MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF VARIANTS OF ALPHA-1-PROTEASE INHIBITOR ISOLATED FROM ASTHMATIC PATIENTS AND SYNTHESIZED BY THE PROCESS OF SITE-DIRECTED MUTAGENESIS

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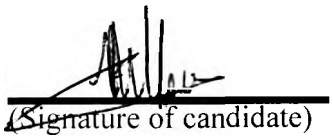
A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2004

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

I further declare that the work presented was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, and granted the clearance number M02-04-22.



(Signature of candidate)

15th day of APRIL 2004

For my parents, Ganesen and Athie, my wife, Nevashini and my brothers Vasen and Deenash, in appreciation of their constant support and encouragement.

ABSTRACT

Asthma is a complex syndrome which has a significant inflammatory basis which results from the complex interactions between heterogenous genetic and environmental factors. Although the environmental allergens are fairly well known, little information concerning the genetic differences between atopic and non-atopic individuals is available. Alpha-1 antitrypsin is the archetypal member of the **ser**ine **p**roteinase **in**hibitor or **serpin** superfamily and the most important proteinase inhibitor in the lung with specificity to neutrophil elastase. Genetic deficiency of the protein is classically associated with early onset emphysema, bronchiecstasis, panniculitis, rheumatoid arthritis and glomerulonephritis. The S(E264V), Z(E342K), M1 (213 Ala) and M2 (R101H) variants of alpha-1 antitrypsin have been implicated in the pathogenesis of asthma. A novel finding was the identification of 2 new variants, the $M1E_{(JOHANNESBURG)}$ and the $M1N_{(JOHANNESBURG)}$ associated with asthma in individuals from South Africa. The novel polymorphism that we describe in exon II (471) **C**TG to **T**TG was found in the heterozygous state with the common normal M3 base allele in an individual with asthma from the United Kingdom. The superfamily of serine proteinase inhibitors control many biological pathways by irreversibly inhibiting their cognate proteinases. The recent crystal structure of α_1-antitrypsin bound to trypsin showed that inhibition of the proteinase resulted from ester bond formation and translocation of the enzyme from the upper to the lower pole of the molecule. The complex was also stabilised by interactions between Lys328 and Asn314 of α_1-antitrypsin and the enzyme. We have shown that mutating these two residues to alanines **(314Asn→Ala, 328Lys→Ala** and **314Asn/328Lys →Ala)** resulted in a 29-fold increase in the dissociation rate constant of the complex. This effect was not specific to trypsin but was also seen following complex formation with human neutrophil elastase. The crystal structures of reactive loop cleaved α_1-antichymotrypsin, antithrombin, plasminogen activator inhibitor-1 and horse leucocyte elastase inhibitor demonstrate that residues that are equivalent to position 328 or 314 in α_1-antitrypsin are available to form hydrogen bonds with the proteinase. Taken together these data identify additional interactions at the serpin-proteinase interface that are important in stabilising the inhibitory complex. Moreover they demonstrate that the proteinase must be positioned at the lower pole of α_1-antitrypsin in the final complex and not trapped with partial loop insertion.

Knowledge of the molecular basis of variants and polymorphisms, both normal and pathogenic, can be useful for the identification of the residues most important for protein function

Part of the work in this dissertation was the subject of publications and conference presentations.

Publications

Mahadeva R., Gaillard M.C., **Pillay V.**, Lomas D.A., Halkas A. "Characterization of a new variant of alpha-1 antitrypsin E (Johannesburg) (H15N) in association with asthma." Hum. Mutat. 2001 Feb; 17(2):156.

Pillay V., Halsall DJ., Gaillard MC., Lomas DA., Mahadeva R. "A novel polymorphism (471C→T) in alpha-1 antitrypsin in a patient with asthma." Hum. Mut. 2001 Feb; 17(2):155-156

Conference Presentations

Gaillard M-C., **Pillay V.** and Halkas A. "Association of rare variants of alpha-1 antitrypsin with severity of asthma in South Africa." International Federation of Clinical Chemistry and Laboratory Medicine-WorldLab, Firenze, Italy, 6-11 June 1999.

Pillay V., Gaillard M-C., Lomas DA., Mahadeva R. "Characterization of a novel polymorphism and a new variant of alpha-1 antitrypsin in association with asthma." 14th International Federation of Clinical Chemistry and Laboratory Medicine-Euromedlab 2001, Prague, Czech Republic, 26-31 May 2001

ACKNOWLEDGEMENTS

The work for this dissertation was carried out in the Department of Medicine at the University of the Witwatersrand Medical School and in the Department of Medicine and Haematology at the Cambridge Institute of Medical Research (Cambridge University, United Kingdom). It was partly supported by the University of the Witwatersrand Postgraduate Merit Award, the Levenburg Bequest, the Medical Faculty Research Endowment Fund and a South African National Research Foundation Scholarship.

First and foremost, I am indebted to my supervisor, Dr Marie-Christine Gaillard, for her able scientific approach, her very human qualities, and her encouragement, enthusiasm and expertise throughout this study. I am most fortunate to have had the honour of working with such an exceptional person.

I am grateful to: Professor David Lomas for valuable discussions and for his encouragement, enthusiasm and expertise throughout this study; Dr Andrew Halkas for the interest he has shown in the work and for his assistance with the asthmatic patients and collating of patient information; Dr Ravi Mahadeva and Dr Aiwu Zhou for their valuable advice, for willingly sharing their expertise, scientific and otherwise; and the Photographic Unit (Medical School) for their technical expertise on the photography.

TABLE OF CONTENTS

CHAPTER 2 – CHARACTERISATION OF NEW VARIANTS AND A NOVEL POLYMORPHISM OF ALPHA-1 PROTEINASE INHIBITOR

CHAPTER 3 – STABILISATION OF THE SERPIN-PROTEINASE COMPLEX THROUGH CONSERVED INTERACTIONS

CHAPTER 4 - MAJOR FINDINGS AND CONCLUSIONS

LIST OF ABBREVIATIONS

A	:	adenosine
ACT	:	alpha-1-antichymotrypsin
Ala	:	alanine
α_1PI	:	alpha-1-proteinase inhibitor
AM	:	alveolar macrophage
Arg	:	arginine
Asp	:	aspartic acid
α_1AT	:	alpha-1-antitrypsin
BAL	:	bronchoalveolar fluid lavage
bis	:	N, N' -methylene-bis-acrylamide
bp	:	base pair
C	:	cytosine
Ca^{2+}	:	calcium ion
CBG	:	cortisol binding globulin
CD	:	cluster determinant
CF	:	cystic fibrosis
Ci	:	Curie
Cys	:	cysteine
dATP	:	2' deoxyadenosine 5' -triphosphate
dCTP	:	2' deoxycytidine 5' -triphosphate
dGTP	:	2' deoxyguanosine 5' -triphosphate
dTTP	:	2' deoxythymidine 5' -triphosphate
dd	:	double distilled
ddATP	:	2', 3' deoxyadenosine 5' -triphosphate
dCTP	:	2', 3' deoxycytidine 5' -triphosphate
dGTP	:	2', 3' deoxyguanosine 5' -triphosphate
dTTP	:	2', 3' deoxythymidine 5' -triphosphate
DMF	:	dimethyl formamide
DNA	:	deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosohate
ds	:	double stranded
ECM	:	extracellular matrix
EDTA	:	ethylene diaminetetra-acetic acid
EPO	:	eosinophil peroxidase
EtBr	:	ethidium bromide
eT_H	:	effector T helper cell
exo I	:	exonuclease I

$Fc_{\varepsilon}RI$	:	high affinity immunoglobulin E receptor
$Fc_{\varepsilon}RI$-β	:	beta chain of the high affinity immunoglobulin E receptor
$Fc_{\varepsilon}RIII$	:	low affinity immunoglobulin G receptor
FEV_1	:	forced expiratory volume in one second
G	:	glycine
G	:	guanine
G-CSF	:	granulocyte colony-stimulating factor
GER	:	gastroeosophageal reflux
GIF	:	gastric intrinsic factor
Gly	:	glycine
Gln	:	glutamine
GM-CSF	:	granulocyte-macrophage colony-stimulating factor
HCl	:	hydrochloric acid
HDM	:	house dust mite
HETE	:	hydroxyeicosatetraenoic acid
His	:	histidine
I	:	isoleucine
IEF	:	isoelectric focusing
IFN	:	interferon
Ig	:	immunoglobulin
IL	:	interleukin
IL-1	:	interleukin-1
Ir	:	immune response
IPTG	:	isopropyl thio-β-D-alactoside
K^+	:	potassium ion
kbp	:	kilobase pair
KCl	:	potassium chloride
kDa	:	kilodalton
Leu	:	leucine
LPR	:	late phase response
LT	:	leukotriene
MAC	:	membrane attack complex
MBP	:	major basic protein
MC	:	mast cell
$MgCl_2$	:	magnesium chloride
MHC	:	major histocompatibility complex

MIP	:	macrophage inflammatory protein
mL	:	millilitre
mM	:	millimolar
mmol	:	millimole
mRNA	:	messenger ribonucleic acid
mT_H	:	memory T helper cell
NCF-A	:	neutrophil chemotactic factor of anaphylaxis
NE	:	neutrophil elastase
nm	:	nanomole
NO	:	nitric oxide
NO_2	:	nitrogen dioxide
NP40	:	"nonidet" P40
NSAID	:	non-steroidal anti-inflammatory drugs
O_2^-	:	superoxide anion
PAF	:	platelet activating factor
PAGE	:	polyacrylamide gel electrophoresis
PCD	:	physiological cell death
PCR	:	polymerase chain reaction
PG	:	prostaglandin
PMN	:	polymorphonuclear cell
Pro	:	proline
pT_H	:	precursor helper T cell
RFLP	:	restriction fragment length polymorphism
RNA	:	ribonucleic acid
SAP	:	shrimp alkaline phosphatase
SCF	:	stem cell factor
Ser	:	serine
SO_2	:	sulphur dioxide
SRS-A	:	slow-reacting substance of anaphylaxis
ss	:	single strand
T	:	thymine
Taq	:	*Thermus acqaticus*
TCR	:	transforming growth factor
TEMED	:	N, N, N',N' -tetramethylene diamine
TGF	:	transforming growth factor
T_H	:	helper T cell
T_m	:	melting temperature

TM	:	transmembrane domain
TNF	:	tumour necrosis factor
Tris	:	tris(hydroxymethyl) aminomethane
TX	:	thromboxane
µg	:	microgram
µL	:	microlitre
µm	:	micromolar
UV	:	ultraviolet
Val	:	valine
VNTR	:	variable number tandem repeats
w/v	:	weight-to-volume

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 THE SERPIN SUPERFAMILY OF PROTEINS

The existence of proteinase inhibitor activity in human plasma was apparently first noted by Fermi and Pernossi in 1894. Since that time a host of investigations have been made to determine the various inhibitory activities in this tissue derivative, primarily by adding a larger number of proteinases of varying specificities and catalytic mechanisms to plasma and plasma fractions (Travis&Salvesen, 1983). These experiments were performed to determine the number, type, concentrations, and mechanism of action of the various inhibitors, and with the hope of understanding their role in the control of proteolytic events within the body (Travis&Salversen, 1983).

