.14mg and 0.57mg) and SEA1 (2.16mg, 3.12mg, and 4.08mg) peptides. Samples were incubated @ 37°C for 18hrs to optimise peptide insertion and then heated to 65°C for 60min to reproduce optimal polymerization conditions. The samples were analysed using Gradient Native PAGE and Coomassie staining. α_1-antitrypsin and α_1-antitrypsin +BC11 peptide samples were also used as controls to allow determination of peptide effects on polymerization.

2.17.3. *In Vivo* Peptide Analysis

2.17.3.1. Introduction

This study attempted to utilise the microsome 'protein delivery system', described in section 2.13, to study the effects of peptide BC11 on *in vivo* α_1-antitrypsin protein secretion using *Xenopus* oocytes. The methods of Koppleman *et al*,. (1992), were used in an attempt to deliver the peptide, using microsomes, directly to the site of α_1-antitrypsin synthesis and processing – within the ER of oocytes.

2.17.4. Addition of peptide to microsomal membranes

Microsomal membranes (2units) were incubated on ice or at 25°C for 1 hour in the presence of Iμ M synthetic peptide BC11 and then added to an *in vitro* translation system as described in section 2.13. The microsomes from this translation mix were then isolated by sucrose density gradient centrifugation (section 2.13), and injected into oocytes. Protein retrieved from survived oocytes was immunoprecipitated and analysed using Gradient Native PAGE (section 2.14).

As a control, various concentrations (10nM, 100nM, and 1μM), of BC11 peptide were added to a translation mix in the presence of 2μl α_1-antitrypsin mRNA and 1unit microsomal membranes. SDS-PAGE analysis (section 2.10.1), allowed any effects of the peptide on protein translation to be determined.

2.17.5. HEPES/CAPS Treatment of Microsomes

To ensure the presence of peptide within microsomal membranes, the external membrane of a fraction of microsomes was disrupted by a high pH treatment, 200mM HEPES/200mM CAPS, pH9.5, and incubated on ice for 1hr. These permealised membranes were then incubated with 100nM BC11 peptide @ 25°C on ice for 1hr and then re-sealed by titration to pH7 by the addition of 1M PIPES

(pH6.5). Re-sealed microsomes were then added to a translation mix, followed by retrieval of microsomes containing protein and injection into oocytes as described in section 2.13.

2.17.6 Further Experiments

Oocytes were also co-injected with 1μM BC11 peptide and mRNA, and incubated in SOS containing 1μM BC11 peptide in attempts to deliver peptide to site of synthesis and processing of α_1-antitrypsin protein within the oocyte.

2.18 Effects of Iodoacetate on secretion of $Tyr^{38}\rightarrow$Cys variant α_1-antitrypsin

Oocytes were microinjected with $Tyr^{38}\rightarrow$Cys variant α_1-antitrypsin and radiolabelled as described in section 2.11.4. Immunoprecipitation was then performed using surviving oocytes as described in section 2.11.5, but with the addition of 10nm Iodoacetate to immunoprecipitation media. Proteins were analysed via SDS-PAGE and autoradiography.

2.19 Effects of TMAO and Glycerol on polymerisation of α_1-antitrypsin

2.19.1 Introduction

Low molecular weight compounds, including TMAO and Glycerol, have been shown to stabilise proteins that are susceptible to misfolding due to the presence of mutations. An example of this activity has been shown by Brown *et al.* in 1997, who reported a stabilisation effect on the ΔF508 CFTR protein that is the major factor in the development of Cystic Fibrosis. This study examined the effects of these two compounds on the polymerization of α_1-antitrypsin *in vitro* and *in vivo*.

2.19.2 *In Vitro* Effects

6.18mg α_1-antitrypsin protein was incubated in the presence of 100mM or 500mM TMAO or 0.5M or 0.75mM Glycerol overnight @ 37°C. Samples were then heated @65°C for 60min to allow polymerisation to occur. Samples were then diluted 1:1 with Native sample buffer and 2µl Bromophenol Blue and samples analysed by Gradient Native PAGE and Coomassie staining (section 2.14).

2.20 Time Course Experiments

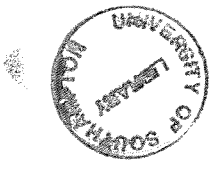
The secretion of protein from *Xenopus* oocytes injected with different variants of α_1-antitrypsin mRNA were analysed using a standard time course.
Oocytes were injected with α_1-antitrypsin mRNA and incubated for 2 hrs in SOS @16°C. Survived oocytes were then radiolabelled and incubated for a further 8 hrs to allow labelling of protein product (section 2.11). The radioactive media was then replaced with non-labelled L-methionine and 12 oocytes were removed at the following time points:

3hrs
5hrs
8hrs
12hrs
24hrs

Protein was analysed via immunoprecipitation and SDS-PAGE.

2.21 Statistical Analysis

Radioactive counts were expressed as a percentage of the total immunoprecipitable material. Statistical significance was assessed using the students t-test and expressed as a probability value where appropriate.



CHAPTER 3

EXISTING AND NOVEL MUTATIONS IN α_1-ANTITRYPSIN THAT AFFECT PROTEIN SECRETION, EXPRESSED IN *XENOPUS* OOCYTES

3.1 Introduction

The pattern of protein secretion resulting from expression of the M, Z and Siiyama α_1-antitrypsin variants has been extensively studied *in ovo*, using the X*enopus* oocyte expression system first demonstrated by Gurdon *et al.*, (1971). Previous experiments have shown that the secretion of both Z and Siiyama protein from oocytes following injection of mRNA mimics that seen from liver cells, with both these deficiency proteins being retained within the oocytes (Foreman *et al.*, 1984, Errington *et al.*, 1985, Sidhar *et al.*, 1995). Therefore, this study examined the secretion of these two variants within oocytes to establish and verify this surrogate secretory system within this laboratory.

The first variant examined in this chapter is the rare (frequency= 1/17 000) F variant, which results from a single amino acid substitution of $Arg^{223} \rightarrow Cys$ (Fagerhol *et al.*, 1965) and is associated with a mild protein deficiency (serum level and function are at least 80% of M). The F variant is presumed to be 'normal' (Billingsley & Cox, 1982) but some studies have reported an increased risk for the development of emphysema when the F allele is expressed in conjunction with Z (Cockcroft *et al.*, 1981, Beckman *et al.*, 1984, Kelly *et al.*, 1989). Brand *et al.*, in 1974, have also reported an FZ patient with cirrhosis but no emphysema, suggesting a possible link (as seen with Z, Siiyama and Mmalton), between accumulation in liver cells and decreased secretion. This chapter examines the effects of the F variant on the secretion of α_1-antitrypsin using the *Xenopus* oocyte secretory system, thus, allowing the effects of a single mutation, $Arg^{223} \rightarrow Cys$, on secretion compared to M variant, to be observed using immunoprecipitation and SDS-PAGE techniques. As Fig.3.1 illustrates, Arg^{223} lies in close proximity to the reactive centre loop. In this structure of the intact model of α_1-antitrypsin (Elliott *et al.*, 1996 (a)), the P5 residue has the potential to play a crucial role in the stabilization of the β-strand loop conformation by forming hydrogen bonds with Arg^{196} and the backbone amide of Met^{226}. These two residues, in combination with a number of other residues (including Arg^{223}, Arg^{281} and Lys^{243}), form a basic pocket around the P5 residue (Glu^{354}), of the

reactive centre loop. It has been suggested that this pocket structure, illustrated in detail in Fig 3.2, may act to 'hold' the loop in its optimal inhibitory position (Chaillian-Huntington & Patston, 1998). This relationship between Arg^{223} and the Glu^{354} residue suggests that the F mutation may act to disrupt the conformation or mobility of the reactive centre loop, which may lead to aberrant secretion of the mutant protein. To examine this possible relationship between the residue at P5 and the F mutation, novel variants, highlighted in Fig 3.1, termed P5 V (Glu^{254}→Val), Q (Glu^{254}→Gln) and S (Glu^{254}→Ser), were constructed and expressed in the oocyte expression system. By studying the effects of these novel mutations, a possible role for the P5 residue in the mild secretory defect produced by the F variant, may be established.

Also examined in this chapter are the effects on secretion of a rare variant (frequency = 0.3-0.67%), resulting in a single amino acid substitution of Arg^{39}→Cys, classified as I (Fagerhol in 1967). Recently, the protein deficiency related to this variant has been examined following the determination of a ZI heterozygote by Mahadeva *et al.*, in 1999. In this case, the presence of a combination of the Z and I alleles, *in vivo*, resulted in a further decrease in the secretion of α_1-antitrypsin when compared to a ZM heterozygote. This supported the theory that I is a mild deficiency allele and that co-expression of this allele may increase the protein secretory defect observed with other deficiency alleles, such as Z. Observations from experiments performed *in vitro* have also indicated that this variant may be associated with the same hepatic accumulatory defect seen with the Z and Siiyama mutations – loop-sheet polymerization. Mahadeva *et al.*, in 1999 determined that, under identical *in vitro* conditions, the I variant of α_1-antitrypsin underwent polymerisation more readily than the M variant, and a mixture of I and Z protein formed polymers more readily than a mixture of M and I protein, identifying this polymerogenic nature of this variant.

Few reports have studied the relationship between the expression of the I allele and α_1-antitrypsin serum levels, but Blundell *et al.*, in 1975 reported IM heterozygotes with an α_1-antitrypsin serum concentration that was only 79% of

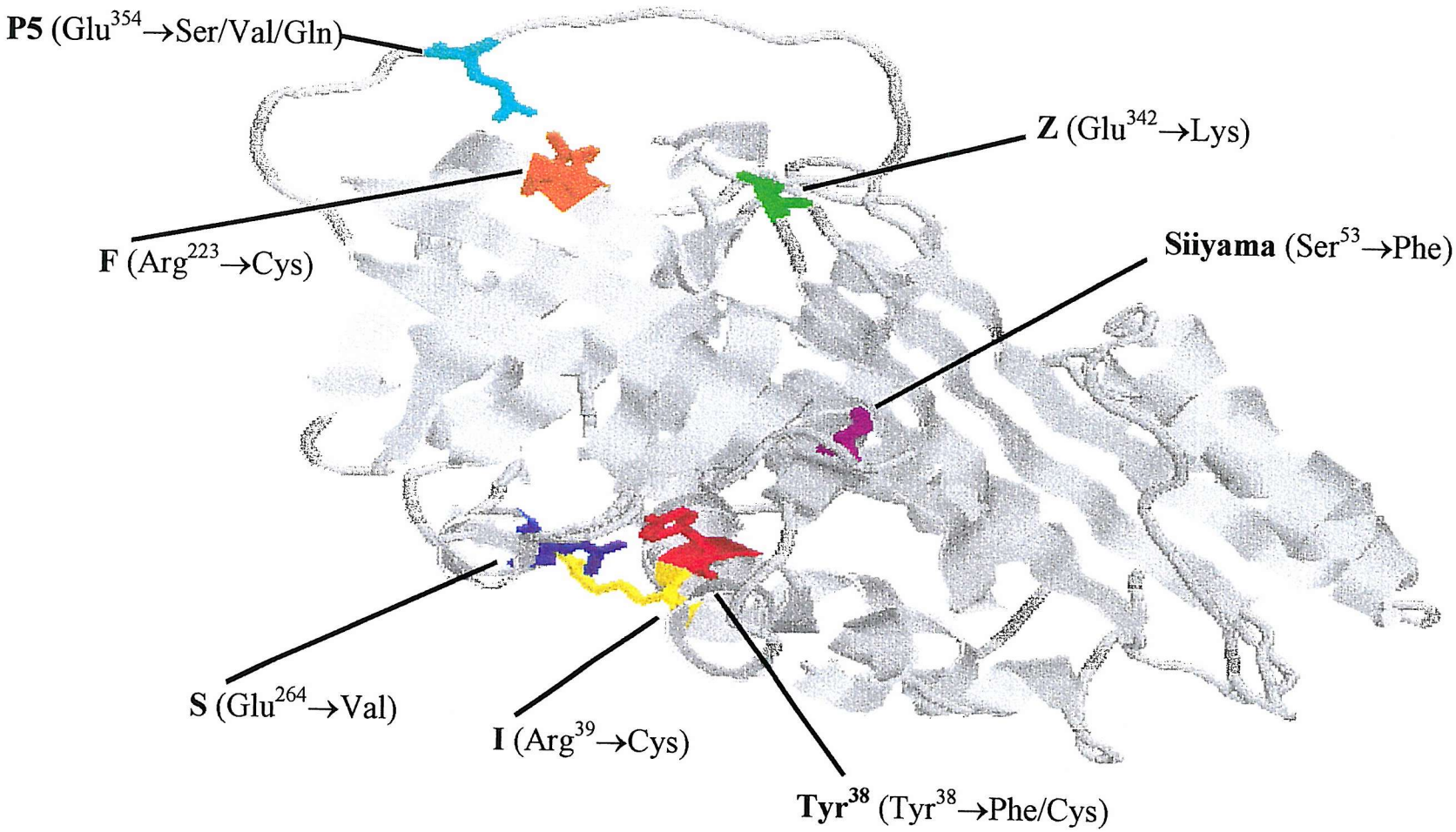


Fig 3.1 RASMOL diagram representing the crystallographic structure of intact α_1-antitrypsin as proposed by Elliott *et al.*, (1996). The single point mutations examined in this study are highlighted as coloured residues.

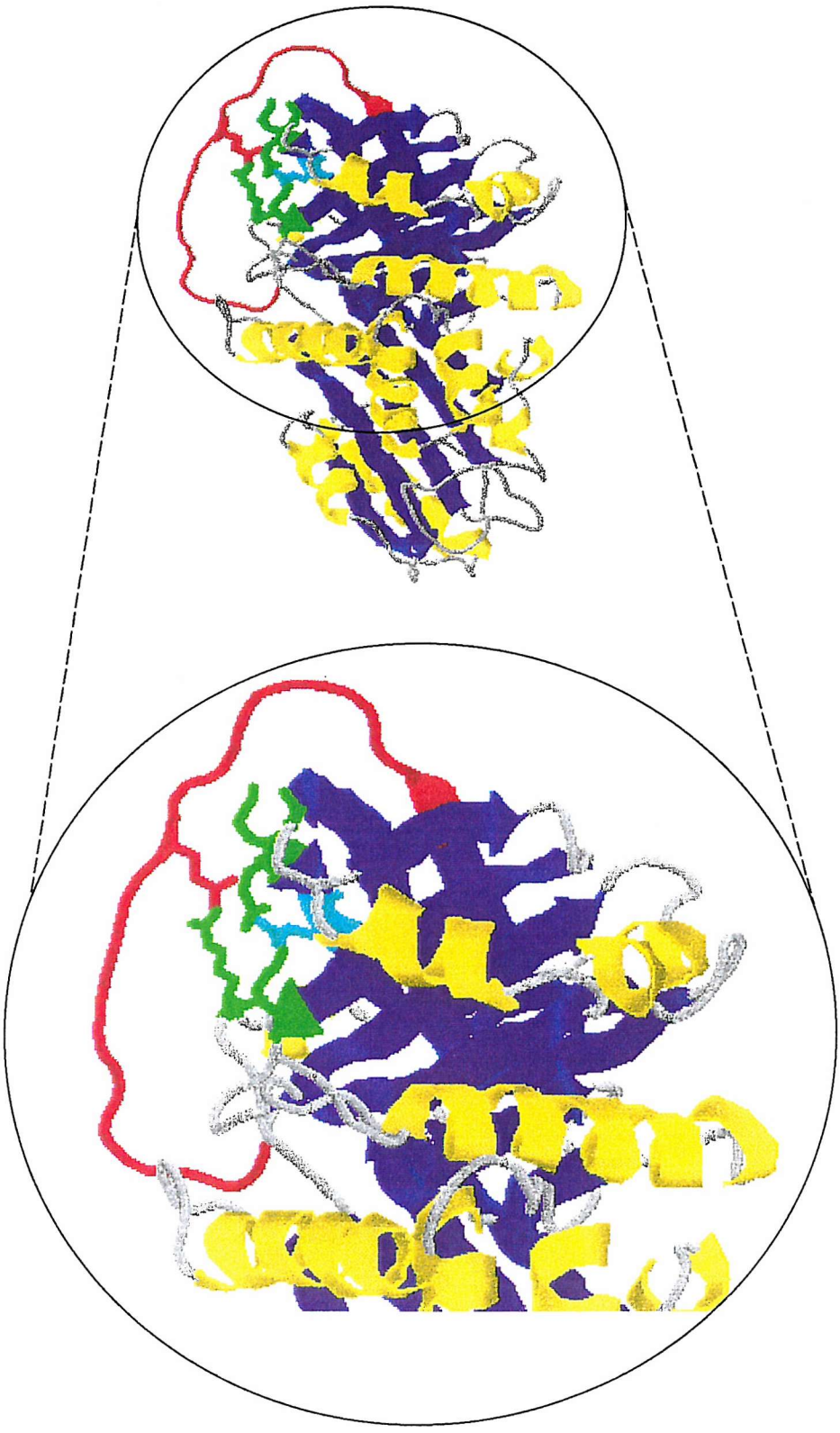


Fig 3.2 RASMOL diagram representing the crystallographic structure of intact α_1-antitrypsin as proposed by Elliott *et al.*, (1996). Enlarged section illustrates, in detail, the relationship between the novel P5 mutation produced in this study (red) and the naturally occurring F mutation (cyan). The residues involved in the formation of a pocket thought to have a role in the retention of the RCL in the active, inhibitory conformation (Elliott *et al.*, (1998)), are clearly shown as a ring of green residues encompassing Arg^{196}, Arg^{281}, Met^{226}, Lys^{243} and the F mutation residue (Arg^{223}), surrounding the central P5 (Glu^{354}) residue.

normal values. Baur & Bencze, in 1987, also reported a mild plasma deficiency and minor impairment of lung function related to the presence of the IM phenotype. This information indicates that IM heterozygote individuals possess a protein level sufficient to protect against emphysema, but the effects of an II homozygote phenotype are still unknown.

This study aims to use the *Xenopus* oocyte secretory system to examine the effects of the I variant on the secretion of α_1-antitrypsin from these cells. Previously, as discussed above, the I mutation has only been studied, *in vitro* or *in vivo*, in combination with the M or Z alleles. Using the oocyte system will determine the effects of the mutation at position 39 alone on protein secretion and give the first indications of the severity of the deficiency that may result from this mutation. The close resemblance between secretion of α_1-antitrypsin variants from oocytes and from *in vivo* liver cells may also provide information about the potential effects of this variant *in vivo*.

As with the F variant, the relationship between the mutation of Arg→Cys at position 39 (I variant), and a mild deficiency in protein secretion is unclear. However, the structure of the intact α_1-antitrypsin molecule (Elliott *et al.*, 1996 (a)), in Fig 3.1. indicates a close spatial relationship between residue 39 and residue 264; the mutation of which from Glu→Val results in the S variant of α_1-antitrypsin. Therefore, the possibility exists that a link between these two residues may be disrupted following mutation of either residue, and this may have an effect on protein secretion (Graham *et al.*, 1989).

As the most common α_1-antitrypsin variant, S forms 7% of all α_1-antitrypsin alleles in North European Caucasians (Owen *et al.*, 1976), and up to 28% of alleles in Southern European Caucasians (Fagerhol, 1976). The S variant results in plasma levels of α_1-antitrypsin that are 60% of normal (Gitlin & Gitlin, 1975) and, thus, like the IM individuals described above, SS individuals do not show enough of a deficiency to develop emphysema. This allele is, however, a risk to health when expressed in combination with the Z deficiency allele. SZ

heterozygotes have α_1-antitrypsin levels that are 'borderline protective' and some of these individuals do develop emphysema (Larsson *et al.*, 1976, Nukiwa *et al.*, 1986, Stein & Carrell, 1995). Experiments performed *in vitro* have observed increased polymer formation, as seen with the I variant, in comparison with the M variant (Mahadeva *et al.*, 1999), but is only mildly polymerogenic compared to the Z protein (Elliott *et al.*, 1996 (b)). The related deficiency has also been attributed, not only to accumulation within the hepatocytes where the majority of the protein is eventually degraded, but also to increased turnover in the plasma. As such, SS individuals do not appear to be of great risk of developing liver disease (Curiel *et al.*, 1989 (a)).

Observations by Loebermann *et al.*, in 1984, had suggested that the secretory deficit caused by the mutation of $Glu^{264}\rightarrow$Lys may be attributed to the loss of a conserved salt bridge to residue Lys^{387}, located on strand 5B. However, results from subsequent work by Brodbeck *et al.*, in 1993, suggested that this salt bridge is not required for normal protein synthesis or secretion and that, therefore, this theory is unlikely. More recent work by Elliott *et al.*, in 1996 (b), has suggested that it is more likely to be the disruption of a hydrogen bond between Glu^{264} and Tyr^{38}, that results in a change in protein conformation and a decrease in secretion.

These observations indicate possible interactions between these three residues, illustrated in Fig 3.3, that, following mutation, may be disrupted resulting in a decrease in protein secretion. This study has used the *Xenopus* oocyte system to determine whether any such relationships may exist. By examining and comparing the effects of the I and S variants and two novel mutations of $Tyr^{38}\rightarrow$Phe/Cys (which result in the loss of an hydroxyl group necessary for formation of a hydrogen bond with Glu^{264}), on secretion of protein from these cells, any possible interaction between these three residues that may be disrupted following mutation can be determined.

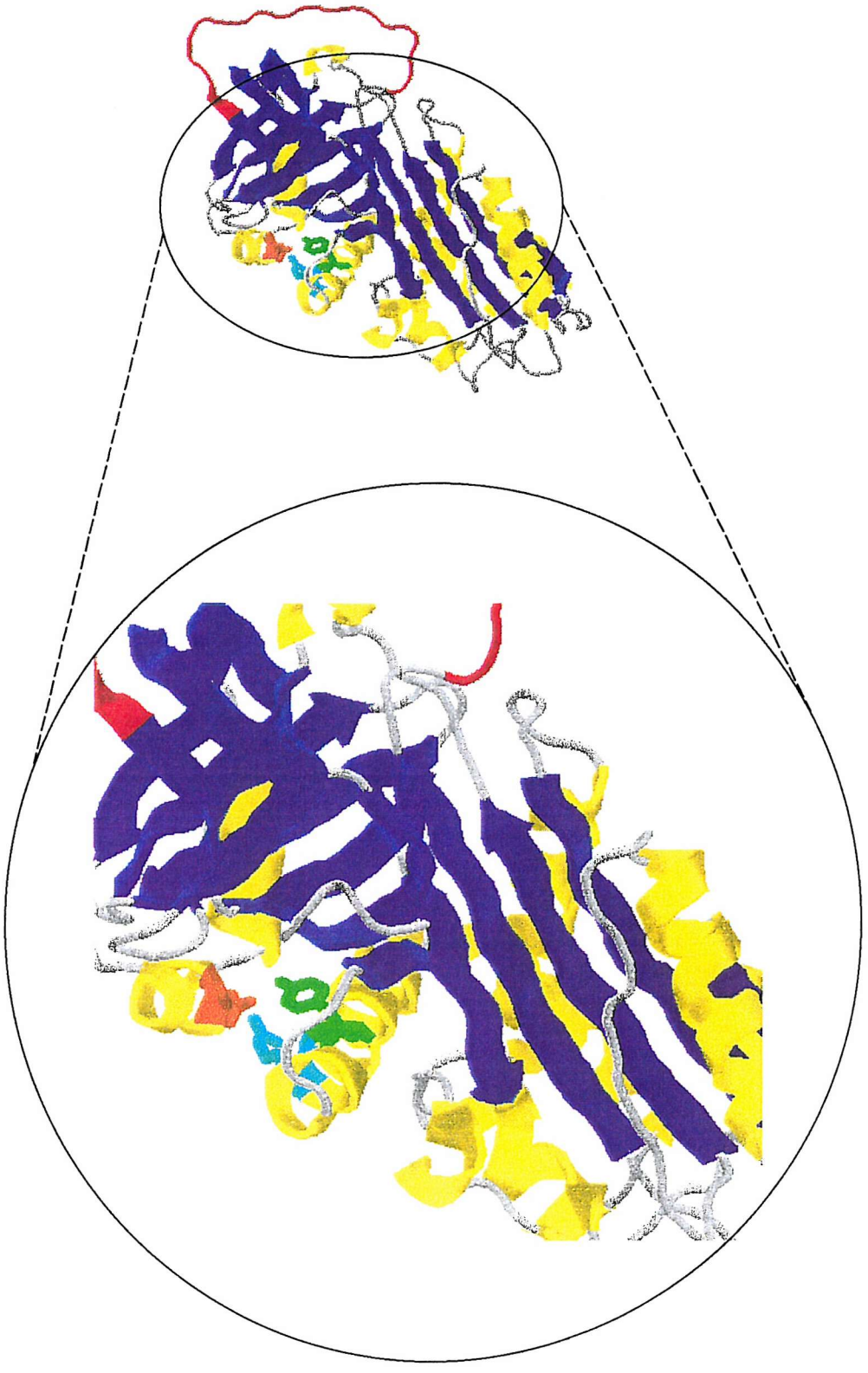


Fig 3.3 RASMOL diagram representing the crystallographic structure of intact α_1-antitrypsin as proposed by Elliott *et al.*, (1996). Enlarged section illustrates, in detail, the relationship between the residues involved in the naturally occurring I (Arg^{39} - cyan) and S (Glu^{264} - orange) mutations. It has been proposed (Graham *et al.*, 1989), that an interaction between these two residues may be disrupted by mutation, leading to a decreased secretion for both variants. The novel mutation (Tyr^{38}→Phe/Cys), produced for this study is shown in green.

3.2 Results

3.2.1 Effect of the Z (Glu^{342}→Lys) and Siiyama (Ser^{52}→Phe) variants on α_1-antitrypsin secretion

The effects on secretion of the Z and Siiyama variants of α_1-antitrypsin have been observed both *in vitro* (Lomas *et al.*, 1992, Kang *et al*,. 1997) and *in vivo* (Lomas *et al.*, 1993, Errington *et al.*, 1992, McCraken *et al*,. 1989). The secretion of these 2 variants from *Xenopus* oocytes has also been established in this laboratory (Wu & Foreman, 1990, Sidhar *et al.*, 1995), and other laboratories (Perlmutter *et al.*, 1985), have also used this system to study the secretion of α_1-antitrypsin. In order to establish and verify the oocyte secretory system for the purpose of this study, the secretion of Z and Siiyama variant proteins were examined and the results compared to those obtained previously.