In 1980 Hunt and Dayhoff noted a similarity between the primary structures of ovalbumin, alpha-1 proteinase inhibitor (α_1-PI) and human antithrombin and proposed the existence of a new superfamily of proteins (Table 1.1). Other proteins belonging to this superfamily (rat angiotensinogen and mouse contrapsin) were soon identified, again based on primary structure similarity (Dolittle, 1983; Hill *et al.*, 1984). In 1985 Carrell and Travis coined the term serpin (an acronym for **ser**ine **p**roteinase **in**hibitor) as a name for these proteins that described the properties of most members of this superfamily as inhibitors of serine proteinases (Carrell&Travis, 1985).

Table 1.1: Plasma Serpins

Inhibitor	μM/L	Mass (kDa)	Target
Antithrombin	2	61	Thrombin, factor Xa
Heparin cofactor II	1	66	Thrombin
Antiplasmin	1	70	Plasmin
Plasminogen activator inhibitor	10^{-4}	50	Plasminogen activator
Protein C inhibitor	10^{-2}	57	Protein C
Antitrypsin	25	52	Neutrophil elastase
Antichymotrypsin	7	58	Cathepsin G
C1-inhibitor	2	104	C1s, kallikrein
Angiotensinogen	10^{-2}	50	Noninhibitor
Thyroxine Binding Globulin (TBG)	0.2	54	Noninhibitor
Cortisol Binding Globulin (CBG)	0.6	53	Noninhibitor

The proteinases released by inflammatory cells act on substrates in the extracellular space. These proteinases can be divided into four main groups: the serine, cysteine, aspartic and metallo-proteinases (Whicher&Evans, 1992). This division is based on the nature of the key catalytic group at the active centre. The choice of serpin as a name for the family has, however, become increasingly inappropriate and confusing as more examples of biologically important, but non-inhibitory, serpins (Zou *et al.*, 1994; Steele *et al.*, 1993) and even serpins that inhibit cysteine proteinases (Ray *et al.*, 1992) have been identified. The name is, however, almost certainly here to stay.

1.1.1 Characteristic Properties of Serpins

There are three principal properties that set serpins apart from other families of protein inhibitors of serine proteinases: (1) Serpins undergo a major conformational change as a necessary part of their mechanism of inhibition of proteinases; (2) They are a family of metastably folded proteins; and (3) Serpin-proteinase complexes are covalent and in almost all cases are effectively irreversible (Gettins *et al.*, 1996).

Serpins require a dramatic partial insertion of the reactive centre as an extra band in one of the three β-sheets of the protein for the inhibitory mechanism to operate effectively (Gettins *et al.*, 1993). Also, in contrast to almost all other proteins, serpins fold in a native conformation that is metastable. This appears to be linked to the need for subsequent conformational change during proteinase inhibition (Gettins *et al.*, 1996).

1.1.2 Occurrence of Serpins

Two of the first serpins identified, antithrombin and α_1 PI, are part of a larger group of human serpins that together represent about 10% of the total protein in plasma. These plasma serpins appear mostly to be involved in regulation of such crucial physiological processes as coagulation, fibrinolysis, inflammation, and the immune response, with consequent pathology in deficiency states (Gettins *et al.*, 1996). Serpins have been identified in many other mammals, amphibians, parasites, insects, plants and even viruses (Table 1.2).

Table 1.2: Species distribution of serpins

SPECIES	SERPIN
Human	α_1-Antichymotrypsin
	α_1-Antiplasmin
	α_1-Proteinase inhibitor
	Angiotensinogen
	Antithrombin
	C1-inhibitor
	Corticosteroid binding globulin
	Heparin cofactor II
	Protease nexin1
	Protein C inhibitor
	Squamous cell carcinoma antigen
	Thyroxine binding globulin
Other Mammals	α_1-Proteinase inhibitor (baboon, dog, donkey, guinea pig, monkey, pig, rabbit, rat, sheep)
	α_1-Antichymotrypsin (cow, goat, pig)
	Angiotensinogen (cow, mouse, rat, rabbit, sheep)
	Antithrombin (cow, mouse, pig, rabbit, sheep)
	C1-inhibitor (guinea pig, rat)
	Collagen binding protein (mouse, rat)
	Kallistatin (rat)
	Leukocyte elastase inhibitor (horse, pig)
Fish	α_1-Proteinase inhibitor (carp)
Amphibians	Ep-45 (Xenopus laevis)
Birds	Antithrombin (chicken)
	Collagen binding protein (chicken)
	Ovalbumin (chicken, quail)
Arthropods	α_1-Antichymotrypsin *(Bombyx mori)*
	Ala-serpin (*Manduca sexta*)
Nematodes	Unidentified function (*Schistosoma heamotobin, S.mansoni, Brugia Malayi*)
Plants	Barley Z protein
	Chymotrypsin inhibitor (wheat)
	Z_4 (barley)
	Z_7 (barley)
Fungi	Corticosteroid binding globulin (yeast)
Viruses	CrmA
	SPI-3 (*vaccinia* virus)
	SERP1 (*myxoma* virus)
Bacteria	None known

1.1.3 Size and Composition of Serpins

Although serpins show some variation in size (figure 1.1), they all contain a core domain of about 360 amino acids that represents the region of high primary and tertiary structural homology. This domain contributes about 40kDa to the total size. The remainder of the serpin is made up of N- and/or C-terminal extensions to the polypetide of different lengths, as well as in many cases carbohydrates attached at non-conserved sites (Gettins *et al.*, 1996). The degree of glycosylation shows a very large range and in the somewhat exceptional case of C1-inhibitor accounts for as much as 40% of the total mass. Other post-translational modifications are posphorylation of the two serines in ovalbumin (Nisbet *et al.*, 1981) and sulfation of two tyrosines in heparin cofactor II (Hortin *et al.*, 1986).

Secreted serpins typically are synthesised as a longer pre-protein containing a cleavable hydrophobic N-terminal signal sequence. Some serpins such as plasminogen activator inhibitor-2 (PAI-2) exist in both secreted and non-secreted forms and may rely on an internal non-cleavable signal sequence, which is a characteristic of the ov-serpin subfamily, for export from the cell (Gettins *et al.*, 1996).

Within the core serpin domain, sequence identity can be as low as 30% (e.g., ovalbumin and α_1 PI) or as high as 92% (e.g., two squamous cell carcinoma antigens occurring at the same gene locus), (Schneider *et al.*, 1995). Although some serpins contain disulphides, others do not. Those that do contain disulphides usually do not show conservation, unless the proteins are highly homologous.

Huber and Carrell have noted 51 residues that are either absolutely conserved in 20 serpins or involve at most change of phenylalanine to tyrosine. All of these conserved residues are either internal or in niches on the surface.

1.1.4 Gene Structure, Chromosomal Location and Evolution

Based on number, size and phase of exons and introns, at least seven distinct serpin gene structures have been shown to exist (figure 1.2). Among these groups of serpins, introns occur at both equivalent and unrelated positions. Whereas some of the exon/intron boundaries occur at positions corresponding to non-structured parts of the serpin polypeptide, others occur within stretches of secondary structure. Woo and colleagues have proposed that a hybrid of the intron deletion and intron insertion models could account for the relationships between serpins. In this model the ancestral gene resembled the α_1-antichymotrypsin gene. Gene duplication over 500 million years ago led to the formation of angiotensinogen, whose protein sequence then diverged from that of the precursor α_1-antichymotrypsin. More recently (between 500 and 250 million years ago) a second gene duplication led to the precursors of (a) α_1-antichymotrypsin and α_1-antitrypsin and (b) ovalbumin and antithrombin. In humans the genes for α_1-antichymotrypsin, α_1-PI, protein C inhibitor and CBG, are despite different functions localised to the same region of chromosome 14 (14q32.1), (Billingsley *et al.*, 1993). They may be expressed co-ordinately during inflammation, since the distinct functions of proteinase inhibition and corticosteroid delivery may be required in concert (Hammond *et al.*, 1990).

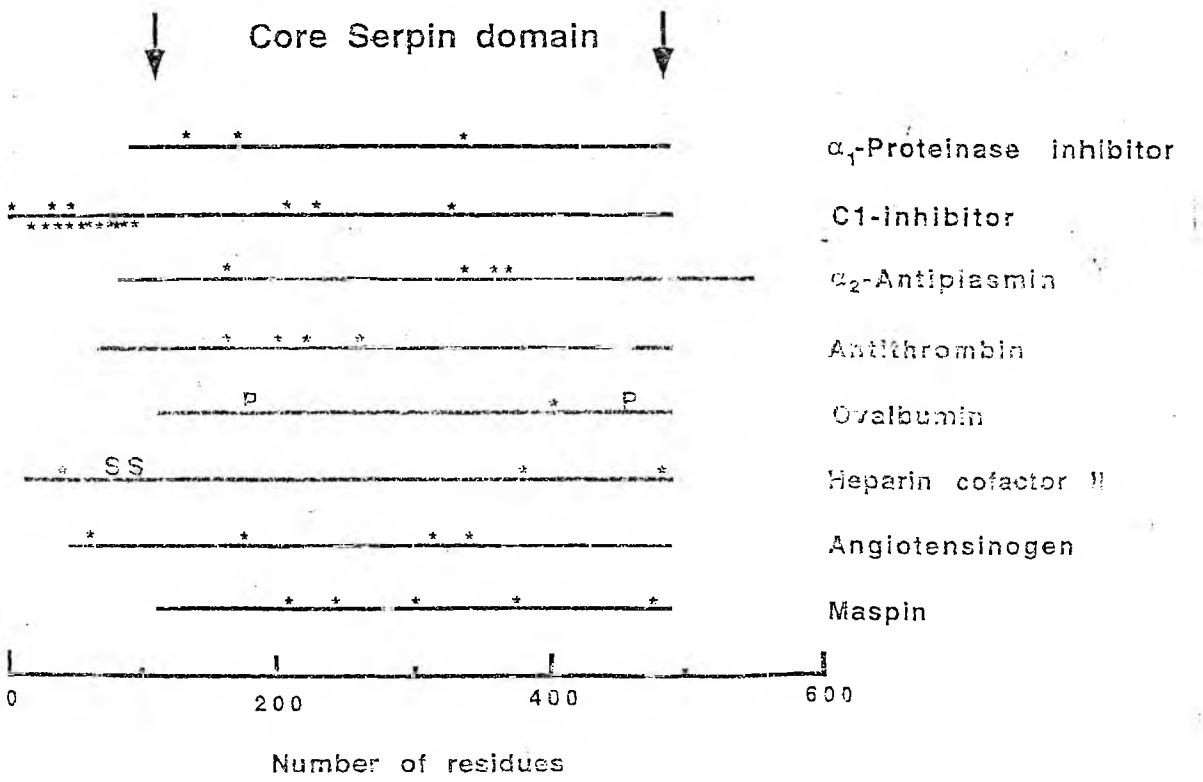


Figure 1.1 Schematic representation of the primary structures of selected serpins to illustrate the size and location of the core serpin domain, and the presence of non-conserved post-translational modifications. Glycosylation sites, either demonstrated to exist or putative, are indicated by *; above the line for N-linked and below the line for O-linked; P represents a phosphoserine and S represents a tyrosine sulphate. For C1-inhibitor, the high density of glycosylation sites makes it difficult to accurately represent all sites present in the N-terminal. (from Gettins *et al.*, 1996).