Fig 3.4 (a) demonstrates the decreased secretion of both the Z and Siiyama variants, compared to M, from oocytes and the quantification of the excised bands of immunoprecipitated protein, in Fig 3.4 (b), indicates the amount of inhibitor secreted as a percentage of total protein synthesised. The values for M α_1-antitrypsin (63.1% secreted ± 2.3) when compared to the Z variant (16.6% secreted ± 1.2) demonstrate that the secretion of α_1-antitrypsin from *Xenopus* oocytes is greatly decreased in the presence of the Glu^{342}→Lys substitution. This result confirms that a significant percentage of Z α_1-antitrypsin is retained within the oocyte, mirroring the retention seen *in vivo* within hepatocytes (Riley *et al.*, 1985, Eriksson *et al.*, 1986).

Fig 3.4 (b) also quantitates the decrease in secretion resulting from injection of the Siiyama variant into oocytes as only 13.7%±1.2 of protein is secreted compared to 63.1% secretion of M α_1-antitrypsin. These results, like those discussed for the Z variant, also mirror the effects of this variants secretion from *in vivo* liver cells (Lomas *et al.*, 1993, Riley *et al.*, 1985), and results for both Z and Siiyama are

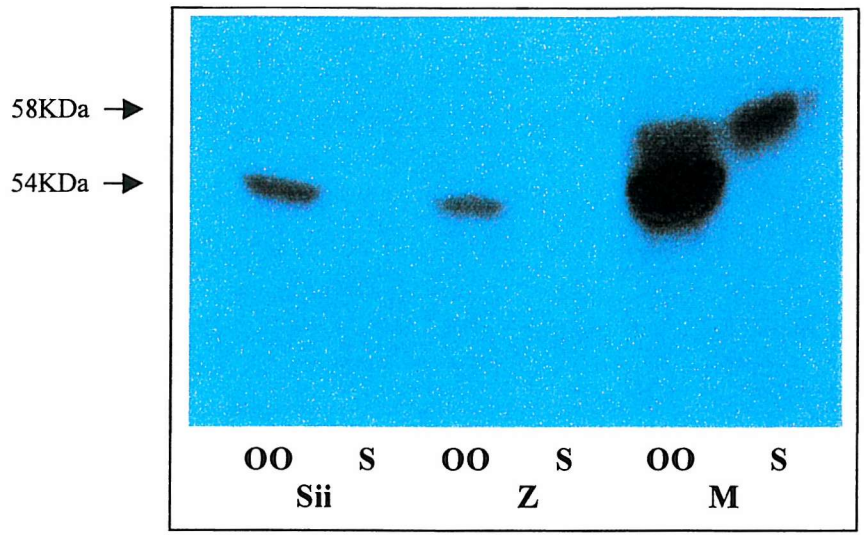


Fig 3.4 (a). Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands compare amounts of protein secreted between oocytes injected with M, Z and S_{iiyama} variant α_1-antitrypsin mRNA. Molecular weights were determined by co-migration of standard molecular weight markers.

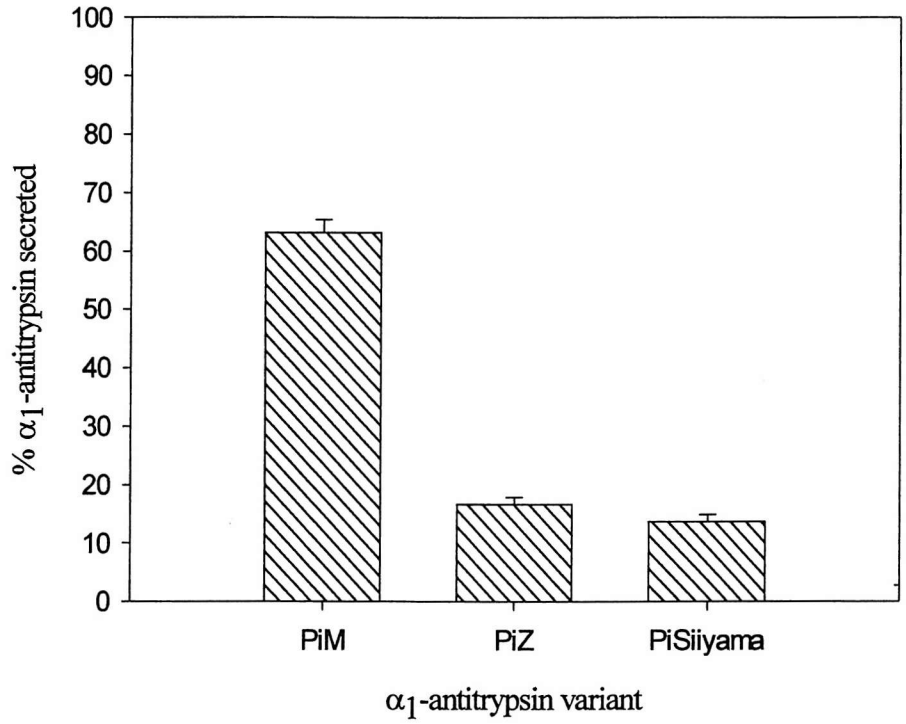


Fig 3.4 (b) Quantitation of the relative amounts of M, Z and S_{iiyama} α_1-antitrypsin secreted from mRNA microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least 5 different experiments, labelling at least 15 oocytes each, using five different animals to eliminate oocyte variation as much as possible. Values are expressed as the arithmetic mean ± the S.E.M.

consistent with previous reports (Foreman *et al.*, 1984, Sidhar *et al.*, 1995), that also demonstrated the decreased secretion of these two variants from *Xenopus* oocytes.

3.2.2 Effect of the F variant (Arg^{223}→Cys) and novel P5 variants (Gln^{354}→Val, Gln, Ser) on α_1-antitrypsin secretion

Previous studies have shown that, *in vivo*, the F variant of α_1-antitrypsin results in a mild deficit of protein and predisposes to the development of emphysema if it occurs with another deficiency allele, in particular Z (Kelly *et al.*, 1989, Cockcroft *et al.*, 1981, Brand *et al.*, 1974, Beckman *et al.*, 1984). Communications with Lomas and colleagues at Cambridge University have also indicated that the F mutation demonstrated a tendency to polymerise, which may result in a decrease in protein secretion, from bacterial cells (unpublished observations). By examining the effects of this variant on the secretion of protein from the oocyte system, an increased understanding of the relationship between the Arg^{223}→Cys mutation and the mild protein deficit observed may be obtained.

Examination of the structure of α_1-antitrypsin, as illustrated in Fig 3.1, demonstrates the close spatial relationship between the Arg^{223} residue and residue P5 of the reactive centre loop of α_1-antitrypsin. As discussed in the introduction to this chapter, the possibility exists that there may be an interaction between these 2 residues, that is disrupted by mutation of residue 223 and results in a destabilisation of the loop, increasing the possibility of protein polymerization. To examine this theory further, various mutations of the P5 residue were constructed and examined to assess the importance of this residue on the secretion of protein.

Fig 3.5 (a) illustrates no visible difference between F and M α_1-antitrypsin secretion from oocytes. Fig 3.5 (b) compares the retained and secreted fractions of variant Z, P5 V and S and normal M α_1-antitrypsin protein from oocytes. Analysis of the excised gel bands in Fig 3.5 (c) shows the amount of inhibitor secreted

expressed as a percentage of the total protein synthesised. The values for M α_1-antitrypsin (63.1% secreted ± 2.3) when compared to the F variant (52.7% secreted ± 3.3) demonstrates a significant difference ($p<0.01$) in the amount of α_1-antitrypsin secreted from the oocytes. These observations suggest that a mild protein deficiency may be related to the presence of the $Arg^{223}\rightarrow$Cys mutation.

The values for M α_1-antitrypsin (63.1% secreted ± 2.3) when compared to the engineered P5 mutations V (62.6% secreted ± 2.2) and Q (60.1% secreted ± 1.7) demonstrates no significant difference ($p>0.01$) in the amount of α_1-antitrypsin secreted from the oocytes.

On close examination of the results comparing the secretion of the F and P5 mutants from oocytes, the percentage secretion can be seen to be almost identical for the F mutation and the P5 S mutation (52.7% compared to 52.8%). These two values are not significantly different ($p<0.99$) and are consistently and significantly ($p<0.004$) lower than the secretion of M, P5 V and P5 Q. This suggests that changes in the nature of the residue at this position of the reactive centre loop can alter the secretory properties of human α_1-antitrypsin in a manner analogous to the F ($Arg^{223}\rightarrow$Cys) mutation. Replacement of the P5 glutamic acid with the bulky, hydrophobic valine or less polar glutamine had little effect while substitution by serine produced an 'F-like' phenotype.

3.2.3 Effect of the I variant ($Arg^{39}\rightarrow$Cys) on α_1-antitrypsin secretion

The effects of the I mutation on secretion of α_1-antitrypsin protein have only been examined *in vitro* (Mahadeva *et al.*, 1999), and *in vivo* (Arnaud *et al.*, 1978, Blundell *et al.*, 1975, Baur & Benzce, 1987), in combination with another allele of α_1-antitrypsin. The use of the *Xenopus* oocyte system allowed the effects of this variant alone to be determined in this system. Fig 3.6 (a) compares the secretion of variant I and normal M α_1-antitrypsin protein from oocytes. This gel illustrates

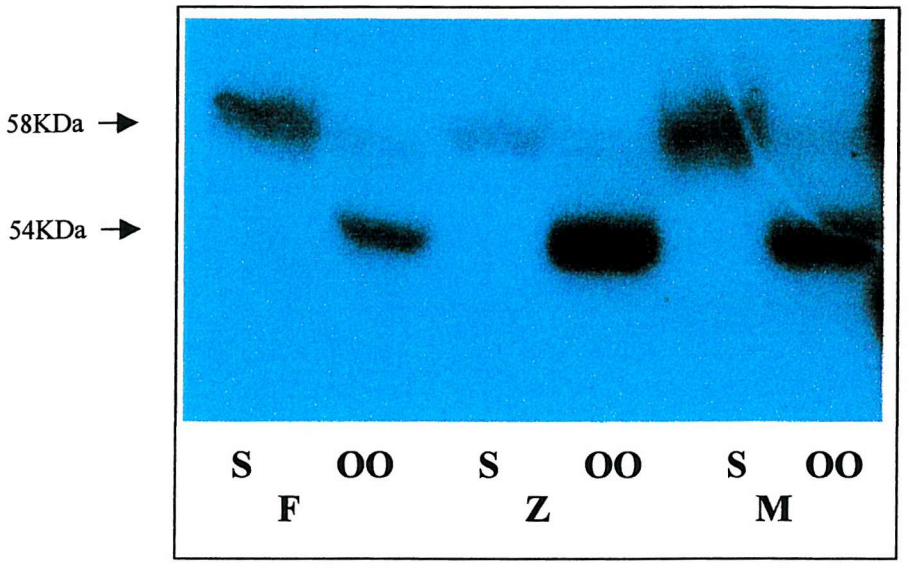


Fig 3.5 (a) Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands illustrate comparison between oocytes injected with M, Z, and F variant mRNA. Molecular weights were determined by co-migration of standard protein markers.

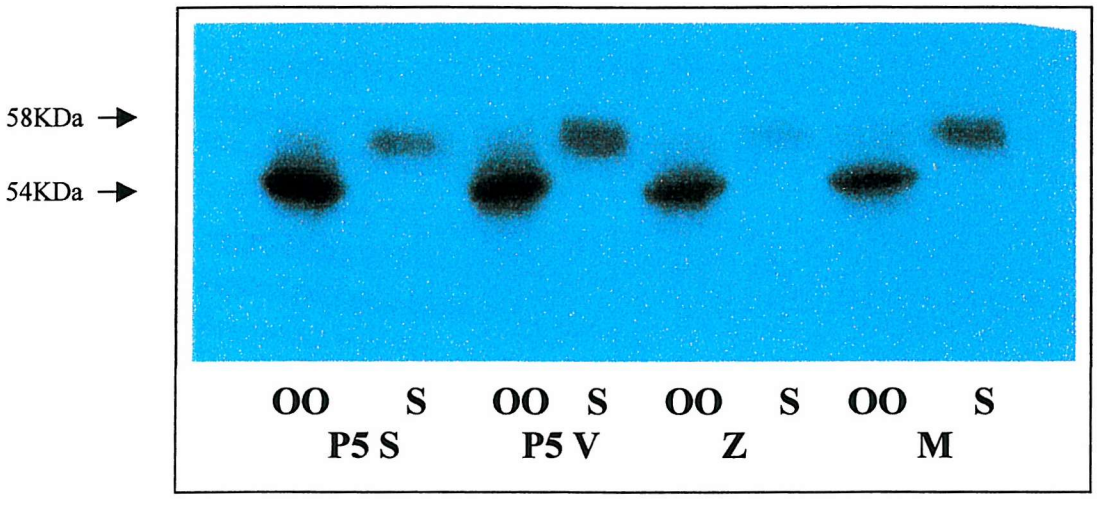


Fig 3.5 (b) Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands illustrate comparison between oocytes injected with M variant, Z and novel P5 mutation S and V α_1-antitrypsin mRNA. Molecular weights were determined by co-migration of standard protein markers.

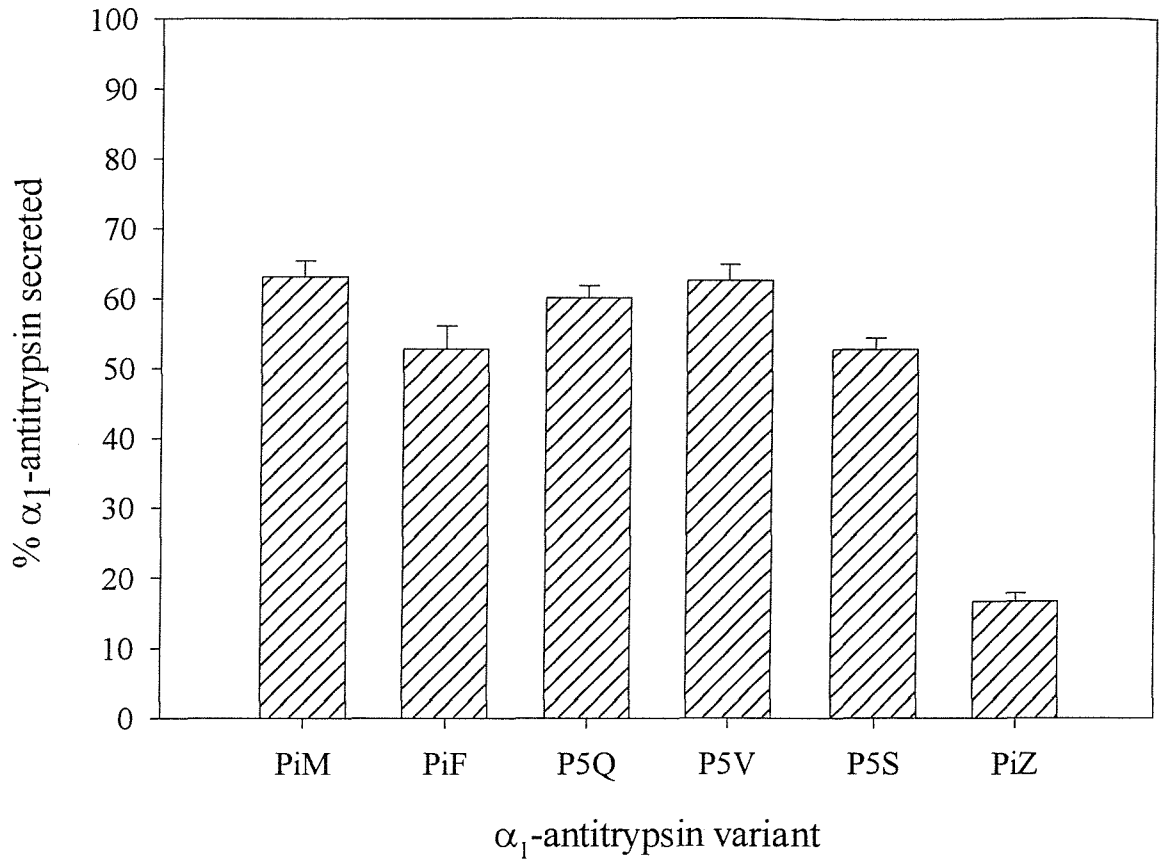


Fig 3.5 (c) Quantitation of the relative amounts of M, Z, F and novel P5 variants (S, V & Q) α_1-antitrypsin secreted from mRNA microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least 5 different experiments, labelling at least 15 oocytes each, using five different animals to eliminate oocyte variation as much as possible. Values are expressed as the arithmetic mean ± the S.E.M.

that the level of I variant protein expression from these cells is lower than that observed with the M variant. This difference in the levels of protein synthesis is most likely related to the concentration of the mRNA injected into the oocytes, that of the I variant being slightly lower than M, but the relative levels of retained compared to secreted protein are not affected by this decreased expression. The results obtained from excised gel bands – Fig 3.6 (c), demonstrates that the secretion of the I variant of α_1-antitrypsin when injected into *Xenopus* oocytes is greatly reduced (26.1% ± 2.6) when compared to M (81.5% ± 3.5).

These results do not indicate only a mild deficiency for this variant (as observed by the results from the *in vivo* studies listed above), but demonstrate that the Arg^{39}→Cys mutation has a substantial effect on the secretion of α_1-antitrypsin. This suggests that, in a homozygous form, the I variant may result in a protein deficiency similar to that observed with the Z and Siiyama variants. This decrease in secretion may be as a result of disruption of the mobility of the A β-sheet, increasing the possibility of protein polymerization. The relationship between mutations in the shutter region of the protein and a subsequent decrease in secretion were further examined by the study of the effects of another mutation in this region of the protein, Glu^{264}→Lys - the S variant.

3.2.4 Effect of the S variant (Glu^{264}→Val) on α_1-antitrypsin secretion

Following the determination of the effects of the Arg→Cys mutation at position 39, within the shutter domain of the protein, the effects of a 2nd, more common, mutation within this region which also results in a decrease in protein secretion were observed. The S variant (Glu^{264}→Val), has been extensively studied *in vivo* (Gitlin & Gitlin, 1975, Curiel *et al.*, 1989 (a), Teckman & Perlmutter, 1996), and observations have demonstrated that the presence of this variant results in a decrease in protein secretion to only 60% of normal levels. As discussed in the introduction, the destabilisation of protein structure related to the I variant, may be the result of a disruption of an interaction between residues 39 and 264. To

establish whether any such disruption between these 2 residues may result in the decrease in secretion observed with both the variants they produce, the effects of the mutation of Glu→Val at position 264 were examined and compared to those determined for the I variant using *Xenopus* oocytes. Fig 3.6 (b) compares the secretion of normal M and variant S α_1-antitrypsin protein from oocytes. Examination of the gel illustrates a decreased secretion of the S variant protein, and the quantification of the excised protein bands in Fig 3.6 (c), determined that the secretion of α_1-antitrypsin from *Xenopus* oocytes in the presence of the S mutation is decreased by 40% (49.6±1.7) compared to M (81.5±3.5).

These results correlate well to the 60% decrease in secretion observed with the presence of this variant *in vivo* (Gitlin & Gitlin, 1975). However, the extent of secretion observed with the S variant does not appear to be related to that seen with the I variant of α_1-antitrypsin. The I variant results in a more severe form of the deficiency, with only ~35% of normal levels of protein secreted, compared to 60% secretion of the S variant protein. This suggests that disruption of a possible direct interaction between these two residues is not likely to be the cause of the decreased secretion observed with either variant. However, in 1996, Elliott *et al.*, (b) suggested that a hydrogen bond between residues 264 and 38 may be altered by mutation to affect protein stabilization and result in the decrease in protein secretion observed with the S variant. To examine this theory, two novel mutations of this residue, Tyr^{38}→Phe/Cys, were created in this laboratory and the effects on protein secretion observed in *Xenopus* oocytes.

3.2.5 Effect of the novel Tyr^{38}→Phe/Cys variants on α_1-antitrypsin secretion

The effects of these two novel mutations can clearly be seen in Fig 3.6 (b), which shows the extent of retention and secretion of these two variants, compared to the M variant of the protein. The gel illustrates that alteration of the tyrosine residue to a phenylalanine leads to a significant decrease in the secretion of protein from *Xenopus* oocytes. Excision and quantification of the protein bands, in Fig 3.6 (c),

determined that the secretion of this variant is 48.6%±3.1, compared to 81.5%±3.5 for the M variant and demonstrates no significant difference ($p>0.001$) from the secretion of the S variant (49.6% ±1.7). These results demonstrate that both these mutations result in secretion of the protein that is only ~60% of normal levels. The similarity in the pattern of secretion between these two variants increases the evidence for the mechanism of decreased secretion of the S variant suggested by Elliott *et al.*, in 1996. The mutation of the 264 residue may disrupt the hydrogen bond between this residue and residue 38, altering the form of the protein and affecting its secretion from oocytes.

The pattern of protein secretion resulting from the mutation of $Tyr^{38}\rightarrow Cys$ is also illustrated in Fig 3.6 (b). This gel clearly demonstrates the presence of a higher molecular weight form of the protein within the oocytes injected with this variant. Excision and quantification of the protein bands, in Fig 3.6 (c), reveals that secretion of the 'normal' processed form of protein following expression of this variant is only 11.2%±2.4, compared to 81%±3.5 for the M variant. The remainder of protein produced appears to be retained within the cell as a higher molecular weight form predicted to be cysteine-linked dimers formed between α_1-antitrypsin molecules within the oocyte.

To test this hypothesis, the oocyte fraction of $Tyr^{38}\rightarrow Cys$ protein was immunoprecipitated in the presence of 10nm Iodoacetate (an inhibitor of cystine bond formation). Fig 3.7 illustrates the results from this experiment and clearly demonstrates the decreased amount of the higher molecular form of this variant protein compared to immunoprecipitation in the absence of Iodoacetate.

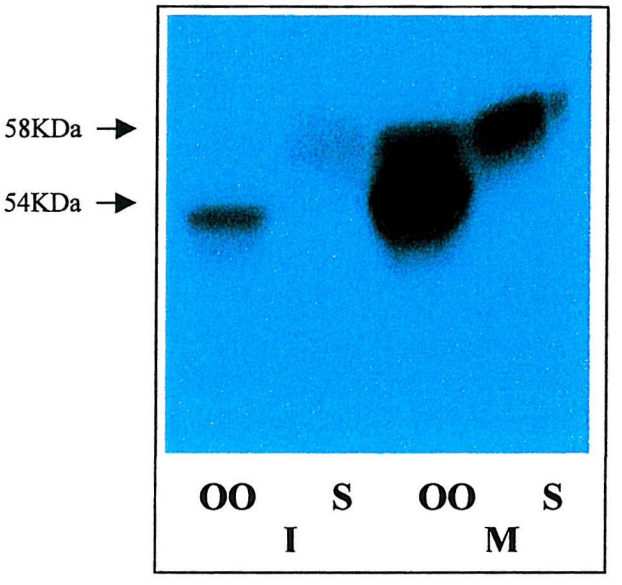


Fig 3.6 (a) Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands show comparison between 'normal' M variant and I deficiency variant. Molecular weights were determined by co-migration of standard protein markers.

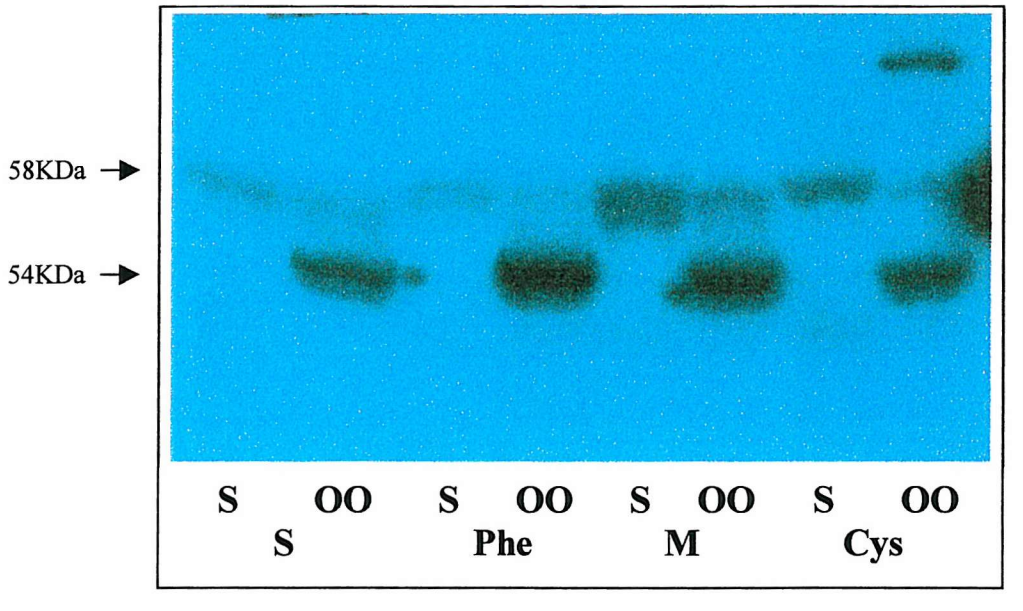


Fig 3.6 (b) Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands show comparison between oocytes injected with M, S, Tyr^{38}→Cys variant (Cys), and Tyr^{38}→Phe variant (Phe) mRNA. Molecular weights were determined by co-migration of standard protein markers.

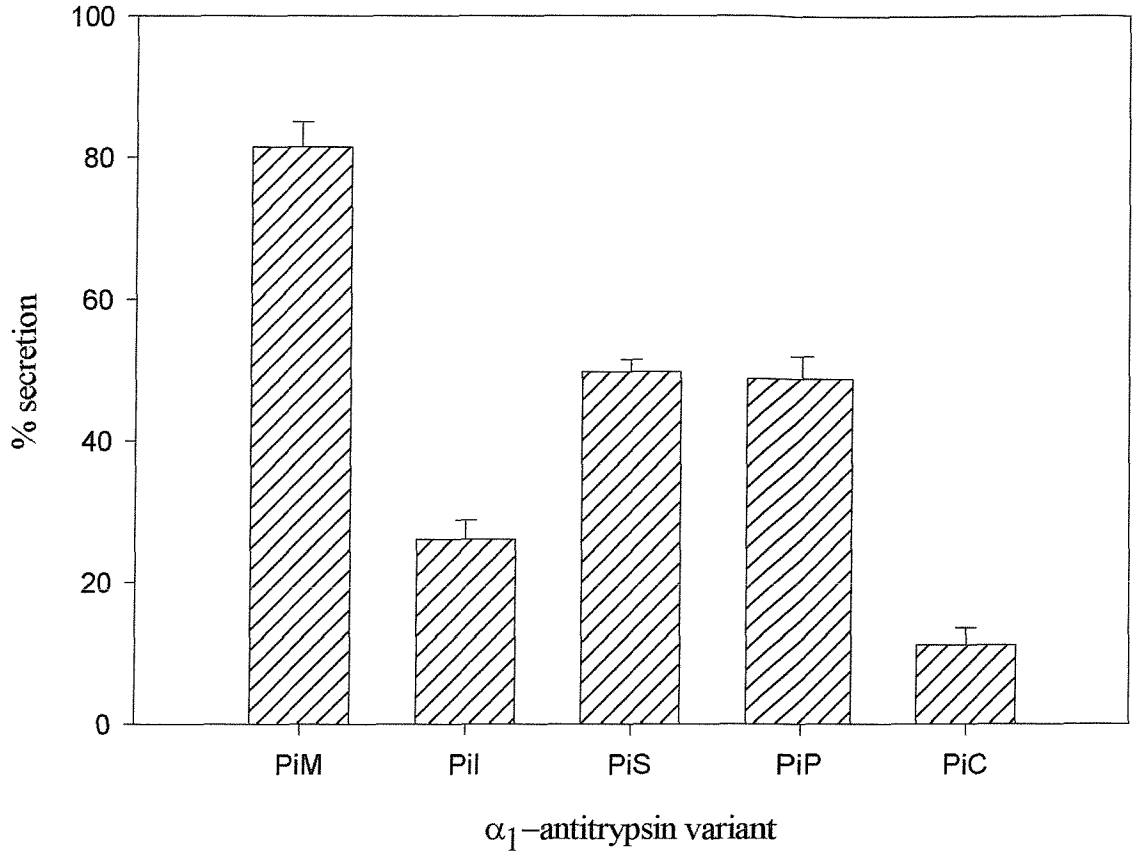


Fig 3.6 (c) Quantitation of the relative amounts of M, I, S and novel variants PiP (Tyr^{38}→Phe) and PiC (Tyr^{38}→Cys) α_1-antitrypsin secreted from mRNA microinjected oocytes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents the mean of at least 5 different experiments, labelling at least 15 oocytes each, using five different animals to eliminate oocyte variation as much as possible. Values are expressed as the arithmetic mean ± the S.E.M.

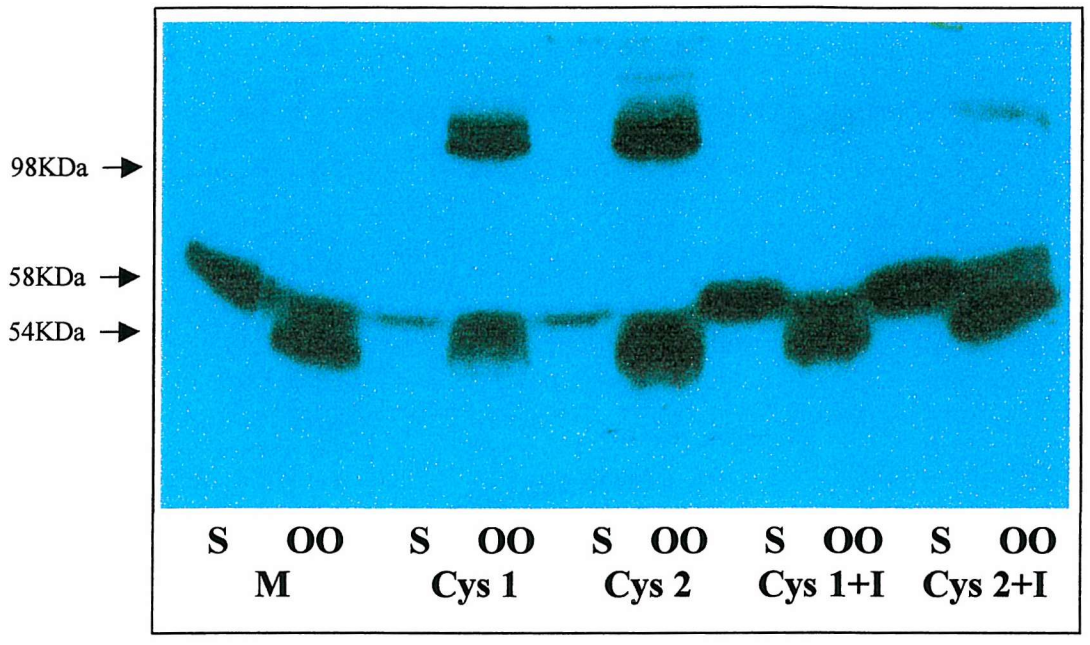


Fig 3.7 Autoradiograph of SDS-PAGE gel showing immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands show comparison between oocytes injected with M, Tyr^{38}→Cys variant (Cys1 and Cys2), and Tyr^{38}→Cys variant immunoprecipitated + 10mM Iodoacetate (Cys1+I and Cys2+I). Molecular weights were determined by co-migration of standard protein markers.

3.2.6 Effect of deficiency variants on the rate of α_1-antitrypsin secretion.

The results presented so far in this chapter have given an increased insight into how specific mutations within the protein structure of α_1-antitrypsin can affect its secretion from oocytes. In an attempt to further examine these effects the rate of secretion of M, Siiyama, F and I variant α_1-antitrypsin from oocytes in relation to the post injection incubation time of these cells was also observed. Oocytes were injected with variant mRNA and incubated in SOS containing ^{35}S methionine as previously described (see methods – section 2.20). A standard number of healthy oocytes were then immunoprecipitated at 3, 5, 8, 12 and 24 hours post-incubation and Fig 3.8 demonstrates the percentage secretion of F, Siiyama and I α_1-antitrypsin from oocytes, compared to M α_1-antitrypsin at each of these time points.

Although the total amount of protein secreted from oocytes following injection of the I and Siiyama variants is decreased compared to M, the rate of secretion of these three variants is comparable as the amount of protein secreted increases, to the levels expected from previous observations discussed in this chapter, and then reaches a plateau at ~12hrs. These results are in agreement with the blockade in secretion observed with the Siiyama and I variants, as discussed in section 3.2.1 & 3.2.3. The rate of secretion over time observed following the secretion of the F variant, however, does not relate to that of the other three variants, as the secretion of this variant is still increasing at 24hrs post-injection. These results suggest that the transport of the F variant protein may differ within these cells to that of the other variants and that secretion of this variant protein may increase to even higher levels following increased incubation time. This theory was examined but the viability of these oocytes post-24 hours was too low to achieve feasible and accurate results.

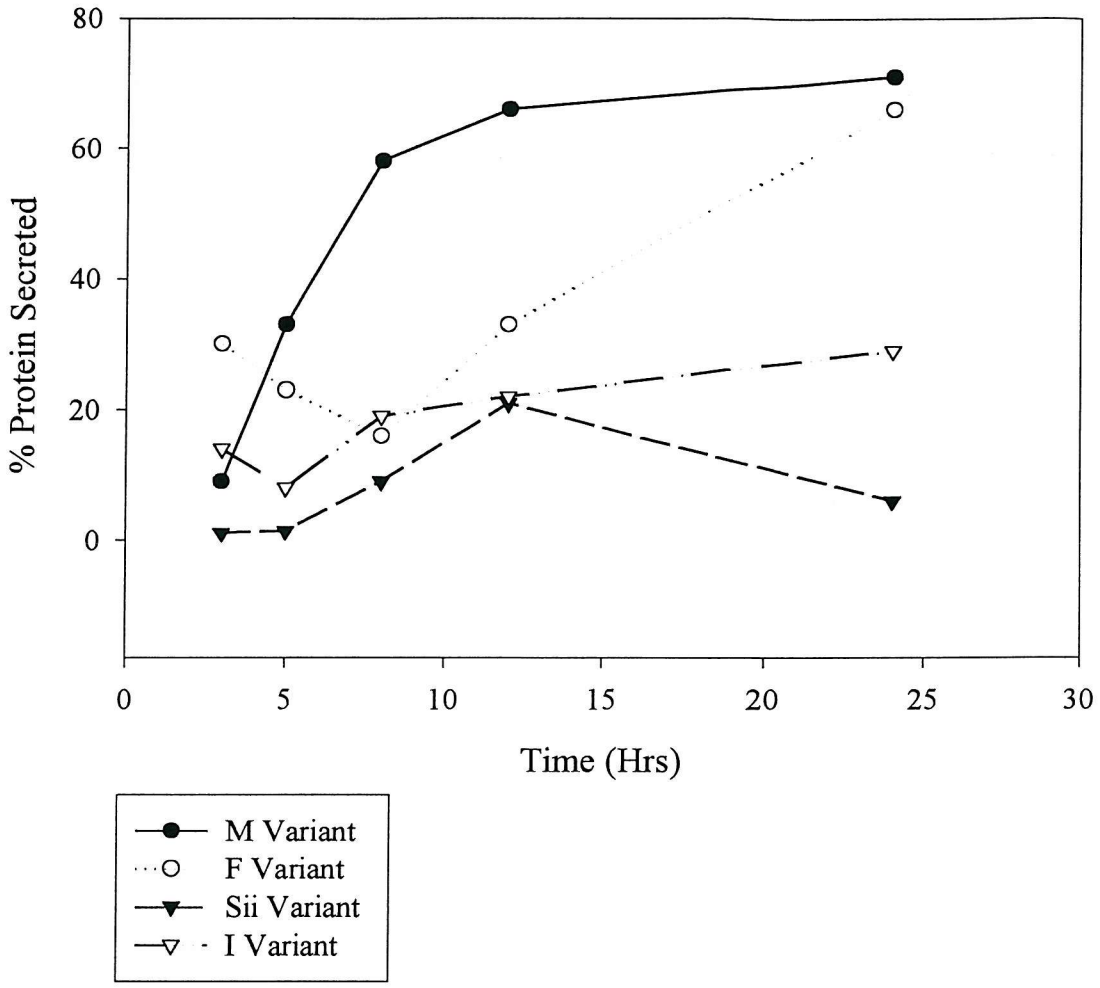


Fig 3.8 Quantitation of the relative amounts of M, Siiyama, F and I α_1-antitrypsin secreted from mRNA microinjected oocytes over a 24 hour time period. At each time interval following injection (3, 5, 8, 12 and 24 hrs), a standard percentage of viable oocytes were immunoprecipitated and analysed via SDS-PAGE. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted protein is expressed as a percentage of the total amount of inhibitor synthesised in oocytes.

3.3 Discussion

The results presented in this chapter demonstrate the effects of a number of existing and novel mutations on the secretion of α_1-antitrypsin using *Xenopus* oocytes. In order to validate the use of this system for the study of α_1-antitrypsin protein secretion within this laboratory, the secretion of M, Z and Siiyama variants of α_1-antitrypsin were first established.

The secreted protein from oocytes injected with M, Z and Siiyama α_1-antitrypsin is quantified in Fig 3.4 (b) and demonstrates that only 16.6% of Z and 13.7% of Siiyama α_1-antitrypsin was secreted from oocytes compared to 63.1% secretion of the M variant. These results are in agreement with previous experiments which observed decreased secretion of these variant proteins following oocyte injection with variant mRNA (Foreman *et al.*, 1984, Sidhar *et al.*, 1995), and also follows the pattern of secretion observed from *in vivo* liver cells (Riley *et al.*, 1985, Carrell, 1986, Eriksson *et al.*, 1986). Due to their clinical significance, the mechanisms behind the effects of these two mutations have been extensively studied and well established. The reason for the decrease in protein secretion caused by expression of a number of other deficiency variants, however, is less clear and, although the clinical effects of these variants may be less severe, the determination of the mechanisms behind the deficiency can only increase the overall understanding of these aberrant proteins.

3.3.1 F & 'P5' variants of α_1-antitrypsin

Recent experiments performed by Lomas and colleagues observed the effects on protein secretion and polymerisation of the very rare, F variant of α_1-antitrypsin ($Arg^{223}\rightarrow$Cys (Fagerhol *et al.*, 1965)). The results indicated a tendency for this mild deficiency variant to polymerise when expressed in bacterial cells (unpublished observations), and, in an attempt to further establish the deficiency

profile of this variant, this study examined its effects on secretion using *Xenopus* oocytes.

Fig 3.5 (c) compares the secretion of protein from oocytes injected with M or F variant mRNA and clearly demonstrates a significant difference ($p<0.01$) between these two variants, as the secretion of F is slightly lower than M (52.7% compared to 63.1%). Previous studies have indicated that F is only a mild deficiency variant (Kelly *et al.*, 1989, Brand *et al.*, 1974), and the small decrease in secretion, compared to M, observed from oocytes also indicates this.

Molecular modelling of intact α_1-antitrypsin (Elliott *et al.*, 1996 (a)), illustrated that Arg^{223} forms interactions with a number of other residues (Arg^{196}, Met^{226}, Arg^{281} and Lys^{243}), in the loop region of the molecule to form a basic pocket centered around one residue – Glu^{254} – located at P5 on the reactive centre loop. Theoretically, if substitution of Arg^{223} disrupts a vital interaction with the P5 glutamic acid residue, then a similar secretory phenotype might be expected from variants mutated at Glu^{254}. To examine this theory, the P5 residue was substituted for glutamine (Q), valine (V) or serine (S) and the secretion of these novel variants from oocytes compared. The data presented in Fig 3.5 (c) indicates that substitution of either valine or glutamine for the glutamic acid normally found at position 354 (P5), shows no significant difference ($p>0.8$ and $p>0.2$ respectively) in secretion of the resultant mutant proteins (62.6% of immunoprecipitable protein secreted for V mutant and 60.1% secreted for Q), compared to the M variant (63.1%). However, the substitution of P5 to a serine residue causes a discernible reduction in relative secretion (52.7%) to a level that is not significantly different ($p<0.99$) to the values determined for the F variant protein (52.8%). Therefore, this specific alteration appears to re-create the mild decrease in protein secretion produced by the mutation of $Arg^{223}\rightarrow$Cys. This suggests an important role for this P5 residue within the putative positively charged 'pocket' structure and the potential for an interaction between the F and P5 residues which, when disrupted, may affect loop insertion and favour intracellular polymerization.

Without detailed structural analysis of the 'P5' variant proteins, it is only possible to speculate on what conformational effects these changes may have. The results presented in this chapter suggest that a charge interaction with surrounding residues, including Arg^{223}, is unlikely to be obligatory as the loss of this charge following mutation to valine or glutamine has no significant effect on secretion. The structures of the amino acids involved in these variants are detailed in Fig 3.9, and illustrates that, as valine and glutamine are of a similar size to glutamic acid, albeit with different electrostatic potentials, substitution of these residues will not create steric effects and this may explain the almost identical secretion of the P5 V and Q variants, compared to M.

The effect of the substitution of glutamic acid to serine at P5 is more informative as it results in a secretory phenotype that is almost identical to the F variant. Comparison of the amino acid structures, in Fig 3.9, shows that arginine, glutamine, valine and glutamic acid all posses long carbon side chains which are absent in both serine and cysteine. The possibility exists that the decreased size of either residue 39 or 254 may decrease the steric constraints and affect the resulting associations of the body of the molecule with the reactive centre loop. Serine and cysteine also possess similar side chain groups (hydroxyl and sulphydryl, respectively) which may permit the formation of inappropriate hydrogen bonds between residues in the reactive centre loop and the body of the protein, altering the position of the loop or preventing the loop from attaining its optimal folding position to allow normal secretion of protein. If the stability of this loop structure is compromised, it may affect the secretion of the protein from the oocyte cell, as the normal processing, secretory or transport elements do not function correctly. A percentage of the protein may be distorted and, therefore, not recognised for secretion and this may lead to the small amount of protein being retained and degraded within these cells.

Observations on examining the rate of secretion of the F variant from oocytes support this theory. Unlike the secretion from variants that are known to polymerize (I and Siiyama), Fig 3.8 illustrates that the secretion of F protein increased steadily over time was still increasing at 24hrs post-

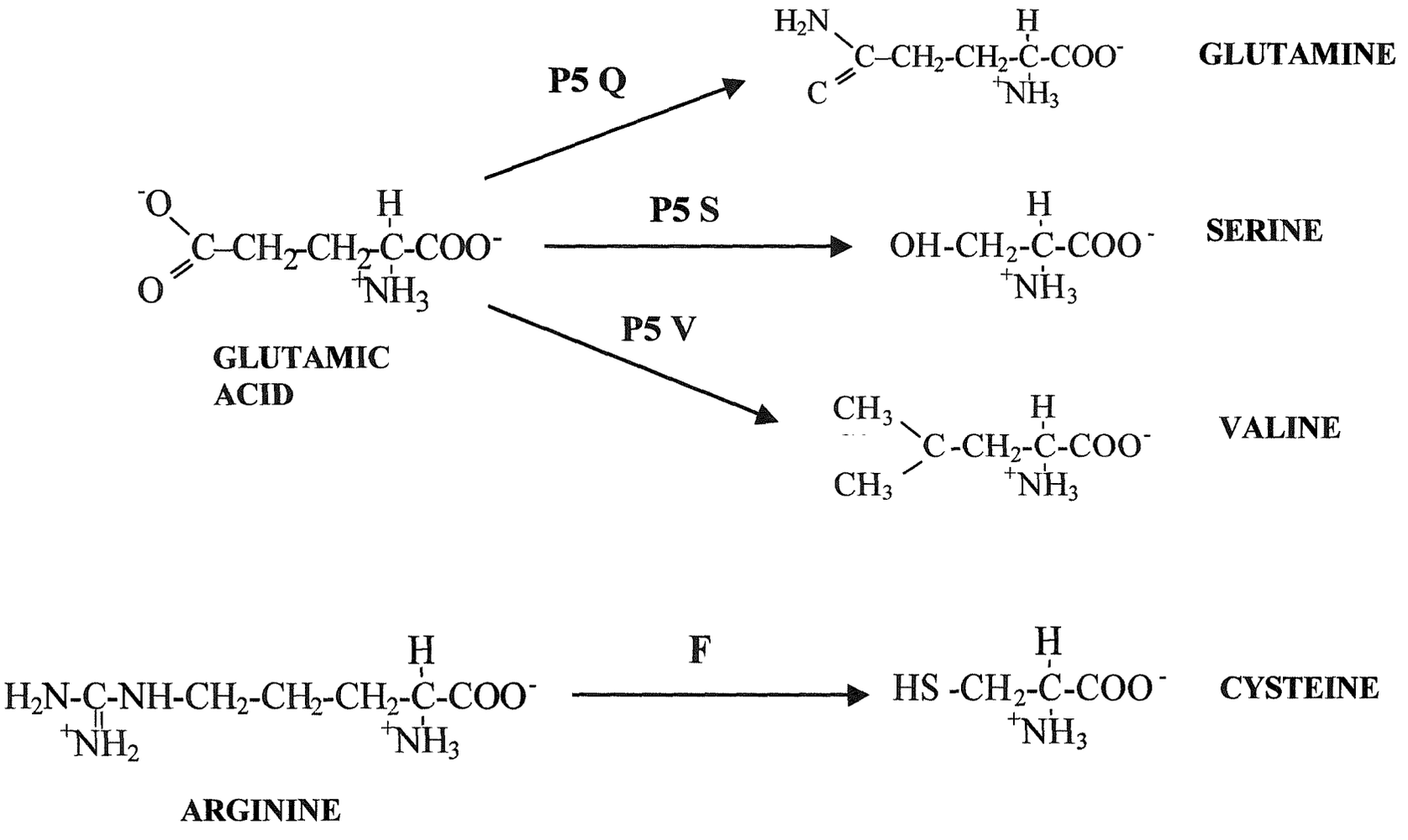


Fig 3.9 Amino acid mutations resulting in the existing F variant, and novel P5 variants (Q, V & S) of α_1-antitrypsin.

incubation. Therefore, it is possible that the presence of the mutation at position 223 may hinder the speed of processing, folding or protein transport of this variant *in ovo*, and this may alter the secretion of the F variant. These results also suggest that secretion of this variant protein may reach even higher levels with increased incubation time and, therefore, the mild secretory deficit observed with the F variant may only be a transient one. This theory was examined but the viability of these oocytes post-24 hours was too low to achieve feasible and accurate results. These results add to the theory that it may be the stabilisation or transport of the F variant protein that is affected within these cells, rather than a blockade of secretion via polymerization.

Although the results presented here do not suggest it, the possibility that this change in loop conformation or mobility makes the protein a candidate for loop-sheet polymerization cannot be dismissed. The alteration of the elements controlling loop structure may fix it in an extended position, away from the body of the molecule, or bind it closer to the protein and increase the chance of insertion of a loop from another molecule into the gap in β-sheet A.

3.3.2 I & S variants of α_1-antitrypsin

The effects on protein secretion of another rare deficiency variant were also examined using the oocyte expression system. Fig 3.6 (c) compares the secretion of protein from oocytes injected with I variant (Fagerhol *et al.*, 1967), and M variant mRNA and indicates that the secretion of the I variant protein is significantly ($p<0.001$) reduced compared to M α_1-antitrypsin injected oocytes (26.1% compared to 63.1%). These results are not in agreement with clinical observations for this variant which suggest only a mild deficiency (~80% of normal levels of secretion (Blundell *et al.*, 1975, Baur & Bencze, 1987, Mahadeva *et al.*, 1999)), but these studies only examined the I mutation in combination with the M or Z allele. Although an II homozygote is yet to be recognised, the results presented here suggest the possibility that a homozygous I phenotype may be

associated with a much severer deficiency than the heterozygous form. However, it is also possible that this variant may be behaving aberrantly in the oocyte system and that the results from these cells are not indicative of the possible *in vivo* effects of this variant protein. A large number of studies (Foreman *et al.*, 1984, Errington *et al.*, 1985, Verbanac & Heath, 1986, Wu & Foreman, 1990, Sidhar *et al.*, 1995), including the work examined here, suggest that the oocyte provides an accurate representation of the effects seen in the liver for every other variant examined. This large body of evidence suggests that the oocyte observations do represent the effects of an I homozygote, but the possibility of an oocyte-mediated effect cannot be dismissed.

Analysis of the secretion of the I variant indicates that, although the total amount of protein secreted from oocytes is increased, the rate of secretion of this variant is similar to Siiyama. In the case of both these variants, the amount of protein secreted increases, to the levels expected from observations discussed in this chapter, and then reaches a plateau at ~12hrs. These results suggest that further secretion, to normal levels, is blocked and this agrees with the theory of the mechanism of loop-sheet polymerization as a major cause of decreased protein secretion (Lomas *et al.*, 1992). The effects of the I variant are not as severe as those of the Siiyama variant (double the amount of protein is secreted following the injection of I variant mRNA compared to Siiyama), and this may have a direct relationship to the position of the mutations in relation to the A β-sheet. Ser^{53} (Siiyama) is located directly under the gap between strands 3 and 5 of the A sheet and mutation to a bulkier Phe residue acts to hold this gap apart and promote loop insertion from another α_1-antitrypsin molecule. Arg^{39} (I), however, lies in the shutter domain of the protein at the base of the sheet and it has been suggested in this study that the mutation of this residue may result in a destabilization of the shutter domain, which may affect sheet mobility. It may be that it is this, more subtle, relationship between mutation and effect that results in the differences in protein secretion between these two variants.

The obvious importance of the nature of the residue at position 39 was examined in more detail based on the suggestion, by Graham *et al.*, in 1989, that the presence of an ionic bond between residues 39 and 264 may have a role in the stabilization of α_1-antitrypsin. A naturally occurring mutation, glutamic acid→valine, related to a decrease in protein secretion to ~60% of normal levels, is already present at position 264 and results in the common S variant of α_1-antitrypsin. Fig 3.6 (c) presents the first results from expression of the S variant in oocytes and demonstrates that secretion of the S variant protein from oocytes is only 49.6%, compared to 81.5% of M protein. This correlates to a decrease in secretion of ~40%, closely mimicking that shown from *in vivo* cells. These results are also in agreement with work by Mahadeva *et al* in 1999, Elliott *et al.*, in 1996 (b) and Curiel *et al.*, in 1989 (a), who, both *in vitro* and *in vivo*, identified that the S variant was only a mild deficiency variant and demonstrated a mild tendency to polymerize.

The difference between the secretion of the I (26.1%) and the S (49.6%) variant protein suggests that, using the oocyte secretory system, it is not simply the disruption of a 'bridge-like' ionic bond between the two residues that results in the decreased secretion of both these variants. However, the effects of the S variant on α_1-antitrypsin secretion have also been linked to another residue – Tyr^{38} (Elliott *et al.*, 1996 (b)). Fig 3.3 shows the proximity of the S, I and Tyr^{38} residues and illustrates the position of Tyr^{38} on helix A within the shutter domain of the protein and how spatial interactions with residue 264 may be possible. To observe the effects of any possible Tyr^{38}-Glu^{264} interactions on the structure and secretion of α_1-antitrypsin, Tyr^{38} was mutated to phenylalanine and cysteine. Fig 3.6 (c) illustrates that injection of oocytes with Tyr^{38}→Phe variant mRNA results in the secretion of only 60% of protein secreted following injection of M mRNA. This also demonstrates a comparable level of secretion, that is not significantly different ($p<0.7$), for the Tyr^{38}→Phe variant (48.6%) as for the S variant (49.6%), of α_1-antitrypsin, when expressed in oocytes.