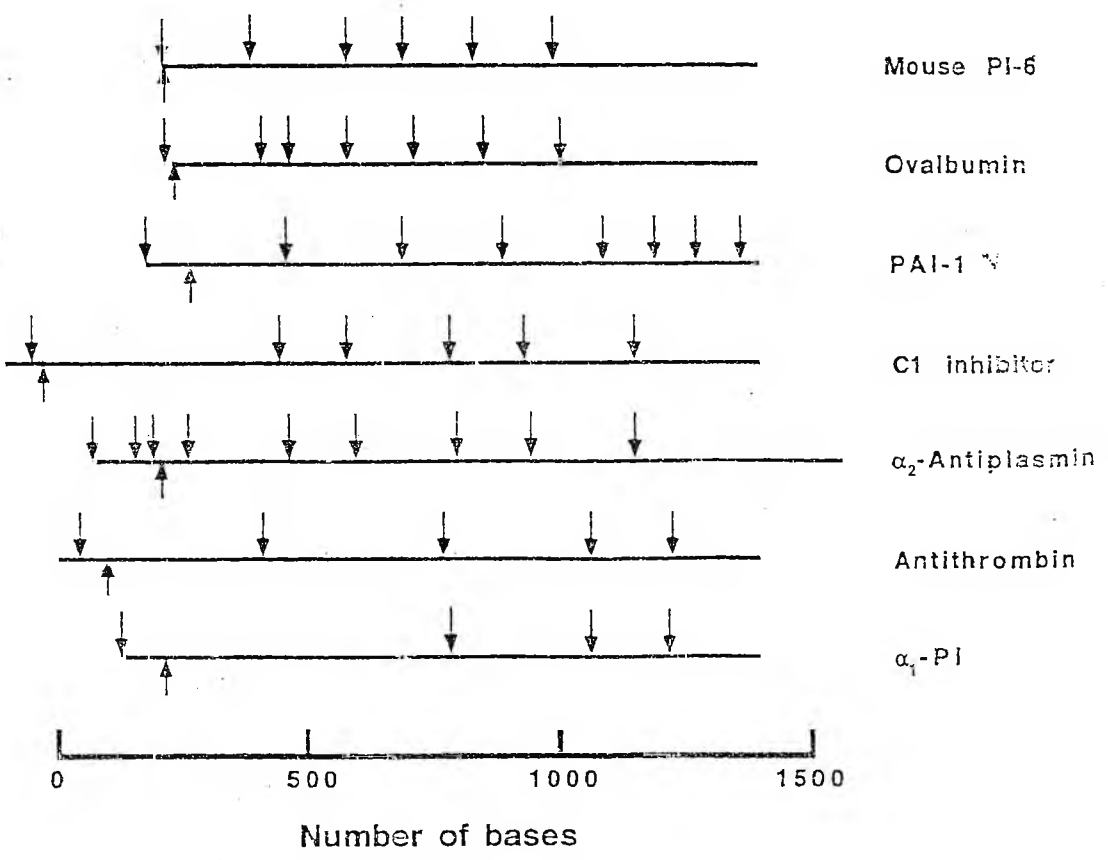


Figure 1.2 Gene organisation of selected serpins to illustrate the seven different patterns of exons and introns so-far identified. The horizontal bars represent the exon coding regions, down arrows indicate the positions of introns, up arrows indicate the start position of the mature protein after processing of any signal (from Gettins *et al.*, 1996).

1.2 THREE DIMENSIONAL STRUCTURES OF SERPINS

The first X-ray structure of a serpin was determined in 1984 and was that of reactive centre-cleaved and therefore inactive α_1-PI (Löbermann *et al.*, 1984). It was a further six years before the next serpin structure was published. That was also of a reactive centre-cleaved serpin, but of ovalbumin, a serpin with no known inhibitory properties (Wright *et al.*, 1990). This structure was quickly followed by that of uncleaved ovalbumin (Stein *et al.*, 1991). After this, new structures appeared much more frequently and led to the first structure of a serpin-protease complex (Huntington *et al.*, 2000). Together these structures of cleaved and uncleaved inhibitory and non-inhibitory serpins have greatly advanced our understanding not only of serpin structure but also of the physical mechanism of their functioning.

1.2.1 The core serpin domain

Although there are striking differences between serpin structures they are all related to the reactive centre loop conformation and its interactions with β-sheets A and C. Even more striking, however, is the almost invariant structure of the core serpin domain. The core domain covers approximately residues 21 to 389 (using the α_1-PI numbering) and is composed of three β-sheets and nine α-helices. The β-sheets are designated A, B and C, and the α_1-helices A through I. β-Sheet A involves the most residues and is composed of 5 strands of polypeptide, followed by β-sheet B which has 6 strands and β-sheet C which has 3 strands (Gettins *et al.*, 1996).

1.2.2 The reactive centre loop

The state of the reactive centre loop (intact versus cleaved) and its conformational state (exposed versus inserted into β-sheet A) together with the associated structural changes of β-sheets A and/or C account for most of the significant structural differences in the core serpin domains (Gettins *et al.*, 1996).

The realisation that the serpins had a flexible reactive site loop first came from the crystal structure of cleaved α_1-PI determined by Robert Huber's group. This structure showed the complete insertion of the proximal portion of the cleaved reactive loop into the central 4^{th} strand position of the A-sheet (Carrell *et al.*, 1997).

The various structural transformations of the reactive centre loop, both definitely known and merely postulated, are shown schematically in figure 1.3.

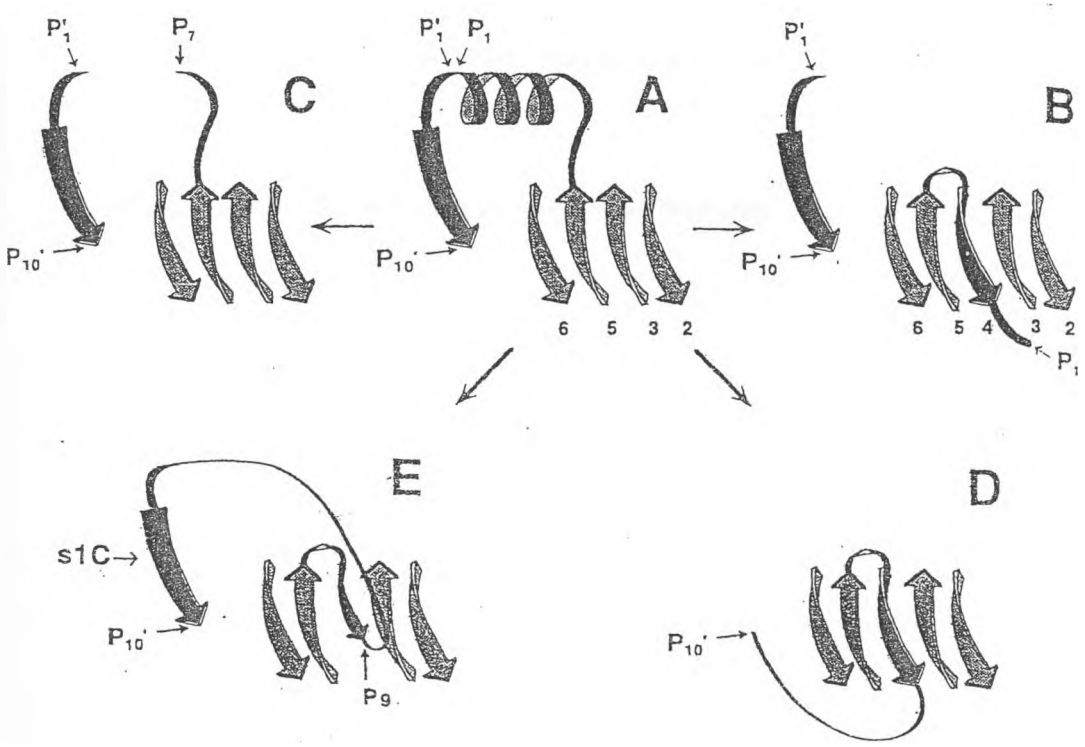


Figure 1.3 Schematic representation of the structural transformations that the reactive centre loop of serpins can undergo. Only the four main strands of β-sheet A (2, 3, 5 and 6) together with the reactive centre region and strand 1 of β-sheet C are shown. (A) The structure of native ovalbumin with the reactive centre in the α-helical conformation. (B) The loop-inserted structure of cleaved α_1-PI and other cleaved inhibitory serpins. (C) The non-loop inserted structure of cleaved on-inhibitory ovalbumin. (D) The loop inserted structure of intact but latent PAI-1. (E) Possible partially loop inserted structure of serpin in complex with proteinase, at the tetrahedral or acyl-enzyme intermediate state, but prior to release of the cleaved new amino terminus (from Gettins *et al.*, 1996).

1.3 Mechanism of action of serpins as suicide substrate inhibitors

1.3.1 Comparison of serpins with other classes of serine proteinase inhibitors

Serpins represent only one of several classes of protein inhibitors of serine proteinases, but one that is distinct from the other classes in a number of important respects.

A) Gross structural and thermodynamic differences

One striking difference between non-serpin serine proteinase inhibitors and serpins, which have molecular weights of 45-100 kDa, is that these proteinase inhibitors are all comparatively small. A second major difference between serpins and other classes of non-serpin serine proteinase inhibitors is in the reversibility of complex formation with the target proteinase. Whereas most non-serpin serine proteinase inhibitors form reversible complexes with proteinases, those with serpins are considered to be irreversible with the possible exception of α_1-antiplasmin interacting with trypsin and chymotrypsin (Shieh *et al.*, 1989).

It has also been shown that spontaneous dissociation of antithrombin-thrombin complexes release only cleaved serpin (Danielsson&Björk, 1983). Related to this apparent irreversibility of most serpin-proteinase complexes is the energy difference between the native and cleaved forms of serpins. For non-serpin inhibitors the energy difference between the cleaved and native inhibitor is close to zero (Estell, *et al.*, 1980), and both cleaved and uncleaved forms of the inhibitors are effective in forming

stable complexes with proteinase and thus acting as proteinase inhibitors. In contrast, not only is there a very large energy difference between cleaved and uncleaved serpins, but the cleaved forms are incapable of acting as inhibitors (Fish *et al.*, 1979). Quantitation of the energy differences between native and cleaved serpins is made difficult by the very much greater stability of the cleaved forms, which results in them being stable even at 100°C (Gettins *et al.*, 1988).

These marked differences in thermodynamic stability between cleaved and uncleaved serpins is a manifestation of the more general difference between serpins and other protein proteinase inhibitors, namely that native serpins fold in a kinetically-trapped metastable state. Indeed serpins belong to a small group of proteins that fold in a native conformation that is a kinetically determined local energy minimum rather than a thermodynamically determined global energy minimum (Baker&Agard, 1994). This metastability can lead to spontaneous change to a more stable conformation such as in the formation of the "latent" form of PAI-1. This metastability plays an essential role in the inhibitory mechanism.

B) Structural differences in the reactive centre loop

The binding interaction between non-serpin serine proteinase inhibitors and proteinases is well understood from a combination of kinetic and X-ray structural studies. Although there are differences in the precise position of the contact interaction between inhibitor and proteinase, the very high affinity of the complex results in all cases from multiple non-covalent interactions.

Most commonly these involve complementarity between residues in the P3-P3′ region interacting with the S3-S3′ subsites on the proteinase (Gettins *et al.*, 1996). In these instances the portion of the inhibitor reactive centre that interacts with the proteinase adopts an extended β conformation that has been termed the "canonical conformation" (Bode *et al.*, 1987).

For some inhibitors, such as chymotrypsin inhibitor-2, there are high temperature factors associated with the reactive centre loop regions, reflecting some mobility that might be beneficial for adapting to the active site structures of different enzymes, with consequently lowered affinity for proteinase. However, even in these cases, the change in structure of the reactive centre loop itself upon complexation is minor (Hubbard *et al.*, 1991). These structurally different classes of serine proteinase inhibitor thus present a common conformation of reactive centre loop residues to the proteinase that could be said to represent an optimised substrate binding conformation. Ironically analysis of actual substrate cleavage sites by trypsin in a number of proteins has shown that the conformation at, and surrounding, the cleavage site is far from this optimal conformation, thus requiring considerable change in local conformation and large changes in loop position relative to the remainder of the structure to interact with the proteinase active site (Hubbard *et al.*, 1991).

On the basis of this comparison the (non-serpin) inhibitor-proteinase interaction has been described as the classic "lock and key" fit of Fischer, whereas true substrate-proteinase interactions are better described as "induced fits." Such flexibility in substrates but not in inhibitors is required to permit adoption of a conformation in

which the transition state rather than the ground state is stabilised and thus to promote the proteolytic reaction rather than inhibition. For the rigid reactive site loop of the inhibitors only ground state stabilisation is favoured, with consequent inhibition (Gettins *et al.*, 1996).

C) Importance of reactive centre residues in determining specificity

Both serpin and non-serpin serine proteinase inhibitors usually show primary proteinase specificity determined by the P1 residue. A consequence of this is that changes at this position, whether through species variation, natural mutation or *in vitro* site directed mutagenesis, can result in loss of inhibitory properties or a change in inhibitory specificity. An example of this is the methionine to arginine change in the serpin α_1-PI (Pittsburgh variant), which converts the serpin from an inhibitor of elastase to an inhibitor of proteinases involved in blood coagulation (Owen *et al.*, 1983; Scott *et al.*, 1986; Wachfogel *et al.*, 1994).

In the non-serpin inhibitors, change of P1 from lysine or arginine to phenylalanine or tryptophan resulted in change from a good inhibitor of trypsin to a good inhibitor of chymotrypsin (Jering&Tschesche, 1976). In addition to the role of P1 in both types of inhibitor, interactions beyond this are also important for effective inhibition. In the case of non-serpin inhibitors there is an extended region that can involve residues on either side of the P1 residue, from as far away as P6 to P3′ (Read&James, 1986). In serpins it has also been shown that changes beyond P1 can change the proteinase specificity (Hopkins *et al.*, 1995; Olson *et al.*, 1995). However, whereas other

mutations have additive effects in non-serpin inhibitors (Wells, 1990), this appears not to be the case for serpins (Hopkins *et al.*, 1995).

D) Inhibitory mechanism of non-serpin inhibitors

For non-serpin inhibitors the portion of the inhibitor reactive centre loop that interacts with the proteinase adopts an extended β-conformation. The extent of interaction is variable, but always results from multiple contacts, involving residues on both sides of the P1 primary specificity-determining residue. Complementarity involves both the side chains and the peptide backbone and is usually between the P6-P3 ′ region of the inhibitor and the S6-S3′ subsites of the proteinase. There is little alteration in conformation necessary to bind optimally to the target proteinase, resulting in little or no loss of binding energy to modify the conformation. The interaction is thus a good example of a lock-and key fit and thus resembles a Michaelis complex (Gettins *et al.*, 1996). The stabilisation of the ground state relative to the transition state ensures that a substrate cleavage reaction is not favoured, with the resultant inhibition of the interaction. In contrast, good substrates of the same proteinase are more flexible and are better described as induced fits. Flexibility in substrates is required to permit adoption of a conformation in which the transition state is stabilised rather than the ground state, with resulting promotion of the proteolytic reaction. Due to the non-covalent Michaelis-like nature of these non-serpin proteinase complexes, they are readily reversible unlike the serpins that almost always give irreversible complex formation (Gettins *et al.*, 1996).

1.3.2 Serpin reactions with cysteine proteinases

There are now a number of well-documented instances of inhibition of cysteine proteinases by serpins (Ray *et al.*, 1992; Hook *et al.*, 1993; Takeda *et al.*, 1995). While there is still a shortage of firm structural evidence for the operation of the same mechanism of inhibition of cysteine proteinases by serpins (as compared to that available for serine proteinase inhibition), it seems that the same mechanism could accommodate all the conditions necessary for effective inhibition of cysteine proteinases (Gettins *et al.*, 1996).

According to Gettins *et al.,* (1996), "the most critical attributes for serpins to function as inhibitors rather than as substrates are: (a) the ability to loop insert to give more stable structures, and (b) the ability to change conformation in the reactive centre loop in response to covalent intermediate formation." Given the similarities in their mechanism of substrate cleavage which involves nucleophilic attack on the peptide carbonyl to form an acyl enzyme intermediate in both cases, it would seem that serpins should, in principle be capable of inhibiting both cysteine and serine proteinases (Gettins *et al.*, 1996).

1.3.3 The serpin mechanism: advantages and disadvantages

At the level of protein biosynthesis and folding, the serpins possess the unusual ability to undergo facile conformational interconversion and also display features that are needed to direct metastable folding. The ability of serpins to fold into a metastable

state, and to subsequently undergo insertion of the reactive loop into β-sheet A, in an essential part of the proteinase inhibition mechanism and is probably one reason that serpins are so much larger than other proteinase inhibitors (Gettins *et al.*, 1996).

A means of regulating the activity of serpins that may have either positive or negative consequences is proteolytic inactivation by cleavage in the reactive centre loop by a non-target proteinase. Thus human neutrophil elastase, in the presence of heparin, is an effective inactivator of antithrombin (Jordan *et al.*, 1987; Jordan *et al.*, 1989), through cleavage at the P4-P3 bond (Carrell *et al.*, 1985). Of potential benefit in regulation of tissue remodelling and vascularisation, α_1-PI can be inactivated by matrix metalloproteinases such as matrilysin (Sire *et al.*, 1994).

A major disadvantage of the serpin mechanism results from the very conformational lability that enables serpins to function as inhibitors. The need to fold as a metastable state and the subsequent ability to undergo loop-to-sheet transitions makes serpins sensitive to mutations that affect one or both of these processes. One of the most dramatic examples of such a variant is the Z variant of α_1-PI which, as a result of problems during folding (Yu *et al.*, 1995), undergoes polymerisation into sheets of linked α_1-PI molecules, which are accordingly not secreted into the circulation and instead build up inside hepatocytes (Lomas *et al.*, 1992). It has been conclusively shown that polymers of α_1-PI form by insertion of the reactive loop of one molecule into β-sheet A of the next (Sivasothy *et al.*, 2000).

1.4 Serpin biosynthesis, clearance and receptors

1.4.1 Serpin biosynthesis

As is found with many plasma proteins, the liver is likely to be the major site of biosynthesis of circulating serpin. However, may extrahepatic tissues produce serpins presumably for the regulation of localised proteolytic pathways. α_1-PI is a major plasma protein, but is also produced in a number of extrahepatic locations, for example by monocytes, neutrophils (du Bois *et al.*, 1991), cornea, kidney and intestine (Kelsey *et al.*, 1987; Molmenti *et al.*, 1993). The finding that the gene is under the control of tissue specific promoters (Perlino *et al.*, 1987) and transcriptional initiation sites (Hafeez *et al.*, 1992) suggests that there are important regulatory mechanisms that needs to be elucidated.

The endoplasmic reticulum (ER) transmembrane protein calnexin, which is a type of molecular chaperone, has been shown to associate transiently with newly expressed α_1-PI and α_1-antichymotrypsin in the ER of HepG2 cells (Ou *et al.*, 1993). Calnexin appears to bind to incompletely folded glycoproteins and involved in the regulated release of these proteins from the ER. Both α_1-PI and α_1-antichymotrypsin are acute phase reactants. In response to an inflammatory stimulus or infection the plasma concentrations can increase up to 4-fold (Koj&Regeoczi, 1978; Sehgal *et al.*, 1989). The main site of antithrombin biosynthesis is the liver (Owens&Miller, 1980; Fair&Bahnak, 1984) but biosynthesis can also occur in the kidney and endothelial cells (Chan&Chan, 1981; Lee *et al.*, 1979).

Protease nexin 1, which is important in regulating proteolytic pathways in the brain, is produced by both astrocytes and glial cells (Guenther *et al.*, 1985; Vaughan, 1993). In the case of glial cells, synthesis is increased by cytokines such as interleukin-1, tumour necrosis factor-α and transforming growth factor-β. Protease nexin 1 is also produced by HepG2 cells (Kazama *et al.*, 1993) and fibroblasts (Farrell *et al.*, 1988).

1.4.2 Serpin-proteinase complexes clearance

In humans the half-life for serpin-proteinase complexes in the circulation is very much shorter than for native serpins, with the serpin-proteinase complex being only a few hours compared with over 24 hours for the native serpins. Studies on the clearance of radiolabelled (I^{125}) human serpins injected into experimental animals showed that there was about a 10-fold faster clearance of serpin-proteinase complex than of native serpins (Gettins *et al.*, 1996).

In addition, competition studies which examined clearance of several serpin-proteinase pairs, including thrombin-antithrombin complex; α_1-antichymotrypsin-cathepsin G complex and α_1-proteinase inhibitor-trypsin complex, showed that a common receptor appeared to be involved. In the case of α_2-antiplasmin-plasmin complexes, binding may involve a separate receptor indicating the possibility of two serpin-proteinase complex receptors. The clearance of many serpin-proteinase complexes by the same receptor, and the specific recognition of complexes over native or even cleaved serpin indicates that the determinates for rapid removal are associated with the whole complex instead of then just the serpin moiety (Gettins *et al.*, 1996).