These observations present the first experimental evidence which supports the structural modelling hypothesis suggested by Elliott *et al.*, in 1996 (b), to explain the mechanism of S α_1-antitrypsin deficiency, who predicted that hydrogen bonding between residues 264 and 38 may be crucial to the stability of the protein. The mutation of the Tyr^{38} residue to a phenylalanine in this study, removed the presence of a hydroxyl group and, thus, prevented this hydrogen bond interaction, with the end result being a decrease in secretion of the protein. Conversely, it is also possible that the decreased secretion caused by the mutation of residue 264 from glutamic acid to valine is also related to the lack of this bond due to the loss of the negatively charged oxygen species, in glutamic acid, which is readily available for hydrogen bonding. These residues are located within the shutter domain of α_1-antitrypsin, and the critical steric requirements within this region are strongly supported by the study of mutations within this region (Kwon *et al.*, 1994, Stein & Carrell, 1995, Sidhar *et al.*, 1995, Lee *et al.*, 1996). Elliott *et al.*, (1996 (b)), have identified that the S mutation has no effect on the conformation of the reactive centre loop and peptide complex experiments (Lomas *et al.*, 1995 (a)), demonstrate that this mutation does not interfere directly with the configuration of the A-sheet. These results, and those from this study, suggest that disruption of the bond from Glu^{264} to Tyr^{38} within the shutter region of the molecule leads to a slight conformational instability of the S variant, which may favour the formation of loop-sheet polymers, as observed *in vitro* (Elliott *et al.*, 1996 (b)). These results increase the evidence for the cause of the deficiency observed with the S variant as, in 1993, Brodbeck *et al.*, determined that the loss of a similar bond between Glu^{264} and Lys^{387}, was not, as first predicted (Loebermann *et al.*, 1984, Long *et al.*, 1984), the prime cause of this deficiency. It is possible that the Arg→Cys change involved in the I variant also affects this region of the molecule, although to a greater extent.

Evidence of the extent of structural alteration by mutation in this area of the protein comes from the 2nd novel mutation of Tyr^{38}→Cys. As Fig 3.6 (b) & 3.7 clearly illustrate, the results from this study suggest that *in ovo* processing of this variant resulted in the formation of higher molecular weight forms of α_1-

antitrypsin which, on further examination, presented as possible Cys-Cys dimers of α_1-antitrypsin. However, the nature of these 'dimers' was not determined and it is possible that these higher molecular weight forms may also be mixed dimers with another protein of approximately the same molecular weight as α_1-antitrypsin, which contains a cysteine residue available for S-S interaction. The native structure of α_1-antitrypsin contains only one cysteine residue (at position 232 (Carrell *et al.*,1982)), that is available for SH-SS interchange reactions (Laurell *et al.*, 1975). Previous work by Tomasi & Hauptman, in 1974, demonstrated that α_1-antitrypsin may complex with IgA via S-S disulphide bond formation involving this residue. The presence of a second Cys residue, also present at the surface of the molecule, may, therefore, increase the possibility of such dimer formation. These results are difficult to interpret but the presence of such protein dimers indicates that the structure of α_1-antitrypsin may be altered significantly so as to allow the presentation of this residue to other available Cys residues of other protein molecules.

This chapter has presented the effects on α_1-antitrypsin secretion of a number of existing and novel protein variants using the *Xenopus* oocyte secretory system. Examination and comparison of the extent of secretion between these variants has led to an increased insight into the possible mechanisms by which secretory deficit may occur and, for the majority of deficiency variants discussed in this chapter, the process of loop-sheet polymerization may be a major factor. The following chapter examines this aberrant process in detail to attempt to determine its effects on α_1-antitrypsin protein at a processing level.

CHAPTER 4

MICROSOMAL PROCESSING OF VARIANT α_1-ANTITRYPSIN

4.1 Introduction

The idea of protein polymerization within the ER correlates effectively with the aggregation of secretory-deficient α_1-antitrypsin variants within hepatocytes. The uncertainty as to whether this polymerization is the 1^{O} cause of the secretory blockade, or simply an epiphenomenon, has led to studies that focus more specifically on the mechanism of ER processing and degradation of α_1-antitrypsin within the cell. The accumulation of ZZ α_1-antitrypsin in the microsomal fraction of *Xenopus* oocytes, as shown by Verbanac & Heath in 1986, suggested that α_1-antitrypsin deficiency may be a result of an alteration in the normal secretory pathway. Following this suggestion, extensive research has been carried out in an attempt to determine the exact mechanism by which variant α_1-antitrypsin is retained within the ER and also how this retention may lead to the formation of insoluble aggregates.

Graham *et al.*, in 1990, and, subsequently, Le *et al.*, in 1992, determined that approximately 80% of Z variant protein produced within the ER consisted of soluble aggregates immediately after protein synthesis. The majority of this variant protein was observed to be degraded before entering the Golgi (Blank & Brantly, 1994, Cox, 1995), leaving only ~5% as aggregates within the liver cells, as 10-15% of the protein was secreted as functional monomers. McCracken *et al.* in 1991, had established that normal levels of Z preprotein are produced and transported into the lumen of the ER. Here it was processed and core-glycosylated, as the M variant, but the rate of movement of Z from ER to Golgi was seen to be dramatically reduced. These observations followed from the experiments of Le *et al.*, in 1990, who demonstrated that by adding a tetrapeptide - KDEL (which acts as a signal for sorting proteins from post-ER compartments back to the ER), to the secreted protein, no intracellular degradation was seen. This indicated that degradation of the protein must occur following ER export, and would explain why the movement from ER to Golgi is reduced under conditions where large amounts of variant protein is produced.

The study of the degradation pathway of variant α_1-antitrypsin has focused particularly on this section of the secretory pathway and has led to a number of

theories as to why not all of the variant protein produced is degraded. Experimental evidence has determined that the retained fraction of Z and other (e.g. Null HONG KONG variant), secretory-deficient proteins is removed from the cell via a pathway that is unaffected by inhibitors of lysosomal protein degredation, or inhibitors of ER→Golgi intracellular protein transport (Le *et al.*, 1990, Graham *et al.*, 1990, Le *et al.*, 1992, Teckman & Perlmutter, 1996). This intracellular degradation is, however, prevented by inhibitors of protein synthesis such as cyclohexamide and specific proteasomal inhibitors (Teckman & Perlmutter, 1996, Le *et al.*, 1992, Novoravdovskaya *et al.*, 1998), suggesting a proteasome-mediated pathway for the degradation of variant α_1-antitrypsin within the ER (Qu *et al.*, 1996). However, it is still not clear if this ER degradation is mediated by one proteolytic pathway, several distinct proteolytic pathways, or several pathways that interact.

Further investigation has suggested a role for the molecular chaperone calnexin in the degradation of variant α_1-antitrypsin. Wu *et al.*, in 1994 and Qu *et al.*, in 1996, both identified interactions between accumulated Z protein and calnexin within the ER. The possibility exists that this interaction may be necessary for initiation of ER degradation or to direct the molecule through the degradation pathway. Genetic traits or environmental factors may also have a role in the degradation or accumulation of variant α_1-antitrypsin. The fact that only 10-15% of ZZ homozygotes develop liver disease leads to the theory that there may other factors involved in the retention of the variant protein. It has been suggested (Wu *et al.*, 1994, Teckmann & Perlmutter 1996), that some subjects may be more susceptible to the aggregation of variant protein. A less effective protein degradation pathway, a defective or less efficient calnexin mediated effect or any other factor that may delay intracellular protein degradation could result in the aggregation of variant protein which would, under normal circumstances, be removed and degraded.

The observations from previous experiments, as discussed above, have presented a number of theories as to why the blockade in secretion of variant α_1-antitrypsin within the liver cell may result in retention and aggregation of this abherrant protein, rather than its degradation and removal from the cell. It is still not clear, however, whether one or a number of different mechanisms are involved in this process (Loayza *et al.*, 1998). Closer examination and manipulation of the ER and

Golgi processing and transport mechanisms of variant α_1-antitrypsin proteins is required to determine the relationship between the polymerization of protein and aggregation formation within the hepatocyte. It may then be possible to attain why a percentage of aggregates of variant protein are not degraded and if the extent of polymerization of the protein has any role in its retention.

The experiments presented in this chapter have been performed in an attempt to closely examine the ER and Golgi processing mechanisms of normal and variant α_1-antitrypsin in relation to polymerization. The use of canine microsomal membranes (a fraction of closed vesicles derived from the RER), in both cell-free *in vitro* (Eriksson *et al.*, 1981, Katz *et al.*, 1977), and *in ovo* systems has provided a novel means of dissecting this processing pathway and allowed close analysis of the ER processing of normal and secretory-deficient variants α_1-antitrypsin. The addition of these microsomes to a cell-free translation system results in the formation of a protein that has undergone a high degree of processing (up to pre-Golgi stage) (Verbanac & Heath, 1986). The protein produced in this *in vitro* system can be manipulated and studied to this stage in the secretory pathway whilst addition of this partially processed protein to a *Xenopus* oocyte system allows the late and post-ER section of the pathway to be studied. Techniques such as this may allow a definitive examination of the relationship between polymerization and secretion of mutant α_1-antitrypsin and, therefore, suggest the mechanism at work in other secretory deficiency disorders.

4.2 Results

4.2.1 Production of canine microsomal membranes and effects on protein processing of α_1-antitrypsin mRNA in an *in vitro* translation system

In vitro translation of M and Z α_1-antitrypsin transcripts in the presence of canine microsomes indicated that the processing capability of these commercially produced microsomes was very low (only 27% of the protein produced was fully glycosylated). Therefore, for the purpose of this study, canine microsomal membranes (microsomes) were isolated and prepared as described by Walter & Blobel in 1983 (see methods – section 2.12).

Fig 4.1 shows the processing of commercial microsomes (2 units per 10µl translation mix) compared to the equivalent number of units of microsomes produced in this lab. The results clearly illustrate the increased percentage of M α_1-antitrypsin protein that was fully glycosylated following the addition of prepared microsomes (72.25% ± 1.44) compared to commercially produced microsomes (27.75% ± 4.59). This high processing ability of the prepared microsomes provided an ideal tool to study the effects of protein processing *in vitro* and *in vivo*. The quantity of microsomes added to the cell-free translation system was then varied to determine the number of units required for the highest percentage and, therefore, most efficient glycosylation of the protein. Fig 4.2 demonstrates the amount of fully glycosylated M α_1-antitrypsin protein produced in a cell-free translation system in the presence of varying concentrations of microsomes (0.5, 1, 2 and 3 units), produced in this study and 2 units of commercially produced microsomes (Promega UK). The high percentage of glycosylation (72.25%), indicates that addition of 2 units of microsomes in a standard translation mix results in optimal processing of protein in this *in vitro* system.

Fig 4.3 provides a visual representation of the processing efficiency of increasing concentrations (0.5, 1 and 2 units), of microsomes in the presence of both M and Z variant α_1-antitrypsin. Examination of this gel clearly illustrates the presence of fully processed, high molecular weight protein (52kDa) following the addition of microsomes to the translation system as well as the presence of non-glycosylated

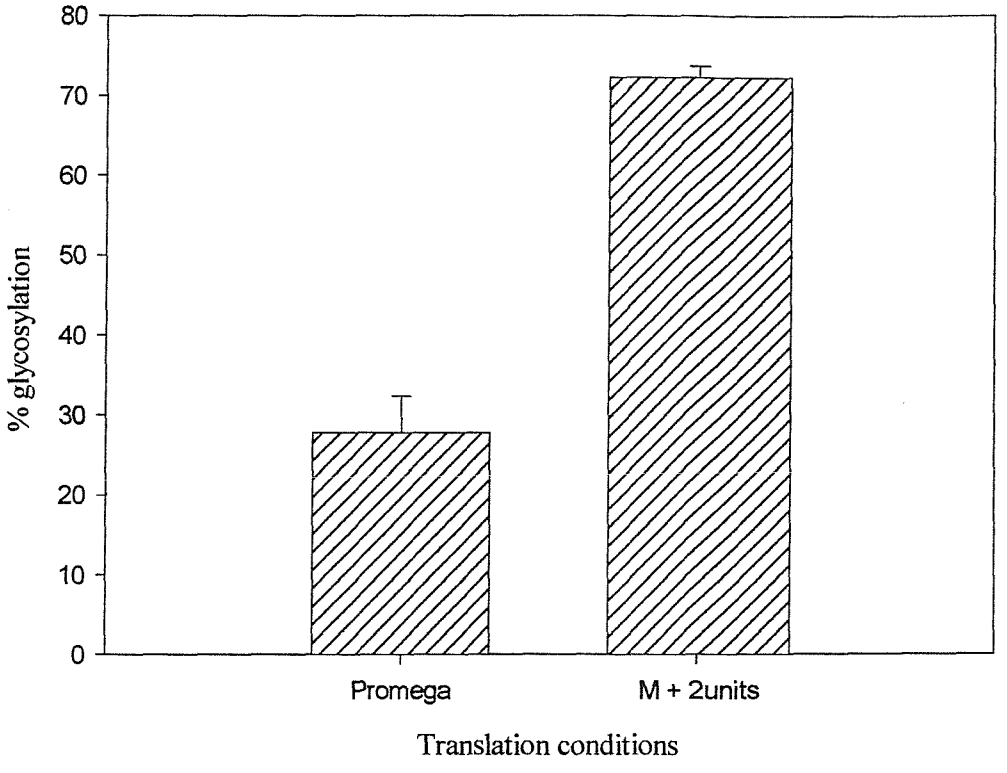


Fig. 4.1 Quantitation of the relative amounts of percentage total glycosylation (3 sites) of translocated M variant α_1-antitrypsin in the presence of manufactures recommended quantity of microsomal membranes (Promega) and an equivalent quantity of microsomes produced in this laboratory (M + 2units). Values are expressed as the arithmetic mean ± the S.E.M and N=>3 for each data set.

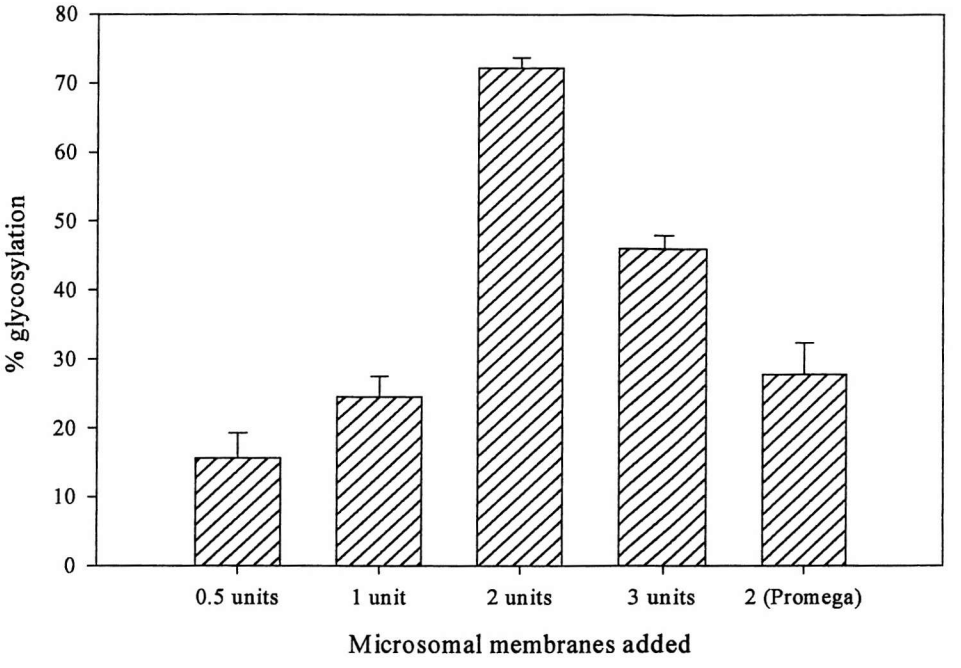


Fig. 4.2 Quantitation of the relative amounts of percentage total glycosylation (3 sites) of translocated M variant α_1-antitrypsin in the presence of manufactures recommended quantity of microsomal membranes (Promega) and varying quantities of microsomes produced in this laboratory (0.5, 1, 2, & 3 units). Values are expressed as the arithmetic mean ± the S.E.M and N=>3 for each data set.

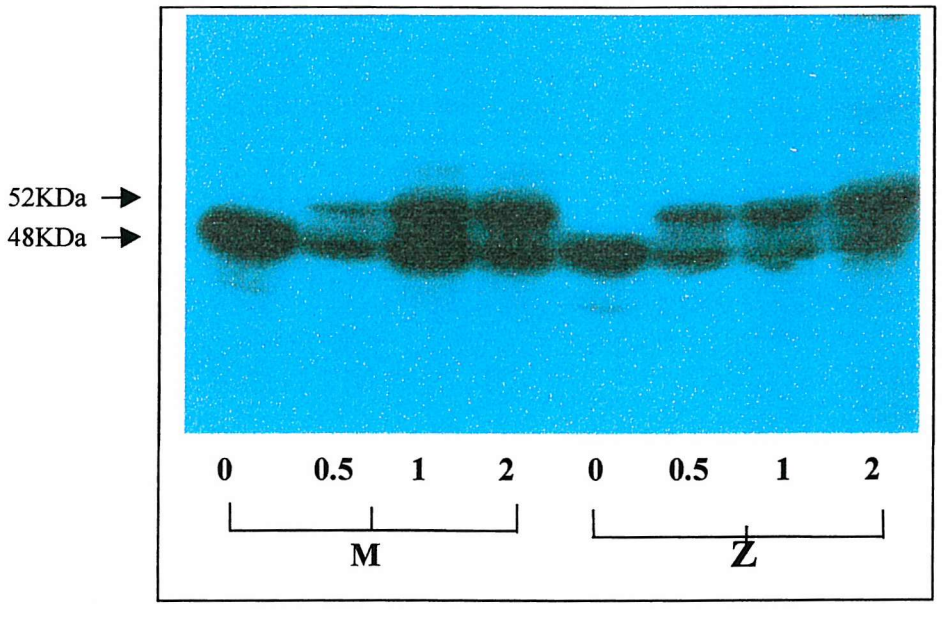


Fig 4.3 Translation of α_1-antitrypsin mRNA in a cell-free reticulocyte lysate system in the absence (0) and presence (0.5, 1, 2), of varying concentrations of microsomal membranes. Numbers relate to units of membranes added to a standard reticulocyte lysate mix. Bands show comparison between translation of M and Z variant α_1-antitrypsin mRNA. Molecular weights were determined by co-migration of standard protein markers.

(48kDa) protein. Both M and Z variants are equally well translated and sequestered in this *in vitro* system.

This pattern of secretion is consistent with expected microsomal processing of the primary translation product which involves removal of the proteins signal peptide and the addition of core oligosaccharide side chains (Lodish *et al.*, 1983). As the concentration of microsomes added increases so does the quantity of the high molecular weight, glycosylated form (52kDa) compared to the unglycosylated (48kDa) form of the protein. This glycosylated material was determined to be resistant to proteolysis by Proteinase K, but not following pre-treatment with Triton X-100 (data not shown), indicating that the protein is sequestered within the microsomal membranes.

In an attempt to further increase the accuracy of the results obtained from studying the processing efficiency of prepared microsomes *in vitro*, the membranes were pre-treated with nuclease to deplete the rough microsomes of any endogenous mRNA activity, following the methods of Pelham & Jackson in 1976. Fig 4.4 compares the protein processing of M and Z α_1-antitrypsin in the presence of Nuclease-treated microsomes, non-treated microsomes and in the absence of microsomes. The results demonstrate that, in this study, the nuclease pre-treatment has abolished the processing capabilities of the microsomes in this *in vitro* system as the higher molecular weight, glycosylated, protein is not present, compared to the non-treated membranes.

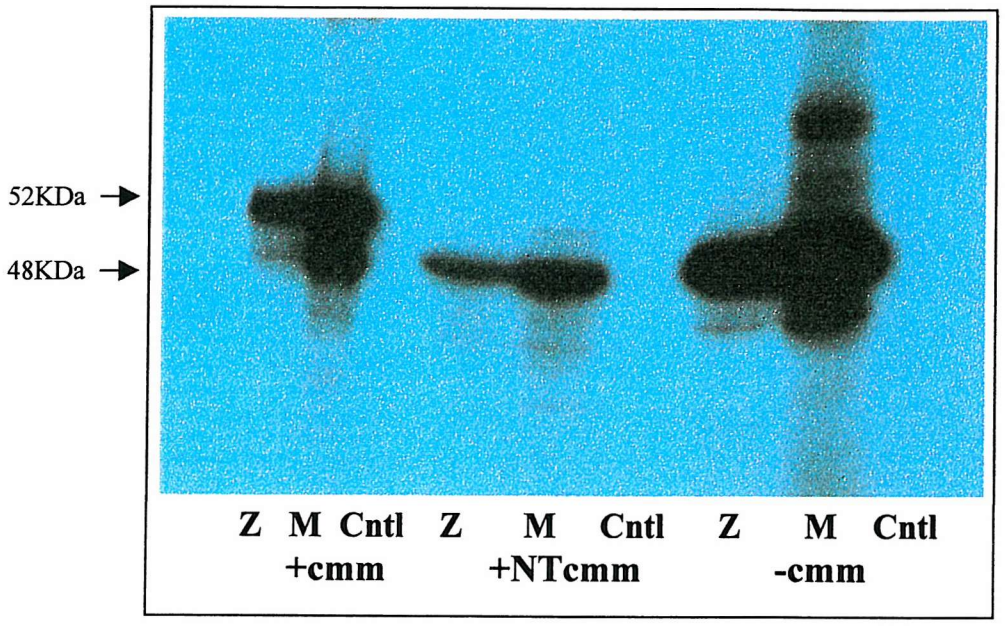


Fig 4.4 Autoradiograph illustrating translational efficiences of M and Z variant α_1-antitrypsin RNA in the presence of canine microsomal membranes (+cmm), nuclease treated cmm (+NTcmm), and in the absence of cmm (-cmm). Cntl bands represent translation in the absence of RNA.

4.2.2 The nature of microsomally sequestered α_1-antitrypsin

Microsomes, containing fully glycosylated α_1-antitrypsin protein, were examined using Native-PAGE to determine the presence of any higher molecular weight forms of α_1-antitrypsin that would suggest the polymerization or aggregation of protein within the microsomes after *in vitro* translation. Microsomal fractions were retrieved from the translation mix using centrifugation through a sucrose gradient. Fig 4.5 indicates no higher molecular weight form of α_1-antitrypsin could be detected within the microsomal fraction containing M or Z translation product suggesting no polymerization occurs at this early stage in protein processing.

The absence of high molecular weight forms within the microsomes suggested that the mechanism of polymerization or aggregation of aberrant protein does not occur under these *in vitro* conditions. However, previous results discussed in this study have demonstrated that a blockade of protein secretion is seen to occur *in ovo* following injection of secretory-deficient variants of α_1-antitrypsin. As the *in vitro* microsomes only represent the processing pathway of the protein to an ER stage, the process of aggregation, that may lead to the blockade in secretion seen in oocytes, may occur following ER processing. A set of experiments were then performed, designed to examine the processing of normal and secretory-deficient variants of α_1-antitrypsin *in ovo*, and observe the state of the protein within the secretory pathway of the oocyte, compared to that observed within the microsomal system.

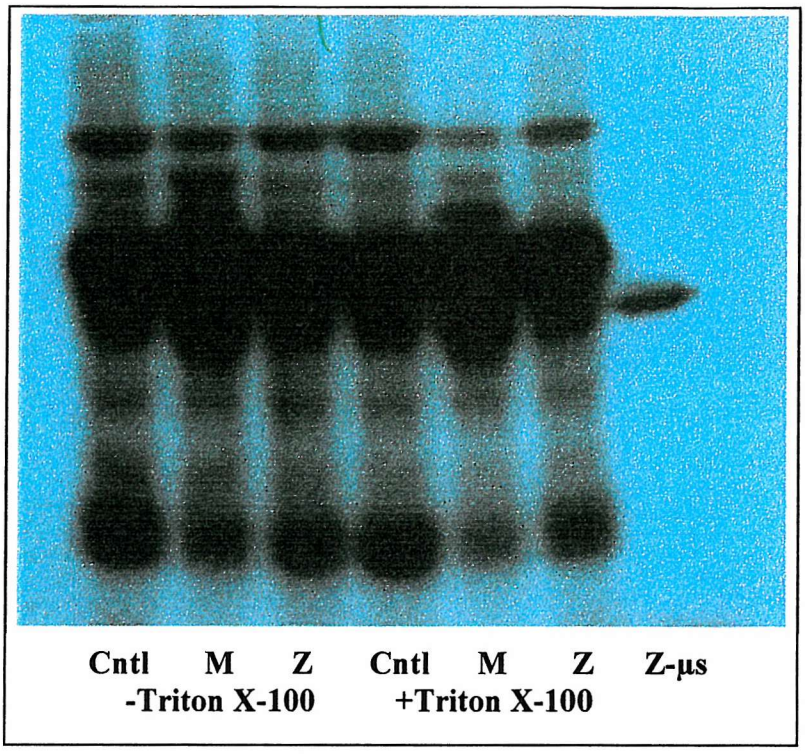


Fig 4.5 Autoradiograph of Native-PAGE gel illustrating the radiolabelled protein contents of microsomal membranes following translation of α_1-antitrypsin mRNA in a cell-free reticulocyte lysate system. Bands show comparison between translation of M and Z variant α_1-antitrypsin mRNA, and also show the effects of gel analysis following treatment with (+) and without (-) Triton X-100, used to permealise the external microsomal membranes. Cntl bands show the effect of Triton on microsomes translated in the absence of α_1-antitrypsin mRNA.

4.2.3 Injection of α_1-antitrypsin RNA and isolation of protein from *Xenopus* oocyte secretory pathway fraction

The secretion of α_1-antitrypsin protein following microinjection of mRNA into *Xenopus* oocytes, as described in Chapter 3, has been observed to closely mimic the protein secretion from liver cells. However, the location and process of this protein accumulation within the secretory pathway of the oocyte itself has not been investigated. Previous experiments in this study have indicated the absence of any accumulated protein following processing of normal and secretory–deficient α_1-antitrypsin protein to an ER stage, using an *in vitro* system in the presence of microsomal membranes. In comparison, the results from mRNA injected oocytes have shown that this protein must accumulate or be degraded within these cells, as secretory deficient phenotypes are retained. In order to determine whether these patterns of secretion were due to oocyte related effects or to a similar process of aggregation that occurs within the *in vivo* liver cell, the contents of the oocyte secretory pathway were isolated from the remainder of the cell and examined. The presence of aggregated protein, combined with the absence of such aggregates following *in vitro* microsomal protein processing, as discussed in section.4.2.2, would indicate that this aggregation occurs at a late- or post-ER stage of the proteins processing.