1.4.3 SEC Receptor

It has been proposed that there is an abundant, high-affinity cell surface receptor on human hepatoma cells and human mononuclear phagocytes that recognise a conformation-specific domain of the α_1-PI-elastase complex as well as of the other serpin-enzyme complexes and has been called the serpin-enzyme complex (SEC), (Gettins *et al.*, 1996). Binding to this SEC receptor activates a signal transduction pathway for increased expression of the α_1-PI gene. In addition, to its role in signal transduction the SEC receptor participates in internalisation and delivery of the α_1-PI-protease complex to lysosome for endocytosis and degradation. Various data indicate that this receptor can be found on monocytes, hepatocytes, neural and other tissue. At present there is not sufficient evidence to suggest that the SEC receptor could be generalised to control the activity of the other serpins other than α_1-PI (Gettins *et al.*, 1996).

1.5 Serpins and disease

1.5.1 Alpha-1 Proteinase Inhibitor deficiency (α_1PI)

The gene for α_1PI has been known to play a role in the pathogenesis of chronic obstructive lung disease (which includes asthma) and may result from a disturbed balance between extracellular matrix (ECM) proteases and their inhibitors (Walls, 1995), resulting in the destruction of alveolar walls (Cox, 1995). Studies have provided evidence for the involvement of this important proteinase inhibitor in

bronchial asthma (Gaillard *et al.*, 1994; Cox, 1995). There are two main alleles associated with α_1PI deficiency, denoted Z and S. A study of 151 children with severe asthma found similar frequencies of the Z allele in the asthma group compared to the control group. However, the children with steroid-dependent asthma contained more Z heterozygotes than the nonsteroid-dependent asthmatics and normal control subjects. This suggests a link between the Z allele and asthma severity (Sandford *et al.*, 1996). There have been other reports concerning asthma and the α_1PI gene. One of the most common alleles at the α_1PI (locus) is known as M_2 and one report has identified an increased prevalence of M_2 in asthmatics (Gaillard *et al.*, 1992). The association of bronchial hyper-responsiveness and S heterozygosity has been detected in a group of asthmatic families (Townley *et al.*, 1990). In addition, there have been reports of an association between asthma and another antiprotease deficiency; that is low levels of α_1-antichymotrypsin. However, the genetic mechanisms have not yet been elucidated (Sandford *et al.*, 1996).

1.5.2 The role of PAI-1 in vascular disease

PAI-1 deficiency is somewhat rare but seems to be associated with bleeding episodes, particularly after trauma or surgery (Schleef *et al*, 1989, Diéval *et al.*, 1991; Fay *et al.*, 1992). More problematic seems the case of an excess of PAI-1. The findings that the plasma concentration of PAI-1 is only 0.4 nM and that expression of the gene is subject to extensive regulation indicates how detrimental high levels of PAI-1 can be. Elevated PAI-1 has been suggested to be a risk factor for myocardial infarction (Hamsten *et al.*, 1985; Hamsten *et al.*, 1987).

1.5.3 Serpins and Alzheimer's disease

There is a growing body of evidence that proteolytic pathways are essential for neural processes such as synaptic plasticity and long term potentiation (Festoff, 1990; Qian *et al.*, 1993; Sappino *et al.*, 1993). Also, serpins have been implicated in neurological diseases such as Alzheimer's disease. PAI-2, C1-inhibitor and antithrombin have been found in amyloid plaques (Kalaria *et al.*, 1993). The most important serpin, however, is likely to be α_1-antichymotrypsin. This serpin is found in the amyloid plaques and is expressed by astrocytes from Alzheimer's disease brain (Pasternack *et al.*, 1989), Although serum α_1-antichymotrypsin does not seem to be a reliable diagnostic marker for Alzheimer's disease ((Hinds *et al.*, 1994). α_1-Antichymotrypsin can modulate the ability of the amyloid β-peptide to form filaments (Ma *et al.*, 1994; Eriksson *et al.*, 1995).

1.5.4 Serpins and cancer

Two serpins implicated in cancer are maspin and squamous cell carcinoma antigen. The acidic variant of squamous cell carcinoma antigen (SCCA2) is expressed primarily by malignant cells and is the major form in the plasma of cancer patients (Gettins *et al.*, 1996). It is also possible that rearrangements of the serpin gene cluster at 14q32.1 are involved in some types of cancer (Byth *et al.*, 1994).

1.6 The genetics of atopy and asthma

Studies of monozygotic and dizygotic twins raised apart show that genetic factors are important in the aetiology of atopic asthma and other allergic diseases. There is little doubt that there is a major hereditary contribution to the aetiology of asthma and allergic diseases. However, the inheritance of asthma and allergy does not follow classical Mendelian patterns that are characteristic of single-gene disorders (Cookson, 1994; Sandford *et al.*, 1996). The inheritance pattern of asthma demonstrates that it is a "complex genetic disorder". A similar complex inheritance is seen in disorders such as hypertension, atherosclerosis, diabetes mellitus and arthritis. All of these diseases show a clear hereditary pattern, but the mode of inheritance cannot be simply classified as autosomal dominant, recessive, or sex-linked (Sandford *et al.,* 1996). Another important feature that distinguishes these complex genetic disorders from single-gene disorders is their prevalence. Atopy in the Western world has a prevalence of 40-50% (Daniels *et al.*, 1996), with asthma occurring in 4-8% of the population, and allergic rhinitis reported in 25% of some populations, as compared to the most frequent Mendelian disorder affecting the lungs, cystic fibrosis (CF), which occurs once in every 2 000 live Caucasian births, which is approximately 100 times less frequent than asthma (Sandford *et al.,* 1996).

Possible reasons that these conditions are not inherited in a simple Mendelian fashion include the following: (a) there may be a number of genes that predispose people to develop these complex traits - either more than one gene in the same individual (polygenic inheritance) or different combinations of genes in different individuals

(genetic heterogeneity); and (b) environmental factors may be necessary for expression of the disease phenotype (Sandford *et al.,* 1996).

Asthma is characterised by chronic inflammation, with infiltration of eosinophils, lymphocytes, and monocytes/macrophages, and is associated with the increased expression of several inflammatory proteins, including cytokines, enzymes and adhesion molecules in the airways (Hart *et* al., 1998). Figure 1.4 illustrates the functional roles of the various candidate genes that have been implicated in the pathogenesis of allergy and asthma.

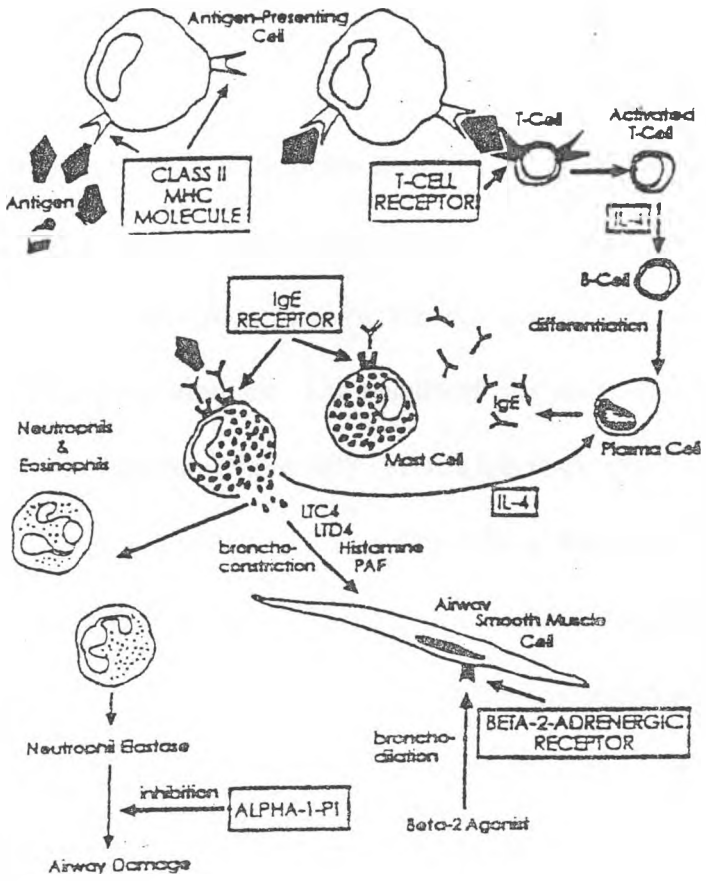


Figure 1.4 Functional roles of various candidate genes that may be involved in the pathogenesis of atopy and asthma. The candidate genes are shown in the boxes: (1) specific MHC/HLA class II antigens allow specific inhaled allergens to be presented more effectively to T lymphocytes by monocytes and other antigen presenting cells. (2) Specific T cell receptor (TCR) types allow more effective T cell responses to particular combinations of MHC/HLA II antigen-allergen complexes. (3) More active, or increased production of IL-4 results in enhanced IgE synthesis. (4) Enhanced binding and signalling by high affinity IgE receptors on mast cells, basophils and other effector cells cause an exaggerated response to allergen-IgE complexes, causing the release of more inflammatory mediators and more IL-4, which in turn further boosts IgE levels by a positive feedback loop. (5) The allergic inflammatory response to mediators and cytokines is increased because of the decreased antiproteolytic activity of alpha-1-protease inhibitor (α_1PI) and other antiproteases, and/or because of a disruption of the protease-antiprotease balance. (6) The smooth muscle response to contractile agonists released from effector cells is enhanced due to defective or downregulated β_2-adrenergic receptors (from Sandford *et al.*, 1996).

Linkage to chromosome 14q and the T Cell Antigen Receptor (TCR)

Linkage has been demonstrated between the α region of the TCR locus and specific IgE responses (Moffatt *et al.*, 1994). The evidence for linkage was assessed using 312 sib-pairs. The linkage pattern suggested a recessive genetic effect that was detected in two separate population samples. The results of this study showed that a gene or genes in the TCR-α region might modify specific IgE responses in atopic individuals (Moffatt *et al.*, 1994). The region also contains the δ chain TCR genes, which are found within the α locus; therefore, they are also candidates for linkage (Sandford *et al.*, 1996). Although this result has not been replicated independently, it suggests that polymorphisms within the TCR genes may limit an individual's ability to respond to specific antigens (Sandford *et al.*, 1996). Chromosome 14q contains many genes that could contribute to the susceptibility of IgE-dependent diseases. These include the immunoglobulin heavy chain genes (IGH) with important functions in immunoglobulin class switching in B cells, the nuclear factor kappa B inhibitor (NfκBI), the transforming growth factor beta 3 (TGF-β) and the transcription factor FOS (figure 1.5). Polymorphisms in such genes or other unidentified genes in the region may modulate total serum IgE and predisposition to allergic disease (Mansur *et al.*, 1999).

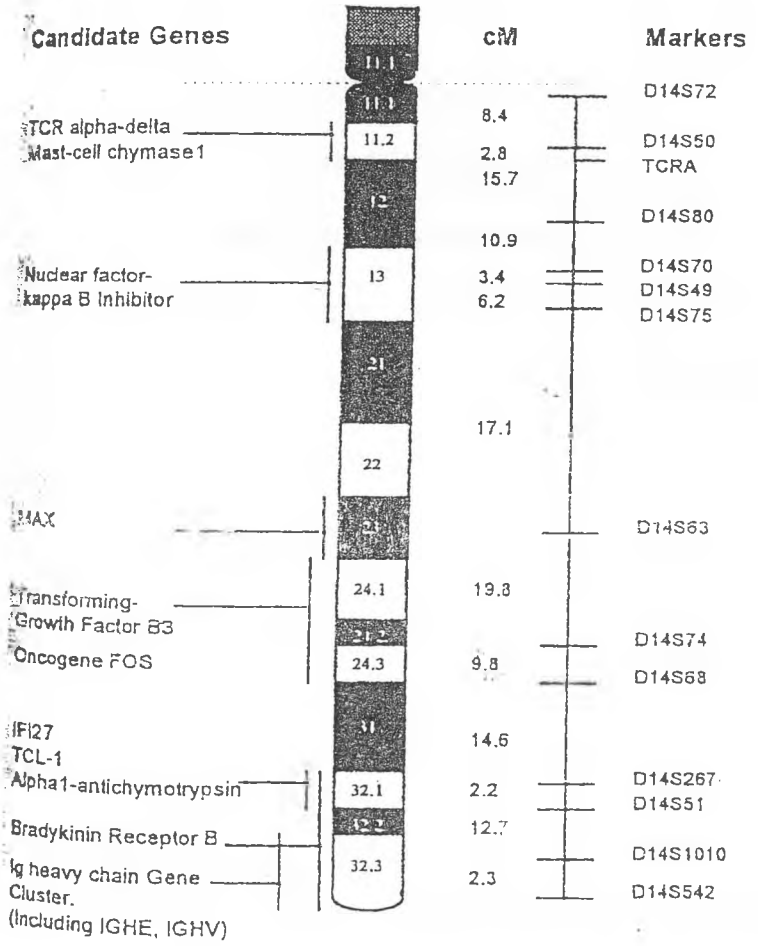


Figure 1.5 Chromosome 14 map showing the cytogenetic regions, the corresponding relative position and order of the markers and the candidate genes mapped to this chromosome. The (sex-averaged) distance in centimorgans and the order of the markers generally correspond to published maps. The relative positions of the candidate genes are based on data obtained from the Genetic Location Data Base (LDB). Abbrevaitions: MAX=oncogene *myc*-associated protein; IF127=interferon-alpha inducible factor 27; TCL-1=T-cell lymphoma factor 1; IGHE=immunoglobulin heavy chain epsilon locus; IGHV=immunoglobulin heavy chain variable locus (from Mansur *et al.*, 1999).

Nuclear Factor-κB and asthma

Asthma is associated with increased expression of inflammatory proteins. The molecular regulatory pathways involved in the induction of chronic cytokine expression and the recruitment and activation of inflammatory cells in asthma are not well understood, but there is an increasing recognition that these processes involve increased transcription of inflammatory genes and that is regulated by transcription factors. One such transcription factor, nuclear factor-κB (NF-κB), regulates the expression of a wide range of genes involved in immune and inflammatory response (Hart *et al.*, 1998).

The observation that some cytokines regulated by NF-κB, such as TNF-α and IL-1β, can also activate NF-κB expression, indicates that this may represent a feed-forward amplifying loop that could form the basis for the persistence of the chronic inflammatory process in asthma. Although NF-κB has thus far been considered as mediating acute inflammatory responses, it has been shown to be chronically activated in chronic inflammatory conditions such as inflammatory arthritis and atherosclerosis (Brand *et al.*, 1996; Marok *et al.*, 1996).

It has been shown that the chronic airway inflammatory process of asthma is associated with an increase of activated NF-κB, which may play an important role in inflammatory and immune responses. NF-κB is not specific for asthma, but may act in synergy with other transcription factors to induce maximal gene expression. The specificity of the inflammatory and cellular responses in asthma may depend on the

interaction of NF-κB with other transcription factors (Hart *et al.*, 1998). Further studies are needed to characterise the roles of the NF-κB family of transcription factors in this interaction in asthma and to determine whether the levels of these transcription factors determine the severity and course in disease.

Linkage to Chromosome 11q13 and the High-Affinity Immunoglobulin E Receptor

Chromosome 11q13 was implicated in the pathogenesis of atopy during a random genome search by Cookson *et al.* (1989), although further evidence exists for non-linkage in many large families (Cookson, 1994). The initial study caused much controversy over its broad definition of atopy and it's failure to replicate linkage in other populations (Hizawa *et al.*, 1992; Lympany *et al.*, 1992). A sib-pair analysis on 743 subjects conducted to reassess the evidence for linkage showed robust evidence for linkage of atopy to chromosome 11q13. This linkage was independent of the definition of atopy and was only observed through the female line (maternal inheritance) (Cookson, 1994). A suggested mechanism for this was the influence of the maternal immune system on the foetus or neonate (Sandford *et al.*, 1996) or imprinting of the paternal copies of the gene (Hopkin, 1995). Imprinting is a process by which a particular gene is differentially activated or silenced (probably by the demethylation or methylation of CPG islands on the gene) depending on the sex of the parent from whom it was inherited (Razin&Cedar, 1994; Sandford *et al.*, 1996). It was suggested that the atopy gene was only expressed when inherited from the mother, indicating that immunological interactions between mother and child, rather than

genomic imprinting, might be responsible for the maternal effect on the inheritance of the atopy gene on chromosome 11q13 (Daniels *et al.*, 1996).

In subsequent studies, investigators have used a maternal inheritance model for the linkage analysis. Shirakawa and colleagues (1996) found linkage to an atopy gene on chromosome 11q13 in a Japanese population. All these observations support the notion that atopic asthma is indeed a polygenic disorder.

The most likely position of the atopy gene was found to coincide with that of the high affinity receptor for IgE (FcεRIβ) (Sandford *et al.*, 1993). Linkage studies of the FcεRIβ locus have generated conflicting results and it has been suggested that FcεRIβ (or another gene nearby) may predispose patients to bronchial hyper-responsiveness, rather than to atopy (Van Herwerden *et al.*, 1995). A variant of the FcεRI receptor (Leu 181) was detected that segregated with atopy when maternally inherited, a finding which supported the hypothesis that FcεRIβ was the gene responsible for the linkage in 11q13. A possible model for the action of this mutation is that it increases the signal transduction activity of the receptor. This change would also increase the level of activation of mast cells which release interleukin 4 (IL-4), which would, in turn, stimulate the synthesis of higher levels of IgE (Sandford *et al.*, 1996). The study by Shirakawa and co-workers (1996), also showed the absence of association between atopy and gastric intrinsic factor (GIF) and CD20 (the 5' and 3' flanking genes of FcεRIβ, respectively), further supporting the candidacy of FcεRIβ as the atopy gene on chromosome 11q13. Another two studies have also shown positive associations between atopy phenotypes and polymorphisms in the 11q13 region; one within the

FcεRIβ gene (Shirakawa *et al.* 1995) and one with a marker from the 11q13 region (Hizawa *et al.*, 1995).

Other candidate genes

There have been studies suggesting that atopy, asthma and bronchial hyper-responsiveness are associated with heterozygosity for cystic fibrosis (CF) of the pancreas (Gerrard, 1985). It was found that heterozygosity for the most common CF allele (±F508) might protect against asthma. It was suggested that this heterozygote advantage could account for the high prevalence of the allele in the Caucasian population, however further studies have not been able to find an association between CF and atopic asthma (Sandford *et al.*, 1996).

Similarly, a possible connection between asthma and familial Mediterranean fever (FMF) has recently been investigated. The high rate of heterozygosity for the FMF gene in some populations suggests that there may be a heterozygote advantage for FMF, however the results of this study were inconclusive (Sandford *et al.*, 1996).

A plausible candidate for corticosteroid resistance in asthmatic subjects is the glucocorticoid receptor gene. A missense mutation in this gene had previously been found to be responsible for familial glucocorticoid resistance (Hurley *et al.*, 1991). In one study the investigators analysed six corticosteroid-resistant and six corticosteroid-responsive asthmatics and compared the data to the wild type gene sequence. As no mutations were found in any of the subjects, this suggested that the cause of

corticosteroid resistance in these asthmatics does not lie in the structure of this gene (Sandford *et al.*, 1996).

The results of a recent study have provided evidence that the gene for interferon-γ (INF-γ) may be involved in the production of the atopic state. INF-γ is a good candidate gene for atopy, since it acts as an antagonist to IL-4 and downregulates the production of IgE from B cells (Tang *et al.*, 1994). The authors found that low cord blood INF-γ levels were predictive of atopic symptoms at age 12 months, suggesting that reduction of INF-γ secretion is a cause rather than a result of the atopic state (Tang, *et al*, 1994).

There are complete genome searches underway that systematically attempt to discover all the genes involved in susceptibility to atopy and asthma. The results of these searches will identify additional candidate genes from those known to be part of the allergic pathway. It is also likely that previously unknown genes will be identified. This search should lead to considerable advances in our knowledge of the mechanisms that lead to asthma and other allergic diseases (Sandford *et al.*, 1996).

1.7 Mechanisms of airway inflammation in asthma

Until recently, asthma has been viewed primarily as a respiratory disease of acute airway obstruction. Thus, research and therapeutic attention focussed principally upon mechanisms leading to acute bronchospasm. Consequently, first-line therapy consisted of bronchodilators to regulate airway smooth muscle contraction. The

contribution of other components of airway obstruction, as well as the potential benefit of anti-asthma therapy directed towards control of these abnormalities, has been largely neglected. However, the approach and focus in asthma therapy and concepts of pathogenesis have begun to change considerably.

There is currently a general consensus that patients with asthma have inflammatory changes in the airway wall, with increased numbers of activated mast cells, eosinophils, macrophages and T-lymphocytes. In addition, there is commonly epithelial shedding. These changes have been observed in bronchial biopsies of even the mildest of asthmatic patients and have been observed in patients with asthma of different clinical types (Saetta *et al.*, 1992).

It is now becoming clear that cytokines released from inflammatory and structural cells in the airway may play a major role in co-ordinating and perpetuating chronic inflammation. Identification of the cytokines involved in asthma and their effects on cell function are now an important focus for research (figure 1.6). Cytokines produce chronic changes in cell responsiveness by influencing the transcription of certain genes that may result in the altered expression of receptors, enzymes and regulatory proteins (Muegge&Durum, 1990).