Fig 4.6 illustrates the form of α_1-antitrypsin protein contained within whole oocyte extract. The isolation of retained α_1-antitrypsin protein (oo) clearly shows the presence of high molecular weight protein in the Z and Siiyama variant α_1-antitrypsin, but not in the M α_1-antitrypsin injected oocytes. These results suggest that aggregates of both the secretory-deficient genetic variants were localised in the secretory pathway of mRNA injected oocytes and suggests that the reduced secretion of the Z and Siiyama variants is due to a form of aggregation similar to that seen in the retention of these variant proteins in liver cells. This pattern of aggregation *in ovo* is also different to that seen *in vitro* within the microsomal membranes, demonstrating that within the secretory pathway of oocytes, a form of aggregation/association of the protein can be detected that is not present when only examining the early part of the pathway reconstructed *in vitro*.

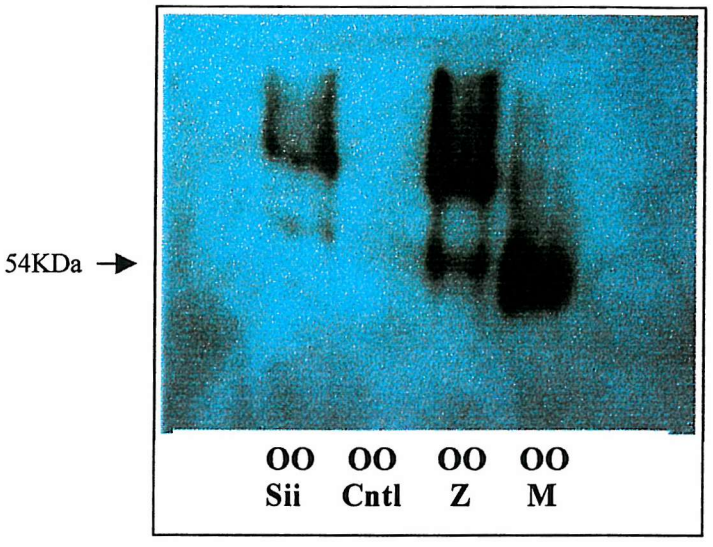


Fig 4.6 Autoradiograph showing Western Blotting of α_1-antitrypsin. Bands show comparison between the contents of the secretory pathway of oocytes (oo) injected with M variant α_1-antitrypsin and deficiency variant Z and Siiyama mRNA. Control lanes show the results from uninjected oocytes.

The secretion of the I and F variants of α_1-antitrypsin was also examined using this technique. Previous experiments, described in Chapter 3, demonstrated that injection of I and F variant mRNA results in a decrease in secretion of α_1-antitrypsin protein from oocytes. Observations following isolation and examination of the secretory pathway of oocytes injected with I mRNA clearly show the presence of some high molecular weight forms, although not as much as observed with Siiyama or Z, within the microsomal fraction of oocytes (as illustrated in Fig 4.7). However, these high molecular weight forms of α_1-antitrypsin are not observed following injection of F α_1-antitrypsin mRNA (shown in Fig 4.8). Therefore, as seen with the Z and Siiyama variants, the decreased secretion of I α_1-antitrypsin protein from oocytes is related to the presence of high molecular weight forms of the protein within the cells secretory pathway. This higher molecular weight form is absent, or not present in as high concentrations, in the F variant, increasing the evidence for the theory presented in Chapter 3, that the decrease in secretion resulting from expression of this variant is not related to polymerization of the protein.

The experiments described so far in this chapter enabled us to establish that elements of the secretory pathway, at a late- or post- ER stage, have an important role in the retention of identified 'secretory-deficient' variants of α_1-antitrypsin. Protein produced *in vitro,* using the microsomal translation experiments, was observed to be in a monomeric, fully glycosylated form for both normal and secretory-deficient variants, whereas the protein retrieved from within the oocytes secretory pathway clearly indicated the presence of protein aggregates related to secretion-deficient variants only. By combining these techniques and injecting protein loaded microsomes into the foreign oocyte system, the progression of α_1-antitrypsin protein can be observed from the non-polymerized, microsomal sequestered material through to the secretion of a mature protein. As the injected protein is known to be monomeric, the presence of aggregates of protein within the oocyte would add further evidence to the theory that these aggregates of protein form at a late- or post-ER stage within the secretory pathway of the oocytes, similar to that suggested to occur within liver cells.

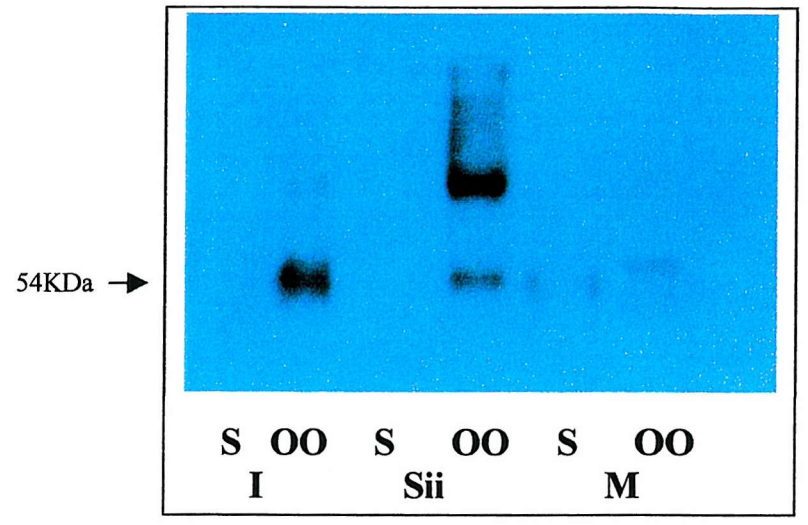


Fig 4.7 Autoradiograph illustrating Western Blotting of α_1-antitrypsin from *Xenopus* oocytes. Bands show comparison between protein detected from whole oocyte extracts (oo) and incubation media (s) from oocytes injected with M and deficiency variant I and Siiyama (Sii) RNA.

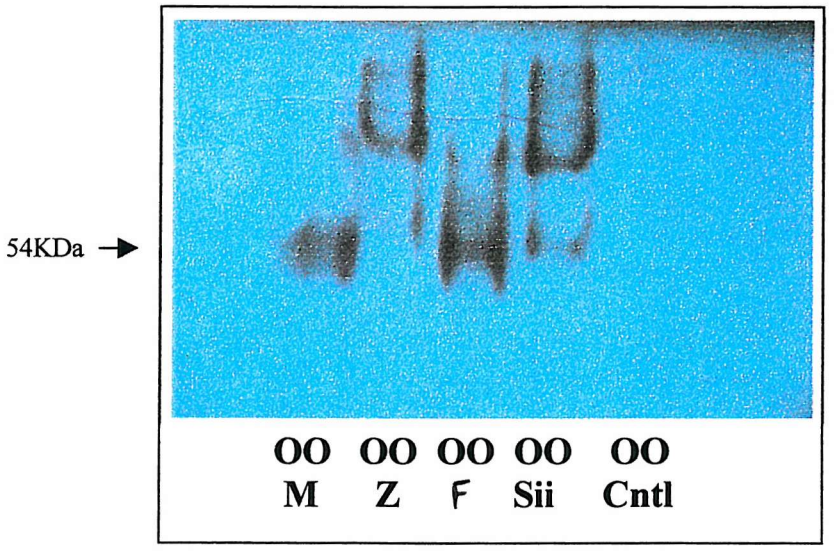
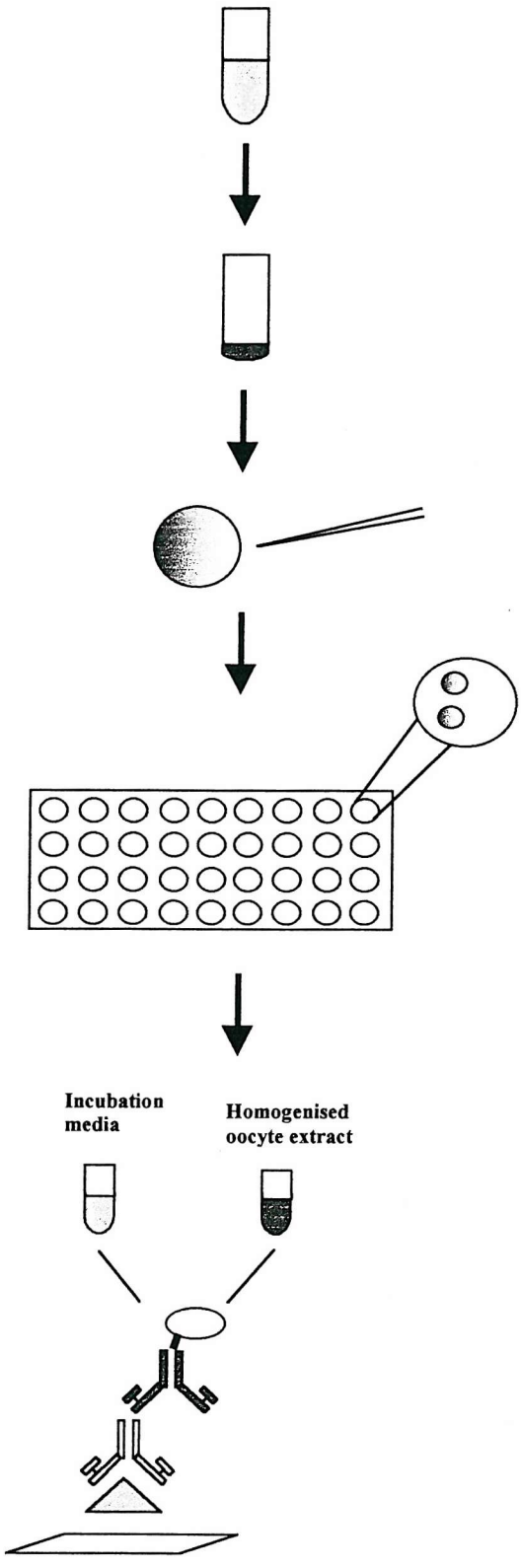


Fig 4.8 Autoradiograph illustrating Western Blotting of α_1-antitrypsin protein retained within the oocyte secretory system. Bands show comparison between the contents of the secretory system of oocytes (oo) injected with M variant α_1-compared to Z, F and Sii variant α_1-antitrypsin mRNA. Cntl lane shows the results from non-injected oocytes. Molecular weights were determined by co-migration of biotinylated standard protein markers.

4.2.4 Injection of microsomal membranes into *Xenopus* oocytes

In 1990, Paiement *et al.*, introduced the technique of injection of intact rat liver microsomes into the *Xenopus* oocyte secretory system. Fragments of RER and Golgi purified from rat liver homogenate were injected into oocytes and the sites of microinjection analyzed by electron microscopy at different times post-injection. Typical ER microsomes were noted to disappear with time to be replaced by flattened cisternae of ER. These results suggest that the injected organelle fragments underwent transformation *in vivo* as a consequence of reconstitution and supported previous observations (Paiement *et al.*, 1989), that dispersed fragments of rat liver Golgi complex (i.e. unstacked vesicles and tubules) also reconstituted into stacked saccules when microinjected into oocyte cytoplasm. These studies suggested that it is possible to inject amphibian oocytes with cellular subfractionations, which can then regain form within the foreign cell. This study aimed to take these experiments further and attempt to produce a functional incorporation of mammalian ER vesicles into the amphibian secretory apparatus, as illustrated in Fig 4.9, thus assembling a functional hybrid secretory system.

Microsomes loaded with α_1-antitrypsin protein, as described in section 4.2.2, were treated with 25mM EDTA (Walter & Blobel, 1983). EDTA treatment was performed to remove ER bound ribosomes and their associated newly synthesised polypeptide chains. Thus, there would be little possibility of mRNA being introduced into the oocyte and being translocated by the oocyte machinery, which could complicate the interpretation of results. Prior to the injection of EDTA treated microsomes into oocytes, it was important to determine any significant effects of EDTA on the processing of α_1-antitrypsin. M, Z and Siiyama variant α_1-antitrypsin was translated *in vitro* in the presence and absence of EDTA (25mm) and Fig 4.10 (a) visually illustrates that the presence of EDTA during translation of α_1-antitrypsin protein has no visible effect on the glycosylation efficiency and processing of microsomes *in vitro*. The quantification of excised gel bands in Fig 4.10 (b), verifies that any affect of EDTA of microsomal processing is not significant ($p>0.5$).



1. Translocation of α_1-antitrypsin + microsomal membranes + ^{35}[S] met.

2. Microsomal fraction isolated by centrifugation though 0.5M sucrose cushion.

3. Recovered microsomes microinjected into *Xenopus* oocytes

4. Incubation in SOS for 16hrs.

5. Collection of incubation media (secreted protein fraction). Homogenisation of oocytes (retained protein fraction).

6. Analysis of protein product via Native-PAGE and Western Blotting with α_1-antitrypsin specific antibodies.

Fig 4.9 Procedure for isolation of α_1-antitrypsin protein secreted from *Xenopus* oocytes following injection with protein loaded microsomal membranes.

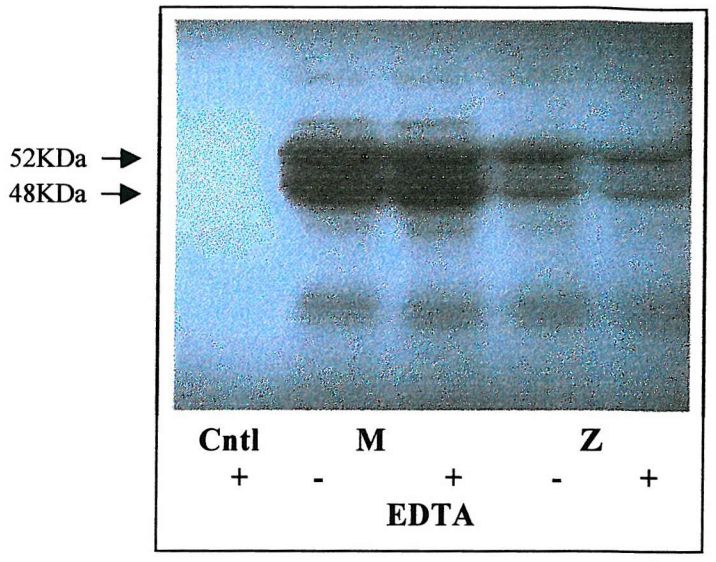


Fig 4.10 (a) Autoradiograph illustrating α_1-antitrypsin protein bands following translation of α_1-antitrypsin mRNA in a cell-free reticulocyte lysate system in the presence of microsomal membranes. Bands show comparison between translation of M variant and Z variant α_1-antitrypsin mRNA, and show the effects of the presence (+) and absence (-) of 25mM EDTA on protein translation and processing. Molecular weights were determined by co-migration of standard protein markers. Cntl bands illustrate incubation of microsomal membranes in the presence of EDTA only.

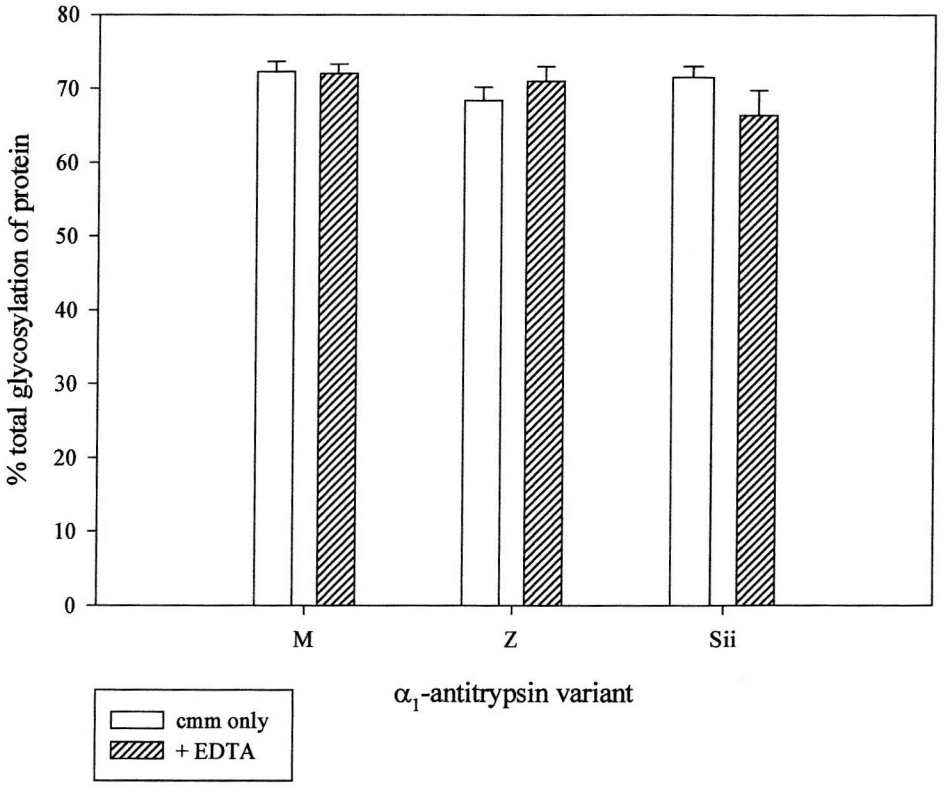


Fig. 4.10 (b) Quantitation of the relative amounts of percentage total glycosylation (3 sites) of translocated M, Z & Siiyama variant α_1-antitrypsin in the absence (cmm only) and presence (+ EDTA) of 25mM EDTA. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Values are expressed as the arithmetic mean ± the S.E.M and N=>3 for each data set.

Radiolabelled, microsomally sequestered α_1-antitrypsin was injected into *Xenopus* oocytes and the secreted and retained protein analysed, as shown in Fig 4.11 (a). Cells were incubated for ~16hrs to allow sufficient time for the injected microsomes to assemble with the oocyte secretory pathway and the protein to be expressed. The results from oocyte cells incubated for longer than this time period were observed to be inconclusive as the integrity of the cells was decreased and leak of cell contents was observed. The secretion of the radiolabelled high molecular weight form of the M α_1-antitrypsin protein suggests that, using this technique, the injected microsomes have successfully integrated into the processing pathway of the foreign oocyte cell at a pre-golgi stage, from where processing is completed and the mature protein is secreted as normal. This figure also demonstrates that radiolabelled Z α_1-antitrypsin protein is detected within the oocytes and a higher molecular weight secreted form is present in the incubation media. Fig 4.11 (b) quantifies the deficiency in secretion produced following injection of microsomes loaded with partially processed Z (17% ± 2.6), and also Siiyama (9%) α_1-antitrypsin protein compared to M (37% ± 4.9). These results follow the pattern of secretion observed in oocytes injected with mRNA, as detailed in Chapter 3, and demonstrates that the secretory defect produced by the Z and Siiyama mutations is retained within this hybrid system, even though the sequestered protein that was injected was determined to be monomeric. The decrease in secretion of M α_1-antitrypsin, compared to mRNA injected oocytes, is predicted to be due to the limitations for protein secretion placed on the cell, as only the amount of protein contained within the microsomal membranes is available for processing and secretion. Total incorporation of the microsomes injected with the oocyte secretory system is also unlikely, reducing the available protein further.

The results from these experiments, combined with those discussed in section 4.2.3, suggest that a mechanism exists that results in the formation of aggregates/polymers of the protein within the oocyte following vesicular fusion. To examine this theory, the secretory pathway of oocytes injected with microsomally-sequestered α_1-antitrypsin was isolated and analysed. Fig 4.12 shows that higher molecular weight forms of Z and Siiyama, but not M, protein are present within these cells. This indicates that the injected material, experimentally determined to be monomeric, is subsequently polymerizing/aggregating within the oocyte.

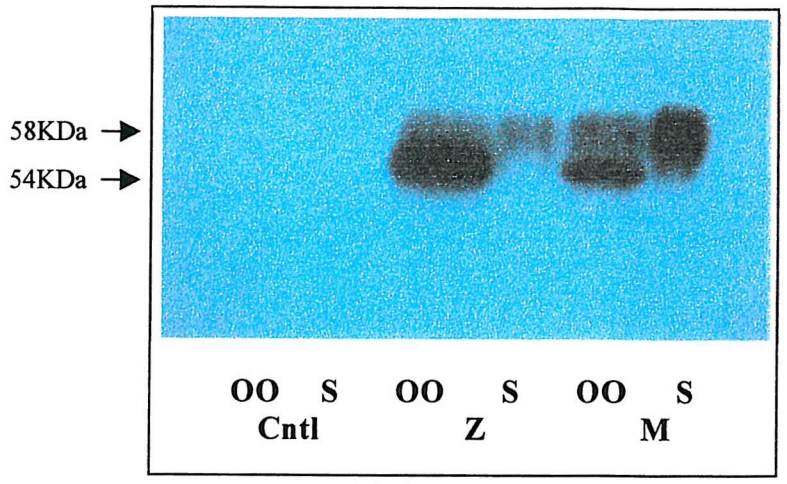


Fig 4.11 (a) Autoradiograph illustrating immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Bands show comparison between oocytes injected with microsomal membranes containing M variant α_1-antitrypsin translated protein and Z variant α_1-antitrypsin translated protein. Molecular weights were determined by co-migration of standard protein markers. Cntl bands illustrate oocytes injected with microsomes containing no α_1-antitrypsin protein.

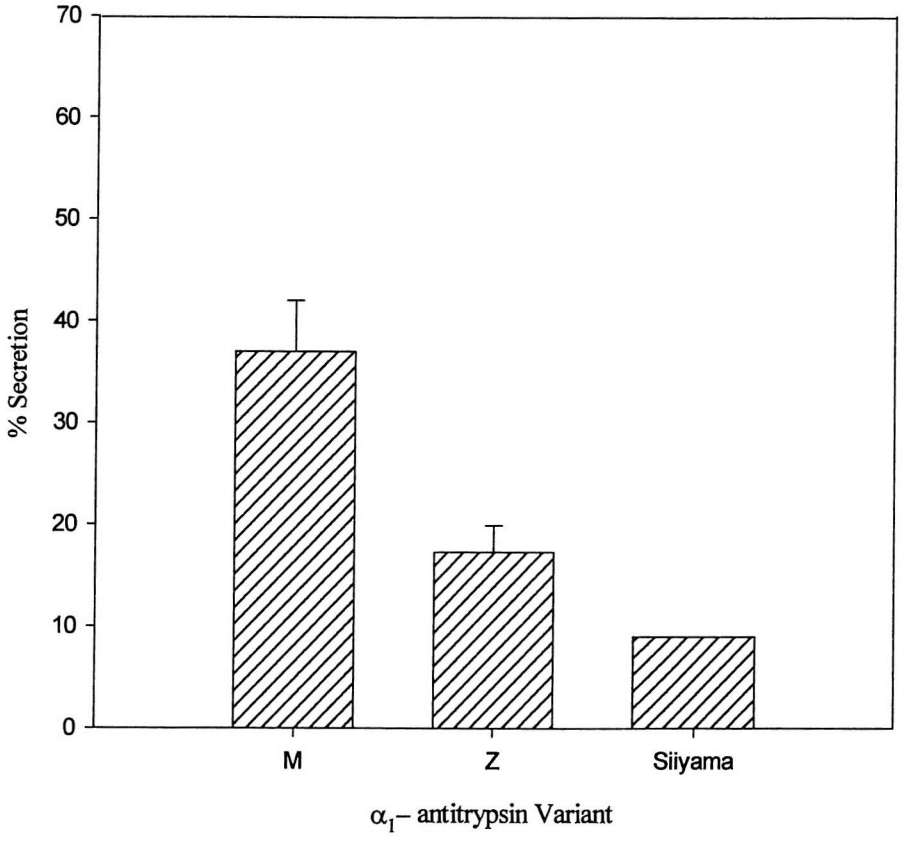


Fig. 4.11 (b) Quantitation of the relative amounts of M, Z and Siiyama α_1-antitrypsin secreted from oocytes injected with protein containing microsomes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of the total amount of inhibitor synthesised in oocytes. M and Z columns represent the mean of at least 5 different experiments, labelling at least 15 oocytes each. Siiyama represents the labelling of 5 different oocytes. Values are expressed as the arithmetic mean ± the S.E.M.

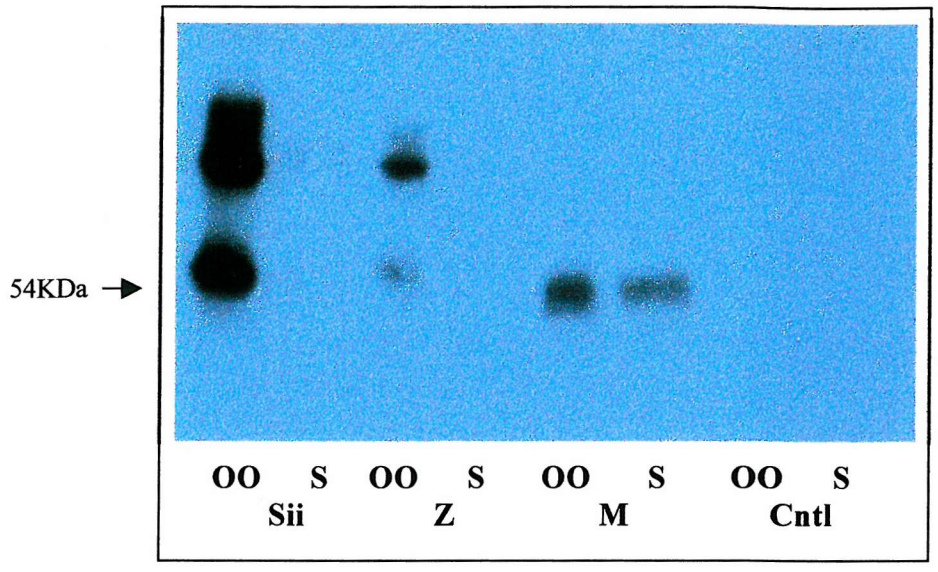


Fig 4.12 Autoradiograph illustrating Western Blotting of α_1-antitrypsin from*Xenopus* oocytes injected with protein loaded microsomes. Bands show comparison between protein detected from whole oocyte extracts (oo) and incubation media (s) from oocytes injected with M variant and deficiency variant Z and Siiyama (Sii) RNA. Control lanes show the results from uninjected oocytes.