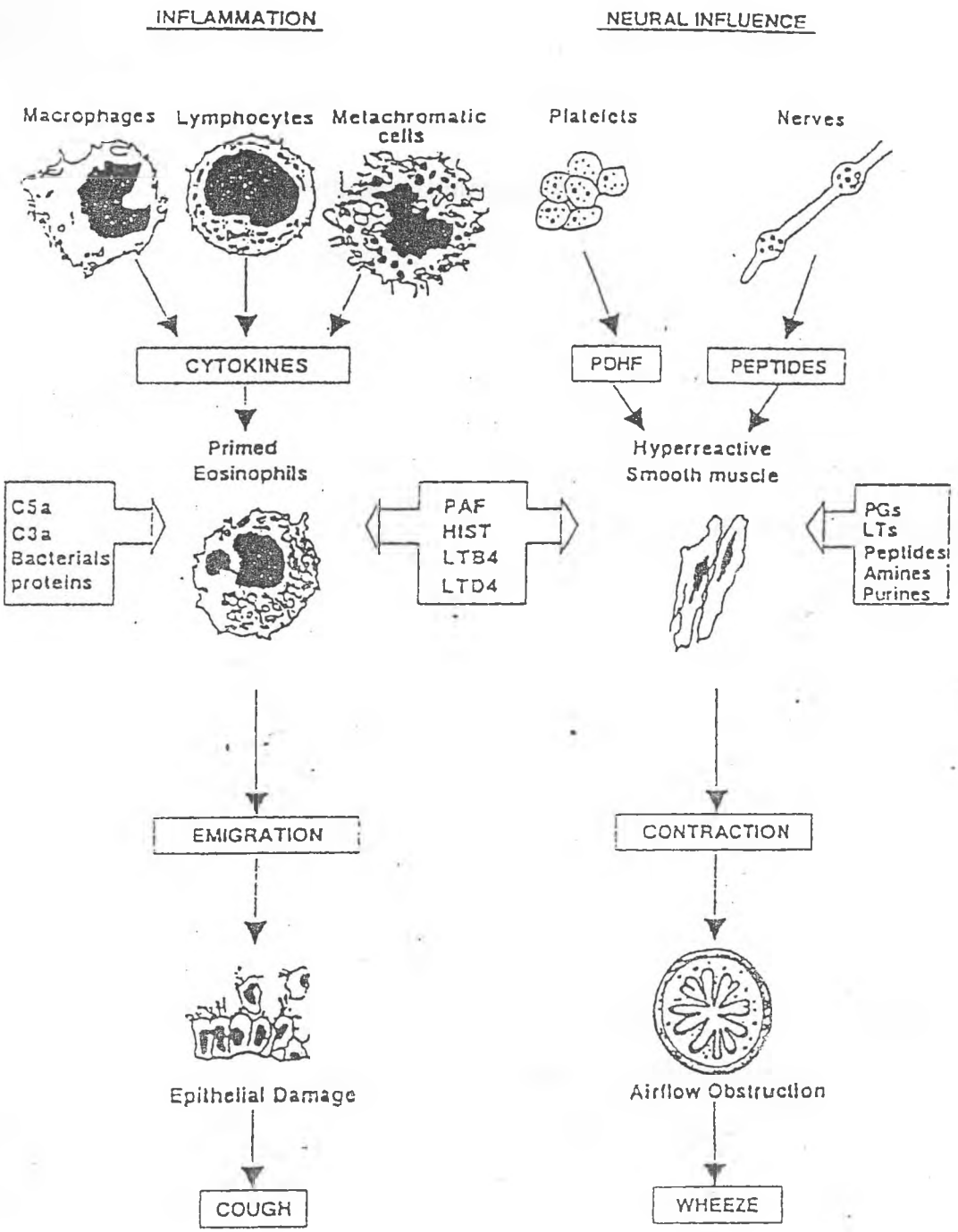


Figure 1.6 Schematic representation of airway hyperreactivity and inflammation in asthma, emphasising a parallel rather than sequential interrelationship (from Chapman *et al.*, 1993).

Elucidating the mechanisms that are involved in the development and perpetuation of the inflammatory and obstruction of the airways that is characteristic of asthma is crucial. The following brief review of the cells participating in allergic airway reactions focuses on the cellular and mediator mechanisms that are believed to be essential for the asthmatic diathesis.

1.7.1 Mast cells

Mast cells (MCs) are high affinity IgE receptor bearing cells that are present in the airway epithelium which appear to be activated in allergic and inflammatory responses. Mast cell mediators such as histamine and tryptase are present in bronchoalveolar fluid. Mast cells may be activated by an inhaled allergen via an IgE dependent mechanism (which involves the cross-linking by allergen, and internalisation of IgE receptors), (Nilsson&Schwartz, 1995). Mast cells possess high-affinity receptors that bind IgE. Cross linkage of cell-bound IgE by binding of allergen results in a rise in intracellular calcium concentration and activation of protein kinase C, leading to phosphorylation of a granule membrane protein. Mast cell degranulation releases histamines and tryptase (Marom, 1991). This IgE-mediated mechanism initiates arachadonic acid metabolism to generate leukotrienes and prostaglandins, which in conjunction with histamine, are undoubtedly involved in the bronchospastic response of their airway smooth muscle contractile properties (Drazen&Austen, 1981). The MCs generation and release of chemotactic factors then sets the stage for recruitment of other inflammatory cells to the airways, and the development of the late asthmatic response (LAR) (figure 1.7).

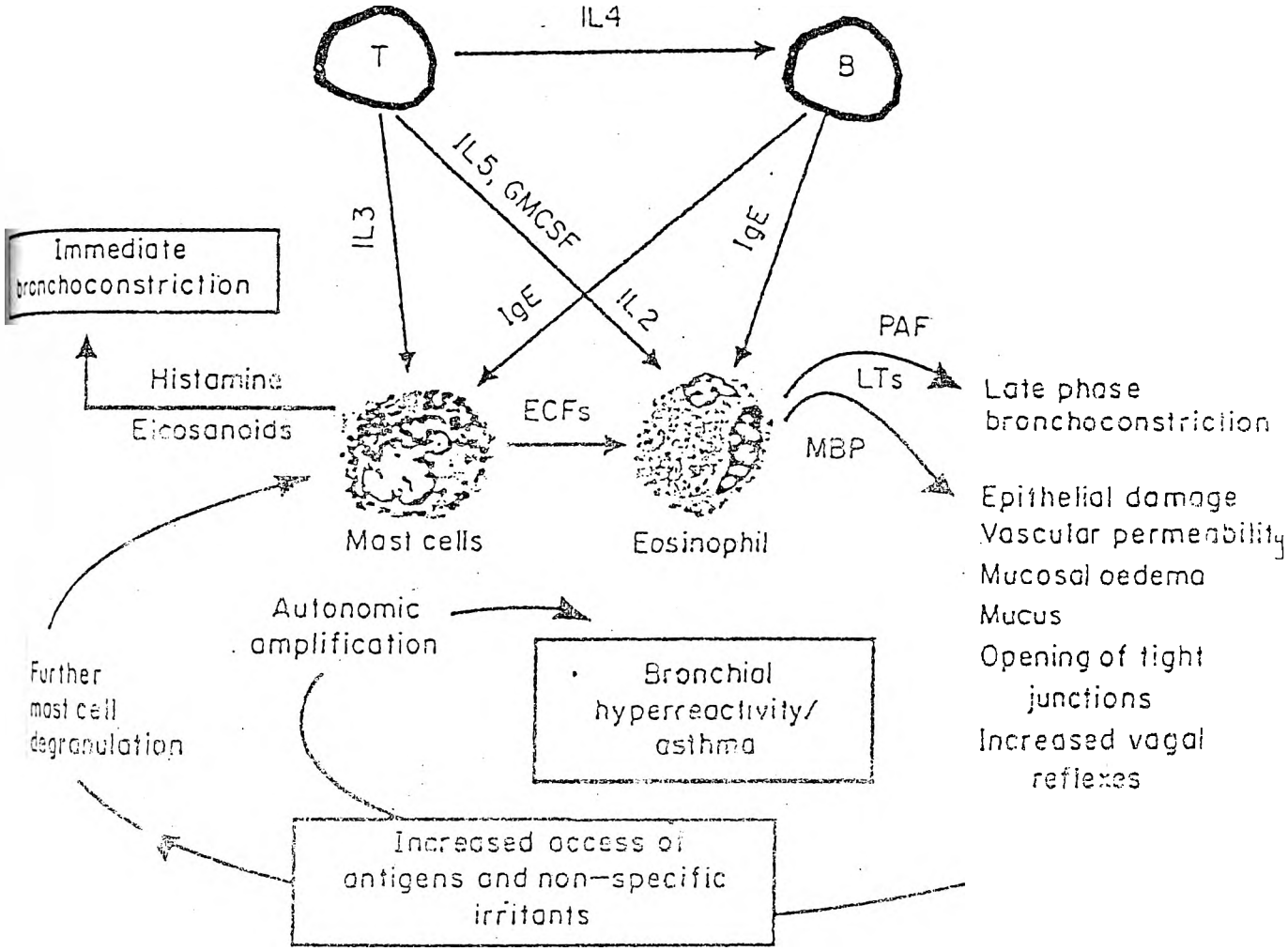


Figure 1.7 Mechanisms involved in the development of airway inflammation and bronchial hypereactivity in asthma (from Richards *et al.*, 1992).

It has been reported that murine MCs synthesise a variety of cytokines upon IgE receptor aggregation and internalisation, and although data on the cytokine production by human MCs are limited, it is possible that MCs are pivotal in recruiting and priming inflammatory cells in sensitised individuals (Arm&Lee, 1992). *In vitro* studies have demonstrated that several different cytokines are produced by MCs, including interleukins (IL)-3, 4, 5 and 6, interferon (INF)-γ and tumour necrosis factor (TNF)-α (Valent, 1994). Both IL-3 and stem cell factor (SCF), a unique activation factor for human MCs, are the major cytokines involved in MC differentiation (Valent, 1994). Furthermore, emerging data suggest a similar MC cytokine profile in humans (Valent, 1994). The ability of MCs to produce such a plethora of cytokines provides them with great potential for interactions with other cell types to modulate homeostatic, allergic, inflammatory and host defence functions. Furthermore, an autocrine role for these cytokines on cell function, growth and differentiation should be considered (Nilsson&Schwartz, 1995).

1.7.2 Basophils

Basophils have long been considered the circulating counterpart of MCs. Their exact function remained elusive for many years, however, recent studies have shed some light on the contribution of basophils to various illnesses. The blood basophil count increases during the pollen season, suggesting that basophilopoiesis may be influenced by environmental factors, such as allergens (Alam&Grant, 1995).

The late-phase allergic response (LPR) induced by antigen challenge in highly allergic patients seems closely related to the naturally occurring reaction in chronic allergic disorders. The number of metachromatic cells increases at the site of LPR.

Since both basophils and mast cells release similar mediators, the exact contribution of each cell type proved difficult to determine (Alam&Grant, 1995). However, it was discovered that the profile of mast-cell/basophil-derived mediators released in the early and late phase allergic reaction is not identical. Histamine is detectable in both phases. Prostaglandin D_2 (PGD_2), a major mediator produced by mast cells, but not by basophils, is detectable in the early but not the late phase allergic reaction (Naclerio *et al.*, 1985).