Microsomes were also treated, pre-injection, with RNase (1μg/ml), to remove any endogenous RNA that may be present on the surface of the membranes following translation and ensure there is no residual translation of mRNA in the oocytes. Fig 4.13 (a) visually compares the secretion (s) and retention (oo) of EDTA- and RNAse-treated microsomes from oocytes and clearly shows that this treatment appears to reduce the total amount of protein secreted from the oocyte cells. Fig 4.13 (b) quantifies the relative secretion of M and Z protein secreted and actually demonstrates that, although the total amount of protein secreted is decreased, the actual percentage secretion of both M and Z α_1-antitrypsin protein is slightly increased (secretion of M protein increased by 25%, secretion of Z protein increased by 15%), following RNAse pre-treatment. This increase in secretion is difficult to interpret and may due simply to oocyte variation or an error in protein quantification due to the very low protein secretion (as illustrated in Fig 4.13 (a)).

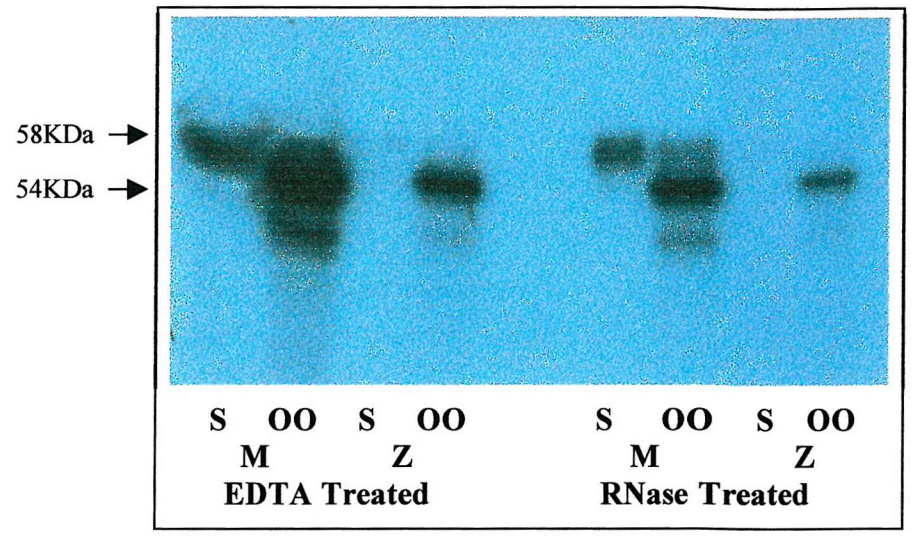


Fig 4.13 (a) Autoradiograph illustrating α_1-antitrypsin protein from *Xenopus* oocytes following injection of EDTA or RNase pre-treated microsomal membranes. Bands show comparison between protein detected from incubation media (s) and oocyte extract (oo) in oocytes injected with M and deficiency variant Z α_1-antitrypsin containing microsomes.

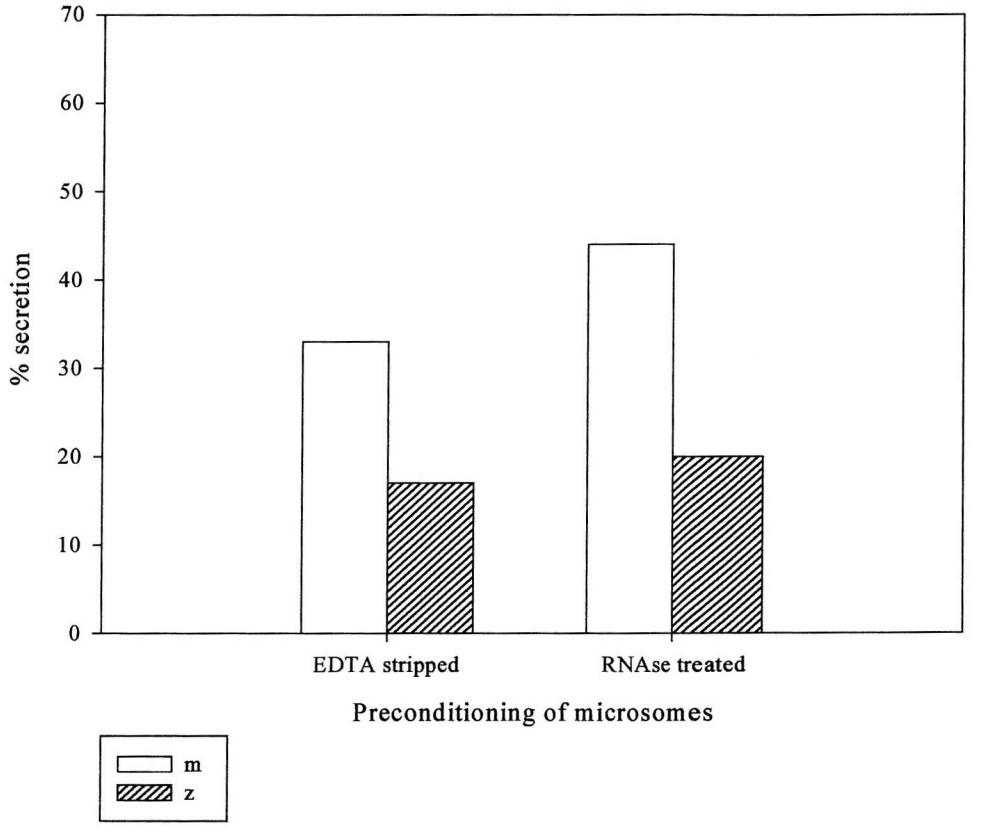


Fig. 4.13 (b) Quantitation of the relative amounts of M and Z α_1-antitrypsin secreted from oocytes injected with protein containing microsomes. Bands were excised from gels and radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of the total amount of inhibitor synthesised in oocytes. Each column represents labelling of at least 15 oocytes. Values are expressed as the arithmetic mean ± the S.E.M.

4.3 Discussion

In this study, the form and presence of normal and secretory deficient α_1-antitrypsin protein has been examined at different stages of the protein secretory pathway using a variety of *in vitro* and *in ovo* techniques.

The findings described in this chapter indicate that the presence of isolated microsomes in an *in vitro* translation system containing α_1-antitrypsin results in the processing of the protein to the expected pre-golgi stage. As illustrated in Fig 4.3 SDS-analysis of translocated M and Z variants of α_1-antitrypsin produced processing to a 52kDa fully glycosylated species. α_1-antitrypsin possesses 3 glycosylation sites and protein was observed at all 3 stages, indicating that the microsomes used in this study were an efficient and valid system for analysis of protein processing *in vitro*. These results also demonstrated that, under these conditions in the presence of microsomes, the processing of Z variant α_1-antitrypsin is like that of the M variant. Therefore, it is observed that, to the ER stage of the secretory pathway, there appears to be no effect of the Z mutation on the stabilization or processing of α_1-antitrypsin protein. As a further comparison between M and Z α_1-antitrypsin, the form of the protein sequestered within the microsomal vesicles was also examined and, as illustrated in Fig 4.5, no evidence of higher molecular weight forms of protein were observed within these vesicles, establishing the protein as a 'normal' monomeric, glycosylated 52kDa species both in the M and Z forms.

These *in vitro* observations suggest that the blockade in the secretion of secretory-deficient variants of α_1-antitrypsin observed in Chapter 3 must occur at a post-ER stage within the cell. This theory was investigated by examining the form of α_1-antitrypsin protein within the secretory pathway of oocytes. The results presented in Fig 4.6 clearly illustrate the presence of high molecular weight forms of protein within the pathway of oocytes injected with Z and Siiyama, but not M, variant α_1-antitrypsin. This suggests that the mechanism of retention of these variants within the oocytes is similar to that seen within *in vivo* liver cells (Carrell *et al.*, 1991, Lomas *et al.*, 1992), and results in the formation of higher molecular weight forms of the protein via a form of aggregation/ polymerization. These results are also in

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5.1 Introduction

The results observed and discussed in Chapters 3 & 4 have provided an insight into the effect of a number of specific variants of α_1-antitrypsin on the secretion of the protein from the *Xenopus* oocyte system. These alterations in secretion have been examined in detail in an attempt to understand why such a protein blockade should occur within these cells. This study has suggested that the aggregation of these variant proteins at a late- or post- ER stage of processing is a major factor in the decreased protein secretion observed following the processing of secretory-deficient variants of α_1-antitrypsin. These results are in agreement with Lomas *et al* in 1993, Elliott *et al.*, in 1996 (a) and Yu *et al.*, in 1995 who observed a similar pattern of secretion from liver cells *in vivo*. Following this investigation into the formation of protein aggregates within the oocyte cells, the next step was to examine the process of aggregation itself and determine what factors may trigger, or hinder this process. An understanding of the mechanism by which serpins aggregate is of great interest not only from a structural perspective but the possibility exists that this understanding may lead to the production of protein mimetics that could control diseases caused by serpin misassembly.

Results from a series of *in vitro* experiments performed by Lomas *et al.*, in 1992, demonstrated that as well as being temperature dependent (polymerization increases in relation to temperature increase), the rate of polymer formation of α_1-antitrypsin is also dependent on protein concentration. The observations from these experiments applied to M as well as Z variant α_1-antitrypsin, indicating that the destabilising effects of temperature on protein structure produced the same results as specific mutations and resulted in an increased tendency for polymerization to occur. This demonstrates that the process of loop-sheet polymerization may be a major factor in the decrease in secretion resulting from the expression of a number of secretory-deficient variant α_1-antitrypsin proteins, and polymerization has also been observed in a number of other variant serpins (Bruce *et al.*, 1994, Lomas *et al.*, 1995 (b), Eldering *et al.*, 1995).

Experimentally determining the specific mechanism by which loop-sheet insertion occurs has proven to be extremely difficult. Over the past decade, a series of experiments have increased the understanding of the mechanism by which loop-sheet polymerization may take place. These experiments involve the complexation of short polypeptides with intact serpins, and are referred to as peptide annealing experiments. The first peptide annealing experiment, performed by Schulze *et al.*, in 1990, involved the complexation of α_1-antitrypsin with a synthetic tetradecameric peptide representing a portion of the reactive centre loop of α_1-antitrypsin (residues 345 (P14) –358 (P1)). Under these conditions the protein was no longer seen to have inhibitory properties, but still underwent substrate-like cleavage at the reactive site on interaction with trypsin. The complex showed a CD spectra and denaturation stability similar to that seen with the cleaved form of α_1-antitrypsin; evidence that the synthetic peptide may actively bind to the protein in the position that the reactive centre loop occupies, strand s4A, in the X-ray structure of cleaved α_1-antitrypsin described by Loebermann *et al.*, in 1984. Subsequently, a range of similar experiments have been performed (Mast *et al.* 1992, Lomas *et al.*, 1992, Chang *et al.*, 1996, Bottomley & Chang, 1999), which have also illustrated that that the addition of similar peptides blocks heat-induced polymerization of protein, increasing the evidence that these peptides act by insertion into the β-sheet A to mimic strand 4A.

The extent of loop insertion into β-sheet A, in relation to inhibitory activity and secretory blockade, has also been determined using similar peptide annealing experiments. These experiments altered the original 13-mer peptide (Schulze *et al.*, 1990) to a series of shorter peptides by progressively shortening the N-terminus by 1 amino acid per peptide and each new peptide was then complexed with intact α_1-antitrypsin. The resulting complexes were tested for inhibitory activity, with the hypothesis that inhibition should occur when the complexed peptide is short enough to still allow the necessary degree of insertion of the proteins own loop. These experiments revealed that insertion of Thr^{345} and part of residue 346 is required for inhibitor function (Schulze *et al.*, 1992). Comparison of the 3D structures of cleaved α_1-antitrypsin (Loebermann, 1984), plakalbumin

(Wright *et al.*, 1990), and, recently, native α_1-antitrypsin (Elliott *et al.*, 1996), supports this model in which α_1-antitrypsin requires the insertion of Thr^{345} into sheet A for activity. This model for the extent of loop insertion is also supported by the work of Mast *et al.*, in 1992, who determined that, following cleavage of the reactive centre loop of intact α_1-antitrypsin at P9 or P10, the protein undergoes spontaneous polymerization. Therefore, insertion of the P1- P8 region of the reactive loop of one molecule into the A-sheet of another is required for polymerization to occur (Mast *et al.*, 1992).

The experiments presented in this chapter examine the effects of time and temperature on the polymerization of α_1-antitrypsin *in vitro*, to establish the optimum conditions for loop-sheet polymerization to occur. This information will then be used to observe the polymerization of α_1-antitrypsin in the presence of three synthetic peptides – BC11 (an 11-mer peptide representing the P14-P4 section of the loop of antithrombin), and two novel peptides with differing properties - SEA1 and REV1, designed for this study.

The BC11 peptide was designed to represent a section of the reactive centre loop of antithrombin that is two amino acid residues shorter than the BC13 peptide designed by Lomas *et al.*, in 1992 and one amino acid residue shorter than the BC12 peptide designed by Chang *et al* in 1996. Following incubation, at elevated temperature, of α_1-antitrypsin in the presence of both these peptides (as discussed previously), polymerization of protein was not observed. The BC11 peptide used in this study was shortened a further amino acid residue to determine the effects of this shorter peptide in preventing the polymerization of α_1-antitrypsin *in vitro*. The effects of the BC11 peptide on the secretion of the Z deficiency variant from *Xenopus* oocytes is also examined.

Both of the novel peptides were modified from the structure of the BC11 peptide in order to examine the effects of alternative 'loop structures' on protein polymerization. The structure of the SEA1 peptide is identical to that of BC11 except for a single amino acid substitution of an alanine at P10 to asparagine. This

introduces a slightly bulkier amino acid, thus altering the 'space' the peptide occupies, but also introduces a possible site for glycosylation into the peptide. This potential glycosylation site may provide a method for localisation of this peptide into the ER of *in vivo* liver cells in sufficient amounts to result in it having an influence on polymerization of protein within these cells. However, if the introduction of the bulkier asparagine residues reduces the effect of the peptide to inhibit polymerization *in vitro*, this potential effect would be of no benefit. Therefore, this study aimed to examine the *in vitro* effects of this novel peptide on the process of polymerization of α_1-antitrypsin.

The REV1 peptide involved a greater change to the original BC11 peptide, which was altered to produce an ovalbumin/antithrombin reactive centre loop hybrid (see Fig 5.1). It is documented (Stein *et al.*, 1989), that the non-inhibitory serpin ovalbumin is unable to undergo the S→R transition like that of the inhibitory serpins. This suggests that the bulky amino acid residues within the P4-P14 region of the reactive centre loop of ovalbumin, that are observed to be smaller residues in the inhibitory serpins, prevent insertion of the loop into the proteins own A β-sheet following loop cleavage. This information would also suggest that ovalbumin does not undergo loop-sheet polymerization, as the mechanisms and movement of structures involved do not occur. By creating a hybrid complex consisting of a section of this reactive centre loop, it may be possible to determine if these bulkier amino acids do prevent loop insertion. Altering only a few of the amino acids within the original BC11 peptide to those of ovalbumin also allows the importance of specific amino acids within this loop, in relation to insertion, to be determined.

	Reactive Loop Sequence P15-P1′															
Residue Position	**15**	**14**	**13**	**12**	**11**	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	**1′**
Amino Acid	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359
α_1-AT	G	T	E	A	A	G	A	M	F	L	E	A	I	P	M	S
Antithrombin	G	S	E	A	A	A	S	T	A	V	V	I	A	G	R	S
BC11		S	E	A	A	A	S	T	A	V	V	I				
SEA1		S	E	A	A	N	S	T	A	V	V	I				
REV1		D	E	V	V	A	S	T	D	V	V	I				
Ovalbumin	G	R	E	V	V	G	S	A	E	A	G	V	D	A	A	S

Fig 5.1 Sequence of peptides examined in this study. Peptide sequences are aligned to α_1-antitrypsin (amino acid number refers to that of α_1-antitrypsin), antithrombin and ovalbumin, to demonstrate the homology between peptide and loop structure of these serpins.

5.2 Results

5.2.1 *In Vitro* polymerisation of α_1-antitrypsin.

In order to study the effects of reactive centre loop peptides on the polymerization of α_1-antitrypsin, the optimal *in vitro* conditions resulting in polymerization were examined. The mechanism of loop-sheet polymerization has been shown to be dependent on both temperature and protein concentration (Lomas *et al.*, 1992), and a higher degree of polymerization was observed following an increase in either of these factors. The effects of increasing temperature were first examined by incubating a constant amount of α_1-antitrypsin protein, at a series of increasing temperatures. Fig 5.2 demonstrates the change in protein polymerization with increasing incubation temperature. As this figure shows, following incubation of α_1-antitrypsin at room temperature (20°C), only minimal formation is observed and an appreciable band representing the unpolymerised material is visible. The results following incubation at 37°C (lane 2), 45°C (lane 3) & 60°C (lane 4), show a gradual increase in the amount of polymerised material in comparison to that which is unpolymerised. At 75°C, there are no monomeric forms of the protein remaining but considerable levels of high molecular weight polymers are retained near the top of the gel, confirming that the process of α_1-antitrypsin polymer formation, *in vitro*, is temperature dependent. Fig 5.3 shows a time course for the polymerization of α_1-antitrypsin following incubation @ 65°C. The results, in Fig 5.2, demonstrated that incubation of protein at this temperature for 60min, resulted in the polymerization of the majority of protein, with no measurable amount in monomeric form. Using the results from these experiments, subsequent polymerization of protein was carried out @ 65°C for 60min.

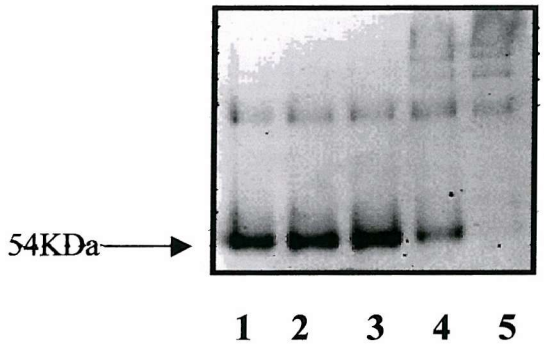

Fig 5.2. Effects of increasing temperature on α_1-antitrypsin polymerization following 60min incubation. 6.2µg of α_1-antitrypsin was heated at a range of temperatures between 20 and 75°C (20, 37, 45, 60 and 75°C – lanes 1-5), for 60min. Samples were added to an equal volume of gel sample buffer and analysed via Native PAGE and coomassie blue staining.

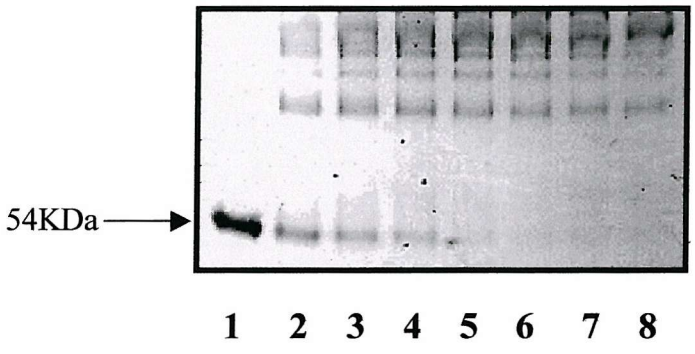

Fig 5.3 Time course for α_1-antitrypsin polymerization at 65°C. 8 samples containing 6.2µg of α_1-antitrypsin were heated at 65°C and the polymerization reaction stopped at each timepoint (lane 1 = 0min, lane 2 = 4min, lane 3 = 10min, lane 4 = 15min, lane 5 = 30min, lane 6 = 45min, lane 7 = 60min, lane 7 = 75min), by incubation of the samples on ice. Samples were added to an equal volume of gel sample buffer and analysed via Native PAGE and coomassie blue staining.

5.2.2 *In Vitro* effects of reactive centre loop peptides on α_1-antitrypsin polymerisation

By first establishing the conditions by which α_1-antitrypsin undergoes spontaneous polymerization, the effect of a series of peptides on this polymerization could be determined. Previous experiments, (Lomas *et al.*, 1992, Schulze *et al.*, 1990, 1992, Mast *et al.*, 1992), have involved the use of short peptides, (between 12-16 amino acids), representing the reactive centre loop sequences of both α_1-antitrypsin and antithrombin, to examine the mechanism of loop-sheet polymerization seen in secretory deficient variants of α_1-antitrypsin *in vitro*. The addition of these peptides, under conditions that would normally result in the spontaneous polymerization of protein, was observed to prevent the loop insertion required for this polymerization to occur. This study examined the *in vitro* effects on polymerization of three peptides with different structures in order to investigate further a possible role for such peptides in the production of normal levels of protein from otherwise deficient variants. Using the polymerization conditions established in section 5.2.1 as a standard, α_1-antitrypsin was incubated in the presence of a molar excess of each of the three peptides and the effects on polymerization compared and related to peptide structure.

The BC11 peptide, structure provided in Fig 5.1, contains 11 amino acid residues, is N-Acetylated to promote insertion (Schulze *et al.*, 1990), and represents the P14-P4 region of the reactive centre loop of antithrombin. α_1-antitrypsin protein was incubated in the presence of a 22x molar excess of BC11 peptide @ 37°C for 18 hours to allow sufficient time for peptide interaction and insertion, followed by incubation for 60mins at 65°C to promote polymerization. Fig 5.4 illustrates the effects of this concentration of the BC11 peptide on the polymerisation of α_1-antitrypsin. This high concentration of peptide was used to promote insertion and to provide a standard to allow comparison with the two other novel peptides. This result demonstrates that, in the presence of this concentration of BC11 (lane 2), a complete blockade of polymerisation is seen compared to α_1-antitrypsin incubated in the absence of peptide (lane 1). This blockade of protein polymerization

correlates well with the results presented in previous studies (Lomas *et al.*, 1992, Schulze *et al.*, 1990, 1992, Mast *et al.*, 1992), which also observed a blockade of polymerisation in the presence of similar peptides. Following the observation that incubation of protein in the presence of this 11-mer peptide completely blocked polymerization, the effect following changes in the sequence of this peptide were examined.

Fig 5.4 and 5.5 illustrate the blockade in polymerisation produced in the presence of 'original' BC11 peptide, compared to that produced following incubation, under the same conditions, in the presence of the two novel peptides designed in this lab – SEA1 and REV1.

The SEA1 (Ac-Ser-Glu-Ala-Ala-**Asn**-Ser-Thr-Ala-Val-Val-Ile) peptide is designed to represent the BC11 peptide, with a single amino acid substitution of alanine to asparagine at P10 (highlighted). This change introduces a bulkier amino acid to the peptide sequence, which may hinder its insertion into the gap in the A β-sheet, but also introduces a possible glycosylation site within the peptide. The presence of the Asn residue may have a role *in vivo* of localizing this peptide to the site of polymerization – the ER of liver cells. To determine the potential benefit of the use of this novel peptide, this study first examined the *in vitro* effects on protein polymerization. Fig 5.4 compares the degree of polymerisation blockade produced in the presence of 22 molar excess of BC11 peptide (lane 2) to that produced in the presence of increasing concentrations (15x, 22x & 28x molar excess) of SEA1 peptide (lanes 6→8). The results from this experiment clearly demonstrate that a similar concentration of the SEA1 peptide (lane 7), did not result in a total blockade in polymerization, as observed following incubation of α_1-antitrypsin in the presence of BC11 peptide. At this concentration of SEA1 peptide, a slight blockade of polymerization is observed (lane 4), and incubation of α_1-antitrypsin in the presence of a higher concentration of this peptide (lane 8), indicates that the degree of polymerization is reduced further as the amount of monomeric protein is increased. It is possible that incubation of protein in the presence of a higher concentration of this peptide might decrease polymerization

further, but this peptide was relatively insoluble, which precluded its use at higher concentrations. To overcome this problem, the peptide stock was dissolved in DMSO, and Fig 5.4 and 5.5 show that the addition of the relevant amounts of DMSO had no effect on α_1-antitrypsin polymerization in these experiments. The results from incubation with the SEA1 peptide suggest that this change from Ala→Asn at P10 has altered the structure of the peptide to such a degree that its ability to insert into the A β-sheet of the protein molecule has been greatly reduced. Therefore, it is possible that the presence of this small Ala residue at P10 may be a prerequisite for loop insertion both in the case of peptide insertion and the functioning of the reactive centre loop itself. This relates to the presence of such larger amino acids at P10-P14 within the loop of non-inhibitory serpins such as ovalbumin, which may account for the proteins lack of inhibitory activity (Stein *et al.*, 1989).

This absence of loop insertion and, therefore, lack of inhibitory mechanism, that is observed with ovalbumin was examined in terms of loop structure by studying the effects of the second novel peptide produced in this study – REV1- on α_1-antitrypsin polymerization *in vitro*.

The REV1 peptide (Ac-Asp-Glu-Val-Val-Ala-Ser-Thr-Asp-Val-Val-Ile), is based on a hybrid antithrombin-ovalbumin loop. The residues altered to those of ovalbumin are highlighted in Fig 5.1 and have been introduced to observe the effects of these bulkier residues, such as valine, on the ability of the BC11 peptide to block the polymerization process. Fig 5.5 compares the degree of polymerisation blockade produced in the presence of a 22x molar excess of BC11 peptide (lane 2) to that produced in the presence of increasing concentrations (0.25x, 8x & 16x molar excess) of REV1 peptide (lanes 5→3). The results from this experiment clearly demonstrate that a slightly lower concentration of the REV1 peptide (lane 3), does decrease the heat-induced polymerization of α_1-antitrypsin protein, although a total blockade, as observed following incubation of α_1-antitrypsin in the presence of BC11, is not seen. It is possible that, as discussed following incubation with SEA1, increasing the concentration of the REV1

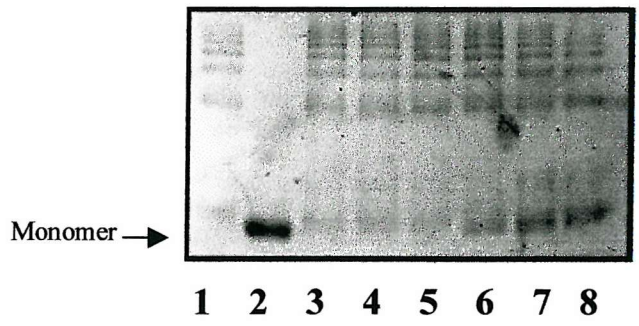

Fig 5.4 Coomassie-stained Native-PAGE gel showing the polymerization of α_1-antitrypsin in the presence of BC11 peptide compared to novel SEA1 peptide. 6.2μg of α_1-antitrypsin was heated at 65°C for 60min in the presence of either 3mg BC11 peptide (lane 2), or 2.16mg (lane 6), 3.12mg (lane 7), 4.08mg (lane 8) of SEA1 peptide. Lane 1 is control α_1-antitrypsin heated in the absence of peptide. Lanes 3→5 illustrate α_1-antitrypsin incubated and heated in the same volume of DMSO used to resuspend the SEA1 peptide. Samples were added to an equal volume of gel buffer and analysed via Native-PAGE and coomassie blue staining.

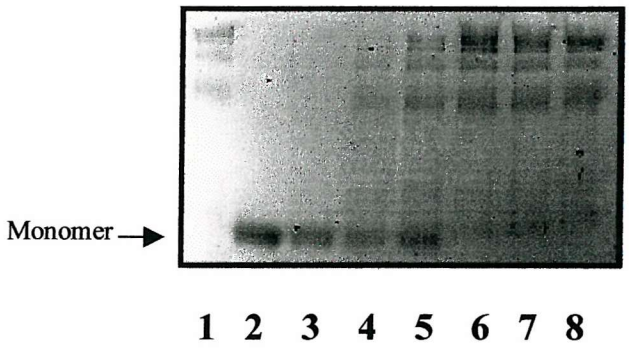

Fig 5.5 Coomassie-stained Native-PAGE gel showing the polymerization of α_1-antitrypsin in the presence of BC11 peptide compared to novel REV1 peptide. 6.2μg of α_1-antitrypsin was heated at 65°C for 60min in the presence of either 3mg BC11 peptide (lane 2), or 2.28mg (lane 3), 1.14mg (lane 4), 0.57mg (lane 5) of REV1 peptide. Lane 1 is control α_1-antitrypsin heated in the absence of peptide. Lanes 6→8 illustrate α_1-antitrypsin incubated and heated in the same volume of DMSO used to resuspend the REV1 peptide. Samples were added to an equal volume of gel buffer and analysed via Native-PAGE and coomassie blue staining.

peptide may increase the effect on polymerization but, with the high molar excess and difficulties solubilising this peptide, the effects of such higher concentrations were not examined here.

The observations that this bulkier peptide had visible effects on protein polymerization, suggests that it may be able to insert into the gap in the A β-sheet of the α_1-antitrypsin protein. These results appear to be inconsistent with the suggestion that the non-inhibitory activity of Sepins such as ovalbumin results from the proteins inability to undergo the Stressed to Relaxed transition as the proteins larger, bulkier loop hinders the mechanism of loop insertion. (Stein *et al.*, 1989).

The analysis of the effects of these three peptides *in vitro* has demonstrated how the structure of the reactive centre loop is important for both the 'normal' process of loop insertion and, consequently, the 'abnormal' process of loop-sheet polymerization. *In vitro*, the effects of such peptides have been extensively studied (Lomas *et al.*, 1992, Schulze *et al.*, 1990, 1992, Mast *et al.*, 1992), however, the ultimate goal is the use of such compounds in the prevention of polymerization *in vivo* to prevent or reduce the effects of α_1-antitrypsin deficiency This study has examined, in detail, the effects of such α_1-antitrypsin deficiency variants using the *in ovo* surrogate secretory system – *Xenopus* oocytes. The ability of the oocyte to mimic the secretion of various secretory deficient variants of α_1-antitrypsin has been examined in Chapter 3, and the presence of aggregates/polymers of protein within the cells injected with these variants has been observed in Chapter 4. Following the elucidation of the effects of the BC11 peptide on α_1-antitrypsin polymerization *in vitro*, it was important to determine if this block of polymerization was also produced following protein processing *in ovo*. The close relationship between the secretion of α_1-antitrypsin variants from oocytes and from liver cells may then suggest a role for this peptide *in vivo*.

5.2.3 *In ovo* peptide analysis

As discussed in the previous section, incubation of α_1-antitrypsin protein in the presence of BC11 peptide produced a total blockade of polymerization *in vitro*. The effects of this peptide on the secretion of Z variant α_1-antitrypsin from oocytes was then examined. The results presented in Chapter 4 suggested that aggregation/polymerization of the Z α_1-antitrypsin protein occurs at a late- or post- ER stage of the processing pathway within these cells. In an attempt to localise the peptide to this region of the processing system, the methods described in chapter 4, involving the injection of microsomes containing partially processed α_1-antitrypsin protein into, oocytes was adapted. In 1992 Koppelman *et al.*, established an assay to detect the transport of peptides into microsomes. This study used an adaptation of these experiments to attempt to achieve the transport of the BC11 peptide into the microsomal membranes. These microsomes were then added to an *in vitro* translation system in the presence of α_1-antitrypsin mRNA and the resulting protein containing microsomes injected into oocytes. As observed in section 4.2.2, these microsomes would then integrate into the oocytes secretory system – delivering the peptide to the site where polymerization occurs and resulting in an increased secretion of the protein. To perform these experiments, it was first necessary to determine any effects of the BC11 peptide on α_1-antitrypsin processing *in vitro*.

Microsomal membranes, prepared as detailed in Chapter 2, were incubated on ice or at 25°C for 1hr in the presence of 10nm, 100nm or 1μM synthetic peptide, BC11 and then added to an *in vitro* translation system, in addition to either M or Z mRNA. Fig 5.6 (a) demonstrates that the addition of this peptide at various concentrations (10nm, 100nm and 1μM), to the translation mix had no apparent effect on the glycosylation pattern of either M or Z α_1-antitrypsin. The highest dose of peptide was, therefore, used in subsequent experiments to ensure optimum peptide:protein ratio *in ovo*. Fig 5.6 (b) confirms that addition of this peptide, at a concentration of 1μM, to the translation mix had no significant effect ($p>0.5$), on the processing of either M, Z or Siiyama variant RNA in this system.

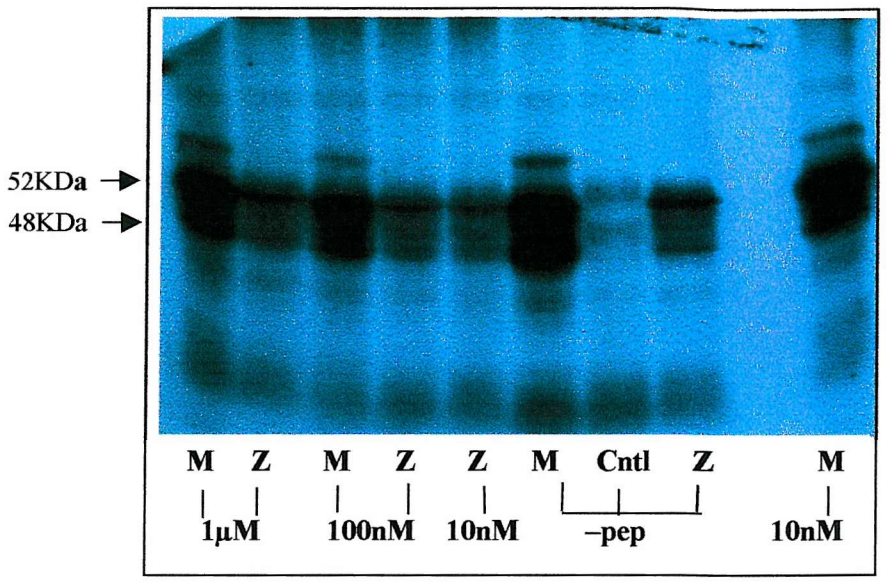

Fig 5.6 (a) Autoradiograph of SDS-PAGE gel showing α_1-antitrypsin protein bands following translation of α_1-antitrypsin mRNA in a cell-free reticulocyte lysate system in the presence of microsomal membranes. Bands show comparison between translation of M variant α_1-antitrypsin mRNA and Z variant α_1-antitrypsin mRNA, and show the effects of the presence and absence (-pep) of varying concentrations (1μM, 10nM and 100nM), of BC11 peptide on protein translation and processing. Molecular weights were determined by co-migration of standard protein markers.

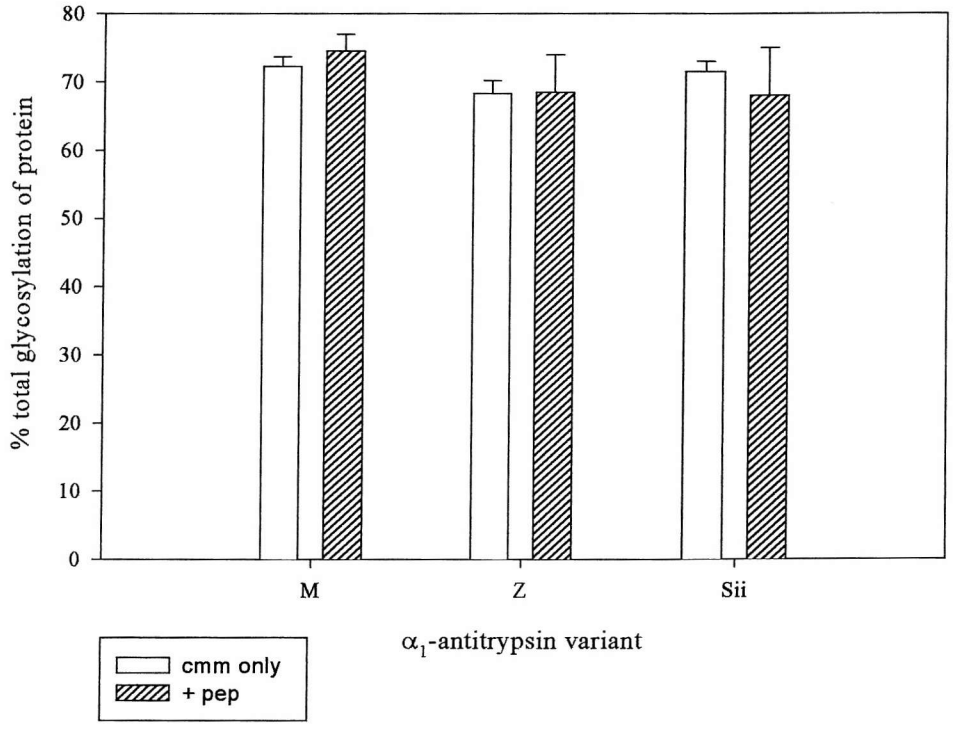

Fig 5.6 (b) Quantification of % total glycosylation of M, Z and Siiyama α_1-antitrypsin protein translated in the absence (cmm only) and presence (+pep) of BC11 peptide. Bands were excised from gels and radioactivity determined by liquid scintillation counting. % total glycosylation is expressed as a total amount of protein glycosylated at 3 sites. Values are expressed as the arithmetic mean ± the S.E.M.

To determine the effects of the BC11 peptide on the retention of Z and Siiyama α_1-antitrypsin *in ovo*, as observed in chapters 3 and 4, following *in vitro* translation, microsomes were recovered via centrifugation through a 0.5M sucrose cushion and microinjected into oocytes (see methods – section 2.13). The contents of oocytes and cell incubation media were then analysed and compared using immunoprecipitation and SDS-PAGE. Fig 5.7 (a) demonstrates that addition of 1μM BC11 peptide (+pep) does not appear to have the same effects on aggregation/polymerization of protein as observed *in vitro*, as the Z secretory phenotype is retained. The low percentage of protein secreted from the oocytes injected with Z α_1-antitrypsin-containing microsomes, indicates retention of the variant protein compared to the M variant. This gel also illustrates that total synthesis of protein from these cells is increased in the presence of BC11 peptide. The reason that the addition of such a small peptide should cause protein synthesis to increase is unclear and further observation of this possible effect in the presence of higher concentrations of this peptide may indicate if this effect is related to the presence of the peptide or simply related to a variable in the methodology. Fig 5.7 (b) quantifies the gel bands in Fig 5.7 (a) and illustrates a slight increase (~6%) in the secretion of Z α_1-antitrypsin in the presence of peptide. This increase is not statistically significant, but may indicate a possible trend that, although the peptide does not have the same effect of completely blocking the polymerization process as *in vitro*, a slight increase in secretion of protein may be observed using this technique. It is important to note that higher molecular weight forms of Z α_1-antitrypsin were visible within the oocyte, but the low amounts of total protein secreted and retained within these cells using this technique resulted in the protein bands not being visible in the photograph of the original autograph in Fig 5.7 (a). Quantification of the excised gel bands in Fig 5.7 (b), therefore, provides a more accurate representation of the relative amounts of retained and secreted protein.

The method involved in these experiments provided no evidence that the peptide was present within the microsomal membranes when injected into the oocytes. Therefore, this method was adapted further in an attempt to ensure the presence of the peptide, which had proved active *in vitro*, within the microsomal membranes.

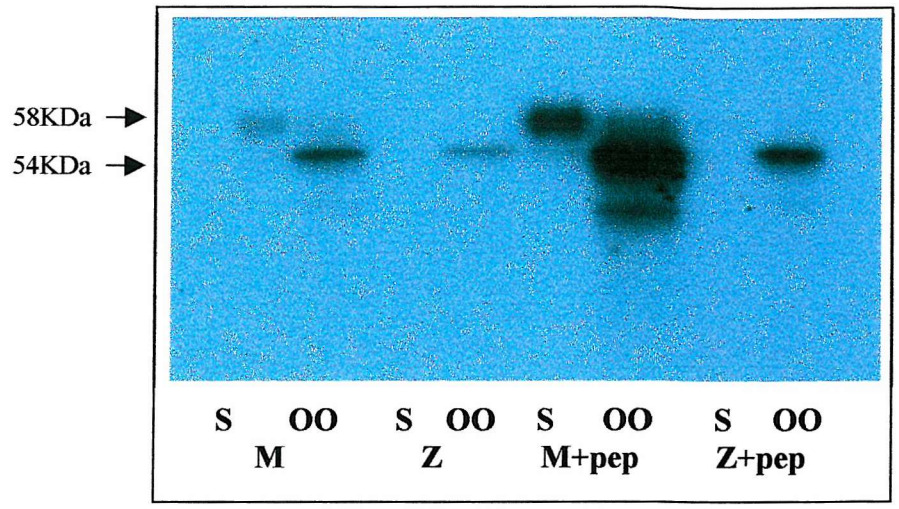

Fig 5.7 (a) Autoradiograph of SDS-PAGE gel illustrating immunoprecipitated protein bands from oocyte extracts (oo) and incubation media (s). Oocytes were injected with microsomal membranes following translation with M and Z α_1-antitrypsin RNA in a cell-free lysate system in the presence (+pep) or absence of 1μM peptide BC11. Molecular weights were determined by co-migration of standard protein markers.

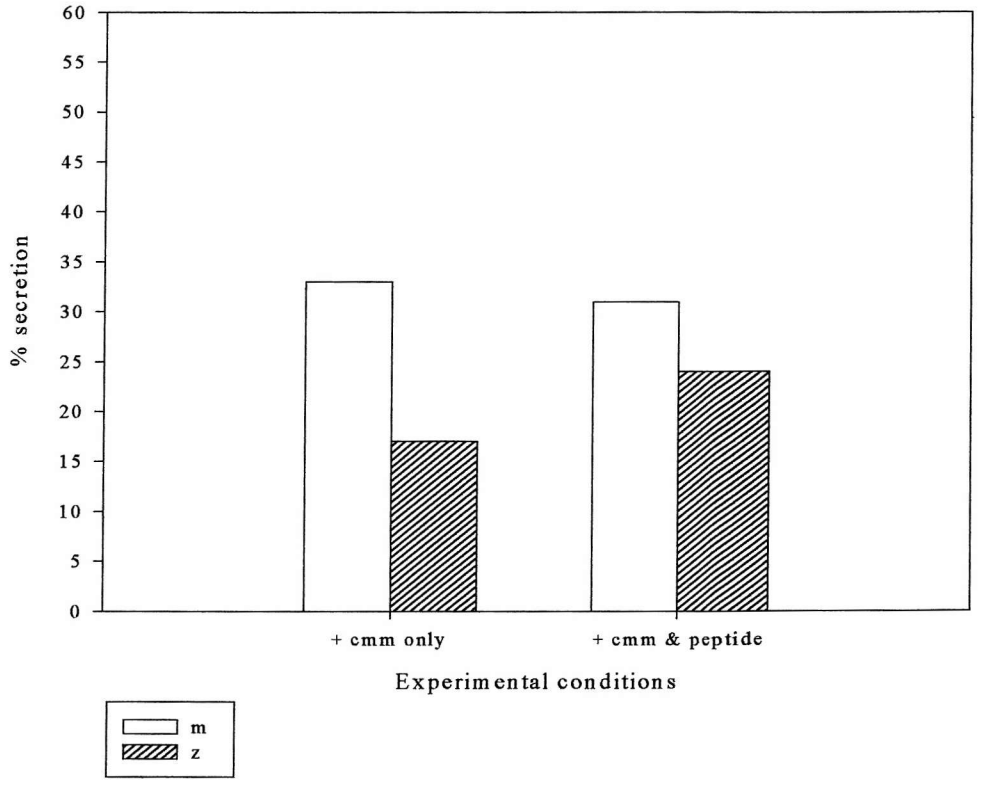

Fig 5.7 (b) Quantification of relative amounts of M, Z and Siiyama α_1-antitrypsin secreted from oocytes injected with protein containing microsomes (+cmm only) alone or following incubation in the presence of 1μM BC11 peptide. Bands were excised from gels are radioactivity determined by liquid scintillation counting. Secreted α_1-antitrypsin is expressed as a percentage of total amount of inhibitor synthesized in oocytes.

To localise the peptide within the microsomes the microsomal outer membranes were disrupted by a high pH treatment, incubated with 1μM BC11 peptide and then resealed (Niccitta & Blobel, 1993). The results from the use of these treated microsomes in an *in vitro* translation system (not shown), demonstrated that, following this treatment, the processing activity of the microsomes was inhibited as the processing pattern observed in the presence of non-treated microsomes was not observed.

A further range of experiments were then performed in order to attempt to ensure integration of peptide within the oocyte. These experiments involved the co-injection of 1μM peptide and mRNA and the incubation of oocytes, post injection, in SOS containing 1μM peptide. Neither of these experimental methods led to an increase in the secretion of the Z variant from *Xenopus* oocytes, compared to the slight increase in secretion observed following the injection of microsomes containing peptide. These results suggest that BC11 peptide does not have as significant an effect on the blockade of polymerization *in ovo* as *in vitro*, although a slight increase in secretion (~6%) is observed.

5.2.4 Effects of TMAO and Glycerol on polymerisation of α_1-antitrypsin

The results discussed in section 5.2.2, present the effects of small, specific peptides on the polymerization of α_1-antitrypsin protein *in vitro*. Results from experiments conducted by Brown *et al.* in 1997 suggested that certain low molecular weight compounds may have a role in the stabilization of proteins that are susceptible to misfolding due to the presence of mutations. This study observed that the addition of compounds including glycerol, deuterated water (D_2O), trimethylamine N-oxide (TMAO) and DMSO increased the transport of the ΔF508 CFTR protein, the defective protein in the genetic disorder cystic fibrosis, from the ER. It was suggested that these compounds may act as 'chemical chaperones' and help 'guide' the protein to its properly folded state, overcoming the effects of any destabilizing mutations that may be present,

resulting in normal secretion. These chaperones may also stabilize the proteins against proteotoxic conditions, such as heat and this study examined the effects of 2 of these compounds to determine if this stabilization property may have an effect on the heat-induced polymerization of α_1-antitrypsin protein *in vitro*.

Following the methods of Brown *et al.*, 1997, α_1-antitrypsin was isolated in the presence of either 100mM TMAO or 0.5M Glycerol and then heated @65°C for 60min to reproduce the ideal conditions for polymerisation of the protein (as determined in section 5.2.2). Fig 5.8 illustrates that incubation of α_1-antitrypsin in the presence of either of these compounds had no visible effect on polymerization as high molecular weight forms of the protein are still visible. Fig 5.9 illustrates that incubation in higher concentrations of these compounds, 500mM TMAO and 0.75M Glycerol, under the same conditions also had no obvious effect. These results suggest that the mechanism of polymerization observed with α_1-antitrypsin is not affected by such stabilizing compounds that have been observed to alter the conformation of defective CFTR protein.

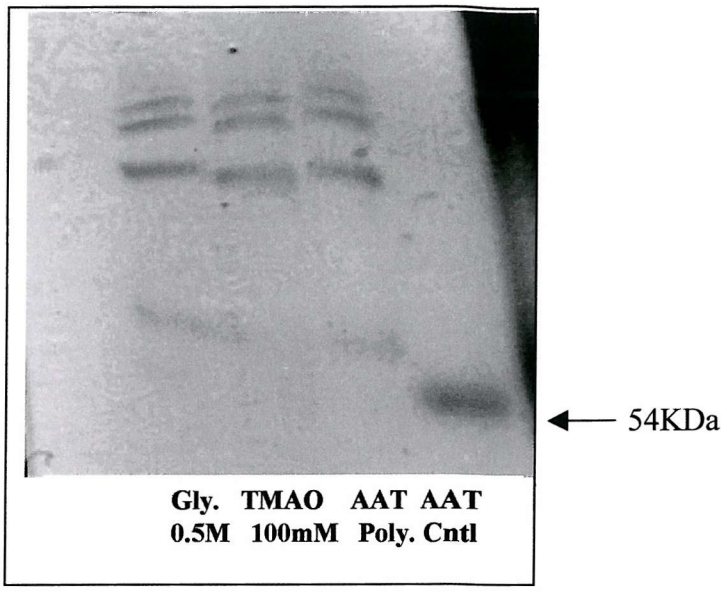

Fig 5.8 Coomassie-stained Native-PAGE gel illustrating the incubation of α_1-antitrypsin protein @65°C in the presence of 0.5M Glycerol (Gly. 0.5M), 100mM TMAO (TMAO 100mM), α_1-antitrypsin protein alone (AAT Poly.), and unheated α_1-antitrypsin (AAT Cntl).

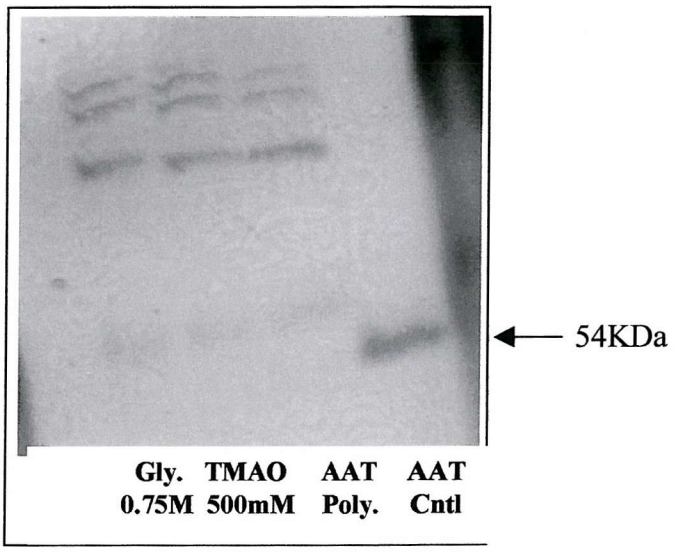

Fig 5.9 Coomassie-stained Native-PAGE gel illustrating the incubation of α_1-antitrypsin protein @65°C in the presence of 0.75M Glycerol (Gly. 0.75M), 500mM TMAO (TMAO 500mM), α_1-antitrypsin protein alone (AAT Poly.), and unheated α_1-antitrypsin (AAT Cntl).

5.3 Discussion

The mechanism of loop-sheet polymerization has been established as a major factor in the retention of an increasing number of secretory deficient α_1-antitrypsin variants at the site of synthesis of the protein. Over the past decade, a number of groups have studied the effects of temperature, protein concentration and small peptides on the *in vitro* polymerization of α_1-antitrypsin (Lomas *et al.*, 1992, Schulze *et al.*, 1990, Mast *et al.*, 1992, Bottomley *et al.*, 1998, Bottomley and Chang, 1999). The results presented in this chapter provide further investigation of the effects of these factors on this aberrant process.

Fig 5.2 and 5.3 illustrate the effects of temperature and incubation time on the polymerization of M α_1-antitrypsin *in vitro,* and indicate that an increase in either of these two factors results in an increase in the amount of protein polymerized to a stage where no monomeric forms are visible. These results are in agreement with those determined by Lomas *et al.,* in 1992, who also observed the temperature dependent nature of α_1-antitrypsin polymerization *in vitro* using both M and Z variants. This suggests that incubation of α_1-antitrypsin at temperatures above the physiological norm results in a similar destabilisation of the protein, leading to polymerization, to that observed in the presence of specific mutations in the protein structure, as occurs with the Z and Siiyama variants. This temperature-mediated polymerization may also suggest an explanation for the individual variation in the severity of such α_1-antitrypsin deficiency. Conditions that, for example, raise body temperature may result in the formation of liver aggregates. This is not so important with regards to individuals who express 'normal' type α_1-antitrypsin, as the results observed in Fig 5.2 illustrate that polymerization of M protein does not occur until ~45°C, but emphasises the importance of control of body temperature in those individuals who express a secretory-deficient phenotype, such as Z, as the rate of polymerization of this protein is greatly accelerated at only 41°C (Lomas *et al.,* 1992).

Over the last decade, a series of novel experiments involving the complexation of small peptides with protein (Schulze *et al.*, 1990, Lomas *et al.*, 1992, Mast *et al.*, 1992, Bottomley & Chang, 1999), have given increased insight into the physical mechanisms of loop-sheet polymerization. Using the observations of these studies as a guide, the experiments discussed in this chapter observe and compare the effects of three structurally different synthetic peptides on the polymerization of α_1-antitrypsin *in vitro*.

The BC11 peptide, was designed to represent an 11-mer (P14-P4) section of the reactive centre loop of antithrombin. Two novel peptides, SEA1 and RAV1, were designed based on the structure of the BC11 peptide to examine the effects of specific amino acid sequence on the activity and insertion ability of this peptide. Fig 5.4 and 5.5 compare the incubation of α_1-antitrypsin, under the standard polymerization conditions, in the presence of the three peptides. Incubation in the presence of the BC11 peptide resulted in a complete blockade in polymerisation, as no higher molecular weight forms of the protein are visible. These results suggest that this synthetic peptide is forming a complex with the protein via insertion into the A β-sheet of α_1-antitrypsin, as a surrogate 'loop', and this hinders the insertion of a loop from a 2^{nd} α_1-antitrypsin molecule. These observations agree with those of Schulze *et al.*, in 1990, and Lomas *et al.*, in 1992, who also showed dramatically reduced rates of polymerisation following incubation of α_1-antitrypsin with slightly longer peptides of similar structure. The results using this shorter peptide also suggest that, although the insertion of this peptide is only as far as P4, this is sufficient to prevent protein polymerization *in vitro*. Observations by Mast *et al* in 1992 determined that loop insertion up to P8 was required for the protein to function as an inhibitor and this confirms that the insertion of the BC11 peptide to P4 would block this 'normal' process of protein loop insertion, thereby also blocking the 'abnormal' process of loop-sheet polymerization.

This extent of polymerization blockade was not reproduced following incubation in the presence of either the two novel peptides. The presence of SEA1 peptide

was observed to cause only a slight decrease in polymerisation of α_1-antitrypsin. Comparison of the peptide sequences illustrates that the SEA1 peptide was designed with only one amino acid change from the BC11 peptide, the substitution of a bulkier asparagine residue for a small alanine residue at P10. The results from these experiments suggest that this single substitution dramatically reduces the ability of the peptide to prevent polymerization. The introduction of the asparagine residue may affect the properties of the peptide by altering the electrostatic forces between residues, forming hydrogen bonds with inappropriate amino acids on the surface of the protein or simply increasing or altering the 'space' this peptide occupies. Any one, or a combination of these factors, may hinder or promote incorrect insertion into the gap in the A β-sheet and these results provide evidence that the peptide sequence is crucial for insertion into β-sheet A. Therefore, peptide insertion may be a more exact process than was first thought, resulting in a close proximity between the annealing peptide and surrounding A-sheet residues. For example, Schulze *et al.*, (1990), observed that a peptide representing the reactive centre loop of α_1-antitrypsin complexed with α_1-antitrypsin but did not combine to similar effect with the related serpin – α_1-antichymotrypsin.

The REV peptide was also based on the structure of the BC11 peptide, but a number of the residues were altered to those present in the reactive centre loop of ovalbumin. The table in Fig 5.1 shows the alignment of these three sequences and highlights the changes made. The results shown in Fig 5.5, demonstrate that, unlike the effects observed with the SEA1 peptide, the polymerisation of α_1-antitrypsin was visibly reduced following incubation with this peptide, as the amount of protein monomers increases. These results were unexpected and are not in agreement with the theory presented for the SEA1 peptide, that suggests insertion into β-sheet A of α_1-antitrypsin may be greatly hindered by the presence of larger amino acids within its sequence. Therefore, other factors must play a role in the unexpected ability of this peptide to inhibit polymerization. The introduction of charge interactions or bond formations may alter the peptides shape or increase its ability to form complexes with α_1-antitrypsin. The increased

bulk of this peptide may also result in it complexing with another region of the A β-sheet and not necessarily inserting into the gap between strands 3 & 5. This may also result in this gap not being accessible to other molecules of α_1-antitrypsin so decreasing the possibility of polymerization. These results can also be discussed in terms of the activity and insertional capability of the proteins own loop structure and suggest that the reason for the non-inhibitory activity of ovalbumin is not simply related to the size of the proteins reactive centre loop (Stein *et al.*, 1989). In 1997, Huntington *et al.*, examined the effects of altering bulky residues in the loop of ovalbumin and observed that loop insertion did occur (as observed with cleaved ovalbumin), but only at a very slow rate and the extent of insertion did not favour inhibitory activity. These results suggest that the size of the loop in non-inhibitory serpins, such as ovalbumin, may not hinder insertion, but it may be interactions or the size of the gap in the A β-sheet that prevent insertion. This theory would explain the ability of the REV1 peptide to prevent polymerization of α_1-antitrypsin, as although the space the peptide occupies is more extensive, the gap in the A β-sheet of α_1-antitrypsin is 'designed' to accept loop structures.

Following the observation that incubation of protein in the presence of BC11 peptide resulted in a blockade of polymerization, the effects of this peptide on the aggregation of Z variant protein *in ovo*, as discussed in Chapter 3, were examined. Protein loaded microsomes, as described in Chapter 4, were injected into oocytes following incubation in the presence of peptide. Fig 5.7 (b) illustrates that only a slight increase, of ~6%, in the secretion of Z protein from oocytes is observed in the presence of peptide. These results suggest that the peptide may have an effect on protein polymerization, *in ovo*, but this is much less pronounced than the *in vitro* effects. This might be due to the comparatively low concentration of α_1-antitrypsin produced within the oocyte cells, compared to the abnormal, highly concentrated amount of protein observed *in vitro*. The major problem with these experiments is the lack of experimental evidence for the amount of peptide present within the microsomes prior to oocyte injection. Further experimentation examined various methods that may increase the localisation of peptide within the oocyte, but this was not measured. The results (not shown) indicated no

significant effect of the peptide on secretion of Z α_1-antitrypsin, and the small increase observed following injection of protein-loaded microsomes containing peptide was also not visible. This suggests that the method of using microsomes to localise BC11 peptide to the processing pathway of α_1-antitrypsin may result in a slight decrease in the polymerisation of secretory-deficient Z α_1-antitrypsin, but these results are only preliminary and require further investigation. The reduced effects of this peptide *in ovo* compared to *in vitro* may be explained by the observations of previous *in vitro* studies examining the effects of similar peptides. These studies (Lomas *et al.*, 1992, Schulze *et al.*, 1990 & 1994, Mast *et al.*, 1992), have all identified that protein:peptide complex formation is strongly dependent on temperature, involving temperatures of 37°C or higher in the presence of up to a 100M excess in peptide compared to α_1-antitrypsin. Schulze *et al.*, in 1990 did observe peptide incorporation at room temperature but the presence of urea was required. The use of the oocyte expression system in this study requires these cells to be incubated at ~17°C for protein expression. It is possible that, even though the peptide was present at high concentrations, this low temperature provided unfavourable conditions for complex formation

CHAPTER 6

GENERAL DISCUSSION

The results from this study are discussed in detail at the end of each relevant chapter. This discussion aims to bring together these results and summarise the main conclusions from this work. This study has used *Xenopus* oocytes as a surrogate secretory system for α_1-antitrypsin. Previous experiments within this laboratory have demonstrated that the secretion of both Z and Siiyama protein from oocytes, following injection of mRNA, mimics that seen from liver cells (Foreman *et al.*, 1984, Errington *et al.*, 1985, Sidhar *et al.*, 1995). Here the technique has been used to examine the effect of further mutations in the α_1-antitrypsin sequence on the secretory properties of the protein.

Recent experiments performed by Lomas and colleagues have observed the effects on protein secretion and polymerisation of the very rare, F variant of α_1-antitrypsin (Arg^{223}→Cys (Fagerhol *et al.*, 1965)). The results indicated a tendency for this mild deficiency variant to polymerise when expressed in bacterial cells (unpublished observations), and, in an attempt to further establish the deficiency profile of this variant, this study examined its effects on secretion using *Xenopus* oocytes. Previous studies have indicated that F is only a mild deficiency variant (Kelly *et al.*, 1989, Brand *et al.*, 1974), and the small decrease in secretion, ~80% of M levels, observed from oocytes in this study also indicates this. Molecular modelling of intact α_1-antitrypsin (Elliott *et al.*, 1996 (a)), illustrated that Arg^{223} forms interactions with a number of other residues in the loop region of the molecule to form a basic pocket centered around one residue – Glu^{254} (P5). To examine the theory that substitution of Arg^{223} disrupts a vital interaction with this P5 glutamic acid, the residue was substituted for glutamine (Q), valine (V) or serine (S) and the secretion of these novel variants from oocytes compared. The alteration to valine or glutamine resulted in no significant difference in secretion compared to M, whereas serine decreased the secretion of α_1-antitrypsin to levels almost identical to the F variant. This suggests that this specific alteration re-creates the mild decrease in protein secretion produced by the mutation of Arg^{223}→Cys, and proposes a potential interaction between the F and P5 residues.

This relationship may be related to the effects of steric constraints in this region of the molecule. Valine and glutamine are of a similar size to glutamic acid and, therefore, substitution of these residues is unlikely to create steric effects, which may explain the almost identical secretion of these variants to M. The F and P5S variants, however, possess smaller residues with similar side chain groups (sulphydryl and hydroxyl, respectively), which may decrease any steric constraints or permit the formation of inappropriate hydrogen bonds between residues in the reactive centre loop and the body of the molecule. This may have the affect of altering the position of the loop or preventing the loop from attaining its optimal folding position to allow normal secretion of protein. A percentage of the protein may be distorted and, therefore, not recognised for secretion and this may lead to the small amount of protein being retained and degraded within these cells.

It is also plausible that this change in loop conformation or mobility may make the protein a candidate for loop-sheet polymerization, by increasing the chance of insertion of the loop from another molecule into the gap in β-sheet A. The polymerization rates for the P5 Q and S mutants, *in vitro,* were presented by Dafforn *et al.*, in 1999, and show similar measurements that are ~ 6X that of wild-type α_1-antitrypsin. These results do not compare with those from this study that indicate the P5Q variant is secreted as normal and, therefore, shows no tendency to polymerise *in ovo*. This suggests that, even though a variant protein may have the ability to polymerise *in vitro*, this may not necessarily relate to its behavior *in vivo*. The difference in the rate of protein secretion from oocytes observed for the F variant, compared to the Siiyama variant also illustrates that polymerization may not necessarily be the mechanism causing the decrease in secretion of this protein. These observations suggest that it may be the stabilisation or transport of this protein that is affected within these cells, as secretion is still increasing at 24hrs post-injection. Therefore, it is possible that the presence of the mutation at position 223 may hinder the speed of processing, folding or protein transport of this variant *in ovo*, and this may alter the secretion of the F variant compared to M. Other mechanisms, such as

interaction with molecular chaperones, may also have a role in the mild secretory defect observed with the F variant. The examination of the rate of secretion for the P5S variant would provide useful information regarding the relationship between these two residues and a decrease in protein secretion.

This study also examined the possibility of a similar interaction between two other residues on the protein structure. Graham *et al.*, in 1989, suggested that the presence of an ionic bond between residues 39 and 264 may have a role in the stabilization of α_1-antitrypsin. Both residues have naturally occurring mutations that effect secretion of protein, arginine →cysteine at residue 39 (I variant) and glutamic acid→valine at residue 264 (S variant). Clinical observations for the I variant suggest only a mild deficiency in secretion (~80% of normal levels of secretion (Blundell *et al.*, 1975, Baur & Bencze, 1987, Mahadeva *et al.*, 1999)), but these studies have only examined the I mutation in combination with the M or Z allele. Using the oocyte system enabled the effects of the I allele alone to be observed and indicated that, contrary to previous observations, this variant resulted in a decrease in secretion to only ~40% of the M variant. The possibility that this result is due to this variant causing an oocyte-mediated effect, such as an alteration in the transport of protein or interaction with a molecular chaperone, cannot be discarded, but this evidence suggests that a homozygous I phenotype may be associated with a much severer deficiency than the heterozygous studies indicate.

Examination of the S variant demonstrated that, in oocytes, protein secretion levels were only ~60% of the M variant. This correlates to a decrease in secretion of ~40%, closely mimicking that shown *in vivo*. These results are also in agreement with work by Mahadeva *et al* in 1999, Elliott *et al.*, in 1996 (b) and Curiel *et al.*, in 1989 (a), who, both *in vitro* and *in vivo*, identified that the S variant was only a mild deficiency variant with a mild tendency toward polymerization. Comparison of the secretion levels of the I and S variant protein suggest that, using the oocyte secretory system, it is not simply the disruption of

an ionic bond, as suggested by Graham *et al.*, in 1989, between the two residues that results in the decreased secretion of both these variants. However, observations from this study do support the structural modelling hypothesis suggested by Elliott *et al.*, in 1996 (b), to explain the mechanism of S α_1-antitrypsin deficiency, who predicted that hydrogen bonding between residues 264 and 38 may be crucial to the stability of the protein. In this study, the mutation of the tyrosine residue at position 38 to a phenylalanine, removed the presence of a hydroxyl group, thus preventing the predicted hydrogen bond interaction to residue 264. This substitution resulted in a level of protein secretion almost identical to the S variant, suggesting that this specific alteration re-creates the decrease in protein secretion produced by the mutation of $Glu^{264}\rightarrow$Val, and proposes a potential interaction between these two residues. Therefore, it is also possible that the decreased secretion caused by the mutation of Glu→Val at position 264, is also related to the lack of this bond due to the loss of the negatively charged oxygen species, in Glu, which is readily available for hydrogen bonding.

These residues are located within the shutter domain of α_1-antitrypsin, and the critical steric requirements within this region are strongly supported by the study of mutations within this region (Kwon *et al.*, 1994, Stein & Carrell, 1995, Sidhar *et al.*, 1995, Lee *et al.*, 1996). Therefore, in relation to its position adjacent to Tyr^{38}, the steric changes that accompany the substitution of Arg→Cys in the I variant may also affect the stability of the protein, although to a greater extent, by altering the spatial interactions within the shutter domain. Observations following the examination of the rate of secretion for I variant protein from oocytes also suggests the mechanism of polymerization may be involved in decreased secretion. The 'pattern' of secretion followed that of the Siiyama variant, with initial, rapid secretion being blocked over time and then remaining at a constant level. These results, and those of Elliott *et al.*, in 1996 (b) suggest that this slight conformational instability may favour the formation of loop-sheet polymers, as observed *in vitro*. Dafforn *et al.*, (1999) demonstrated a faster rate of

once again, may illustrate another mechanism involved in the decrease in secretion with certain deficiency variants. Lomas *et al.*, (1995), demonstrated that the Gly→Val mutation at position 264 did not interfere directly with the configuration of the A-sheet (to allow increased possibility of intermolecular loop insertion), and Elliott *et al.*, (1996 (b)) observed that this mutation also has no effect on the conformation of the reactive centre loop. Therefore, the position of the I and S variant mutations, within the shutter domain, most likely decreases the stability of the molecule to allow an increased chance of polymerization by an indirect effect through interactions with the A-sheet. Mutation of Tyr^{38}→Cys resulted in the formation of higher molecular weight forms of α_1-antitrypsin, retained within the oocyte, which are predicted to be cysteine dimers between α_1-antitrypsin molecules or mixed dimers with another protein of approximately the same molecular weight as α_1-antitrypsin, which contains a cysteine residue available for S-S interaction. Previous work by Tomasi & Hauptman, in 1974, demonstrated that α_1-antitrypsin may complex with IgA via S-S disulphide bond formation involving the cysteine residue at position 232 (Laurell *et al.*, 1975). The presence of a second Cys residue, also present at the surface of the molecule, may, therefore, increase the possibility of such dimer formation. These results are difficult to interpret but the presence of such protein dimers indicates that the structure of α_1-antitrypsin may be altered significantly so as to allow the presentation of this residue to other available Cys residues of other protein molecules.

The findings described in this study suggest that different mechanisms may exist for the decrease in secretion observed in oocytes injected with different variants of α_1-antitrypsin. To study this hypothesis in more detail, the processing of variant α_1-antitrypsin protein, both *in vitro* and *in ovo*, was observed. Examination of the contents of the isolated oocyte secretory pathway illustrated the presence of high molecular weight forms of protein within the pathway of oocytes injected with Z and Siiyama, but not M, variant α_1-antitrypsin. This suggests that the mechanism of retention of these variants within the oocytes is similar to that seen within *in*

vivo liver cells (Carrell *et al.*, 1991, Lomas *et al.*, 1992), resulting in a decreased protein secretion via a form of aggregation/ polymerization. These results are also in agreement with work performed by Verbanac & Heath in 1986, who provided evidence that the majority of Z α_1-antitrypsin that was synthesized remained in the microsomal vesicles of the oocyte cell. The same high molecular weight form of protein was observed within the secretory pathway of oocytes injected with I variant mRNA, again suggesting that the retention of this variant within oocytes occurs via a similar mechanism of aggregation. The isolation of the secretory pathway from oocytes injected with F variant mRNA, however, revealed that no such high molecular weight protein was present within these cells. The mild deficiency related to this variant, in combination with the absence of high molecular weight protein, increases the evidence for the theory that the decrease in secretion observed with this variant is not due to polymerization or aggregation of protein, but may be due to a folding or transport problem.

The presence of high molecular weight protein within oocytes suggests that the mechanism of protein aggregation seen in hepatocytes, is reproduced within this system. Close examination of the formation of these high molecular weight forms was provided by a novel set of experiments designed in this study, to re-construct the secretory pathway of α_1-antitrypsin protein using a combination of *in vitro* and *in ovo* techniques. Microsomes, containing translocated α_1-antitrypsin protein, were injected into oocytes and the secretion of protein from these cells examined. Prior to injection, the form of protein sequestered within the microsomes was determined to be a monomeric, core-glycosylated, 52KDa species, in the M, Z and Siiyama variants. *In vitro* translocation of protein also demonstrated that the processing of Z and Siiyama variant α_1-antitrypsin is like that of the M variant. Therefore, it is observed that, to the ER stage of the secretory pathway, there appears to be no effect of the Z or Siiyama mutations on the stabilization or processing of α_1-antitrypsin protein.

The quantification of secreted protein, following injection of protein loaded microsomes, clearly illustrated that the secretory phenotype was retained, and the presence of high molecular weight forms of Z and Siiyama protein within the secretory pathway of these oocytes provided further evidence for polymerization/aggregation of protein at this late stage of processing. Therefore, these experiments not only illustrate that foreign microsomes containing protein will integrate within the oocyte secretory system to result in the secretion of a fully mature protein, but also present the theory that aggregation/polymerization of α_1-antitrypsin protein, within the secretory pathway of oocytes, occurs at a late- or post- ER stage. This suggests a later stage than research conducted by Le *et al.*, in 1990, and McCracken *et al.*, in 1991, who suggested that the formation of high weight forms of α_1-antitrypsin protein within liver cells may be due to an aggregation of such variant protein at the point of its highest concentration in these cells – the ER. As the transport of protein from ER to Golgi is the rate limiting step during processing (McCracken *et al* in 1991), it is possible that such a build-up of protein at this stage may lead to aggregation/ polymerization of the aberrant protein.

A number of factors have been suggested for the retention of the secretion-deficient variants of α_1-antitrypsin within liver cells, and the same factors may be involved within the oocyte. The α_1-antitrypsin may bind to a form of molecular chaperone following vesicular fusion and transport to the Golgi as a part of the quality control process within the oocyte. It is also be possible that, due to the large amount of Z or Siiyama protein produced that is 'misfolded', this quality control system inside the oocyte is simply 'overwhelmed' resulting in the aggregation and polymerization of protein before it can be degraded. Teckman *et al.*, (1996), demonstrated this effect *in vivo* in homozygous Z phenotypes who developed liver disease. The aggregation of protein is predicated to be due to the presence of defective calnexin or an inefficient degradatory system which is easily overwhelmed by the large volumes of misfolded protein produced. Zhang *et al.*, in 1997, have also illustrated that misfolded proteins may also interact and be degraded via a BiP or a separate proteasome-mediated

pathway if binding to calnexin is not possible. Therefore, Z or Siiyama variant protein may also affect similar pathways within the oocytes, leading to decreased ER degradation of protein which will, due to the concentration dependant nature of loop-sheet polymerization (Lomas *et al.*, 1992), increase the possibility of polymerization within the cells. The interaction of variant α_1-antitrypsin with molecular chaperones may also explain the lack of protein aggregates observed *in vitro* within microsomal membranes. Calnexin exhibits an affinity for monoglucosylated oligosaccharides (Ware *et al.*, 1995), and it is possible that interaction with calnexin is not favoured or there may be an absence of calnexin within these vesicles. Over the last decade, a series of novel experiments involving the complexation of small peptides with protein (Schulze *et al.*, 1990, Lomas *et al.*, 1992, Mast *et al.*, 1992, Bottomley & Chang, 1999), have given increased insight into the physical mechanisms of loop-sheet polymerization. To examine the mechanism of aggregation/ polymerization of protein that was occurring within oocytes, the effects of three short peptides on the polymerization of α_1-antitrypsin was examined *in vitro* and *in ovo*.

The *in vitro* effects on polymerization of peptide BC11, designed to represent the P14-P4 section of the reactive centre loop of antithrombin, and two novel peptides, SEA1 and RAV1, based on the structure of the BC11 peptide, were observed to examine the effects of specific amino acid sequence on the activity and insertion ability of this peptide. The results show that incubation in the presence of the BC11 peptide resulted in a complete blockade in polymerisation, suggesting that this synthetic peptide is forming a complex with the protein via insertion into the A β-sheet of α_1-antitrypsin, as a surrogate 'loop', thus, hindering the insertion of a loop from a 2nd α_1-antitrypsin molecule. These observations agree with those of Schulze *et al.*, in 1990, and Lomas *et al.*, in 1992, who also showed dramatically reduced rates of polymerisation following incubation of α_1-antitrypsin with slightly longer peptides of similar structure. Observations by Mast *et al* in 1992 determined that loop insertion up to P8 was required for the protein to function as an inhibitor and this confirms that the insertion of the BC11 peptide to P4 would block this 'normal' process of protein

loop insertion, thereby also blocking the 'abnormal' process of loop-sheet polymerization.

This extent of polymerization blockade was not reproduced following incubation in the presence of either the two novel peptides. The presence of SEA1 peptide was observed to cause only a slight decrease in polymerisation of α_1-antitrypsin, suggesting that the substitution of a bulkier asparagine residue for a small alanine residue at P10 dramatically reduces the ability of the peptide to prevent polymerization. The introduction of the asparagine residue may affect the properties of the peptide by altering the electrostatic forces between residues, forming hydrogen bonds with inappropriate amino acids on the surface of the protein or simply increasing or altering the 'space' this peptide occupies. Any one, or a combination of these factors, may hinder insertion or promote incorrect insertion into the gap in the A β-sheet and these results suggest that the sequence of the peptide is crucial to allow insertion into β-sheet A to take place. Peptide insertion may be a more exact process than was first thought, with a closer proximity between the annealing peptide and surrounding A-sheet residues. For example, Schulze *et al.*, (1990), observed that a peptide representing the reactive centre loop of α_1-antitrypsin complexed with α_1-antitrypsin but did not combine to similar effect with the related serpin – α_1-antichymotrypsin.

The REV1 peptide involved the substitution of a number of residues in BC11 for those present in the reactive centre loop of ovalbumin. On incubation with this peptide, the polymerisation of α_1-antitrypsin was visibly, and unexpectedly, reduced. Therefore, unlike the suggestion for the SEA1 peptide, it is unlikely that steric constraints play a vital role in the mechanism of this peptide and other factors must play a role in the ability of this peptide to inhibit polymerization. The introduction of charge interactions or bond formations may alter the peptides shape or reduce its ability to form complexes with α_1-antitrypsin. The increased bulk of this peptide may also result in it complexing with another region of the A β-sheet and not necessarily inserting into the gap

between strands 3 & 5. This may also result in this gap not being accessible to other molecules of α_1-antitrypsin so decreasing the possibility of polymerization. These results also suggest that the reason for the non-inhibitory activity of ovalbumin may not simply be related to the size of the proteins reactive centre loop (Stein *et al.*, 1989). In 1997, Huntington *et al.*, examined the effects of altering bulky residues in the loop of ovalbumin and observed that loop insertion did occur, but only at a very slow rate and the extent of insertion did not favour inhibitory activity. Therefore, it may be interactions or the size of the gap in the A β-sheet that prevent insertion. This theory would explain the ability of the REV1 peptide to prevent polymerization of α_1-antitrypsin, as although the space the peptide occupies is more extensive, the gap in the A β-sheet of α_1-antitrypsin is 'designed' to accept loop structures.

The effects of the BC11 peptide on the aggregation of Z variant protein *in ovo* were also examined using protein-loaded microsomes injected into oocytes following incubation in the presence of peptide. A slight increase (6%) in the secretion of Z protein was observed and this suggests that BC11 may have an effect on protein polymerization, *in ovo*, which is much less pronounced than the *in vitro* effects. This suggests that the method of using microsomes to localise BC11 peptide to the processing pathway of α_1-antitrypsin may result in a slight decrease in the polymerisation of secretory-deficient Z α_1-antitrypsin, but these results are only preliminary and require further investigation. The major problem with these experiments is the lack of experimental evidence for the amount of peptide present within the microsomes prior to oocyte injection. Further experimentation examined various methods that may increase the localisation of peptide within the oocyte, but the results indicated no significant effect of the peptide on secretion of Z α_1-antitrypsin. The measurement of peptide uptake into microsomal membranes prior to oocyte injection would validate these results and may suggest a more efficient system for localising peptides to the site of protein aggregation suggested in this study – the late-or post- ER stage of the secretory pathway.

The results presented in this study also the use of *Xenopus* oocytes as a surrogate secretory system for α_1-antitrypsin protein analysis and also present the possibility that the use of this *in ovo* system may not be limited to the study of α_1-antitrypsin secretion. The new technique of dissecting the processing pathway of this protein may increase the ability to study the mechanism of aggregation and loop-sheet polymerization in more detail. As this study has shown, the oocyte system produces results which closely mirror those seen *in vivo* and the possibility exists to use the *Xenopus* oocyte system to study the effects of possible inhibitors/ antagonists of polymerization alongside *in vivo* experimentation within liver cells.

CHAPTER 7

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