Metachromatic granules of basophils contain a large number of pharmacologically active mediators that are released upon basophil activation. Mediators are typically classified as preformed and newly generated. The preformed mediators are histamine, neutrophil chemotactic factor, N-α-ρ-tosyl-L-arginine methyl ester hydrochloride (TAME) esterase and kallikrein, while the leukotrienes LTC_4, LTD_4 and LTE_4 and platelet activating factor (PAF), are the most important lipid mediators that are generated almost immediately after allergen challenge (Busse *et al.*, 1994; Alam&Grant, 1995). It has been well established that viral infection of the airways causes exacerbation of asthma. Although the mechanism of such an exacerbation may be complex, incubation of basophils with viruses results in enhanced mediator release and chemotaxis (Chonmaitree *et al.*, 1991). Thus, the heightened mediator release from basophils may exacerbate the symptoms of asthma.

1.7.3 Eosinophils

Of the effector cells in asthma, the eosinophil has possibly the most important and potentially pivotal role in generating airway inflammation. The biology of the eosinophil is an indication of this cell's potential relevance to asthma (Weller, 1991; Howell, 1995). In 1975, Horn and co-workers examined the usefulness of total eosinophil counts in the diagnosis and management of steroid dependent asthma. They found that eosinophil counts in a cohort of asthma patients paralleled disease severity. Total eosinophil counts were inversely related to pulmonary function and a rising eosinophil count was found to be associated with increasing bronchial obstruction as reflected by changes in specific conductance, forced expiratory volume in 1 second (FEV_1), and maximum mid-expiratory flow rate.

Recent data indicate that eosinophils themselves can elaborate proinflammatory cytokines in allergic reactions. Initial studies demonstrated that human eosinophils express and release transforming growth factor β (TGF-β) and interleukin-1 (IL-1), (DelPozo *et al.*, 1990; Wong *et al.*, 1990). Although the relevance of TGF-β to allergic inflammation is unclear, IL-1 has the capacity to induce adhesion-molecule expression on endothelial cells and is itself an eosinophil activator. Recent studies have suggested that eosinophils have the capacity to synthesise IL-3, GM-CSF and IL-5 (Desreumaux *et al.*, 1992; Kita *et al.*, 1991; Moqbel *et al.*, 1991).

Thus, although the T lymphocyte is considered to be a major source of these cytokines, it is possible that eosinophils themselves also contribute to the overall cytokine

production in allergic airway inflammation. This raises the possibility of an autocrine mechanism whereby stimulated eosinophils may both release and respond to cytokines, such as IL-1, IL-3, IL-5 and GM-CSF. Thus there is the potential for a self-perpetuating cycle, with continuous eosinophil infiltration and activation, and consequently chronic inflammation (Björnsdottir *et al.*, 1995).

The recent observations indicating a dependence on the presence of functional T lymphocytes to produce LAR eosinophilic inflammation of the airways, suggest that a product derived from T lymphocytes will be the "activation" factor for eosinophils. However, further in-depth studies of eosinophil-T-lymphocyte interaction will be required (Björnsdottir *et al.*, 1995).

1.7.4 Neutrophils

The evidence that neutrophils play a critical role in bronchial asthma is controversial. Neutrophils appear to be normal resident cells of the larger airway. Similar numbers of neutrophils have been found in bronchial wash and in bronchial biopsies in asthmatic subjects, in subjects with chronic bronchitis and in control healthy subjects (Kirby *et al.*, 1987; Fabbri&Ciaccia, 1992; Saetta *et al.*, 1992). The fact that the number of cells is no different does not exclude the possibility that cells may have a different degree of activation. For instance, the plasma chemotactic activity for neutrophils is increased and peripheral-blood neutrophils show markers of activation during active asthma, after exercise-induced asthma and during early and late asthmatic reactions induced by allergens and toluene diisocyanate (TDI) (Sastre *et al.*,

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1990).

Interestingly, the percentage increase in plasma neutrophil chemotactic activity correlates with the percentage decline of FEV_1 of the early asthmatic reaction (Sastre *et al.*, 1990). In humans, bronchoalveolar neutrophilia occurs before the onset and during the first few hours of the allergen-induced late asthmatic response and the increase of neutrophils is much larger than the increase of the number of airway eosinophils, the neutrophil increase usually precedes the eosinophil increase, the airway neutrophilia and the increased plasma chemotactic activity and activation of circulating cells are all prevented by anti-inflammatory steroids and cromolyn, which attenuate spontaneous allergen-precipitated asthma attacks, thereby suggesting as association between asthma attacks and the migration and activation of neutrophils in airways. Circulating neutrophil-generated superoxide anion (O_2-) is increased in young asthmatics and correlates with the degree of histamine-induced bronchial hyperesponsiveness (Fabbri *et al.*, 1995).

Even stronger evidence for the role of neutrophils in asthma comes from several studies which have demonstrated that these cells have the ability to synthesise and release immunoregulatory cytokines, which include TNF-α, IL-1β, and the IL-1 receptor antagonist (IL-1Ra), IL-8, transforming growth factor (TGF)-β1 and IL-6, although the evidence for the release of the latter is contradictory. All these cytokines (excluding IL-1ra), as well as granulocyte colony-stimulating factor (G-CSF) and GM-CSF significantly prolong neutrophil survival (Cassatella, 1995).

In addition, the neutrophil is a potential source of a wide variety of mediators, including potent lipid mediators such as prostaglandins, thromboxanes, leukotrienes B_4 and PAF, which may then contribute to airway responses and/or exacerbation of the inflammatory response. From lavage studies, it is known that there is a neutrophilia present before, during and after late (but not early) asthmatic reactions induced by allergens (Metzger *et al.*, 1987).

Although there is substantial evidence that neutrophils may be involved in asthma attacks and asthma exacerbations, and although neutrophil products can indeed alter airway function, the role of these cells in causing the pathologic and physiologic features of asthma remains to be established.

1.7.6 Macrophages and monocytes

Alveolar macrophages (AM) and mononuclear cells express $Fc_\varepsilon RII$ (CD23). The proportion of monocytes and macrophages expressing CD23 is increased in allergic patients compared to normal individuals and is further enhanced following airway antigen challenge (Borish *et al.*, 1991). Macrophages and monocytes are also activated by antigen in an IgE-dependent manner to transcribe and secrete cytokines (IL-1β) and TNF-α (Borish *et al.*, 1991), produce superoxide anion, release β-glucuronidase and other lysosomal enzymes and augment cytotoxicity (Calhoun&Jarjour, 1995).

Upon activation, AM and mononuclear cells release a plethora of compounds which have been implicated in or linked to asthma. These factors can be broadly grouped as: (1) chemotactic factors such as LTB_4, IL-8, complement fragments and a neutrophil chemotactin distinct from IL-8, all of which can serve to recruit granulocytes; (2) cytokines and other cell-activating factors which includes IL-1 β, TNF-α, IL-6 and GM-CSF; (3) smooth muscle and mucous gland activators, including LTC_4, PGD_2 (smooth muscle spasmogen), endothelins 1 and 3 (regulate smooth muscle tone), LTB_4 and IL-1β (promote dose-dependent mucous glycoprotein secretion); and (4) direct inflammogens which include β-glucuronidase, neutral proteases and several lysosomal enzymes, which, as mentioned previously, are released following IgE receptor cross-linking, and all of which are believed to be instrumental in amplifying the inflammatory response (Arm&Lee, 1992; Calhoun&Jarjour, 1995; Drazen *et al.*, 1995).

1.8 Objectives of the present investigation

The striking conservation in the sequences, probable mechanism and structure of serpins from organisms as diverse as viruses, barley, insects and mammals clearly show how remarkable the serpins are. Studies at both the protein and genetic level have much to tell us about the phylogenetic relationships between organisms as well as the evolution of different serpins within a particular organism. The advances in molecular genetics and the increased understanding of the biochemistry of serpins have done much to enhance our understanding of the role of serpins in human disease and will continue to do so.

This area is likely to expand both diagnostically and therapeutically, not only for serpin-deficient states but also as the role of serpins in the pathogenesis of disease caused by viruses and parasites in elucidated. With this in mind the present study was undertaken in order to: (a) characterise new variants of α_1-proteinase inhibitor in association with asthma; and (b) assess the stability of the newly characterised serpin-proteinase complex.

CHAPTER 2

CHARACTERISATION OF NEW VARIANTS AND A NOVEL POLYMORPHISM OF ALPHA-1 ANTITRYPSIN

2.1 Introduction

Alpha-1 antitrypsin (α_1-AT) deficiency is an autosomal hereditary disorder characterised by low serum and lung levels of α_1-AT, a high risk for the development of emphysema in the 3rd and 4th decades, and a lesser risk for the development of liver disease, particularly in childhood (Crystal *et al.*, 1990). This disorder is an extraordinary example of the power of modern genetics (Crystal *et al.*, 1990). It was first recognised in 1963,Laurell and Eriksson discovered the disease α_1AT deficiency by noting a marked reduction of the α_1-globulin band in approximately 1 of 500 serum protein electrophoresis patterns of a random Swedish population.

α_1-AT is an inhibitor of serine protease's, including neutrophil elastase, trypsin, chymotrypsin, cathepsin G, thrombin, tissue kallikrein, factor Xa and plasminogen (Carrell *et al.*, 1986; Travis&Salversen, 1983). For this reason, it is sometimes referred to by the more general term α_1-proteinase inhibitor (α_1PI). α_1-AT deficiency, one of the most common lethal hereditary disorders of Caucasians of European descent, is an autosomal recessive disorder characterised by reduced serum levels of α_1AT, a 52-kDa glycoprotein that functions as an antiprotease (Crystal *et al.*, 1989;

Carrell *et al.*, 1982). The deficiency state is caused by mutations in the α_1-AT gene, a pleomorphic 12.2-kb, 7-exon gene on chromosome 14 at q31-31.2 ((Long *et al.*, 1984). The major clinical manifestations of α_1-AT deficiency relate to the function of α_1-AT and where it is made. α_1-AT serves as an inhibitor of neutrophil elastase (NE), a powerful, destructive proteolytic enzyme stored in neutrophils. The liver is the major site of α_1-AT gene expression, releasing 2g of α_1-AT into the circulation daily. α_1-AT diffuses into most organs, where it protects extracellular structures from attack by NE released by activated or disintegrating neutrophils (Crystal, 1990).

The lower respiratory tract is particularly vulnerable to a deficiency of α_1-AT, which normally represents > 90% of the anti-NE protective screen of the alveolar walls (Gadek *et al.*, 1981; Wewers *et al.*, 1987). When serum α_1-AT levels are < 11 μM, there is insufficient α_1-AT to protect the lower respiratory tract from its burden of NE, permitting progressive destruction of the alveoli (Crystal, 1990).

2.1.1 Structure of α_1-AT

The α_1-AT protein is a single chain of 394 amino acids with three complex carbohydrate side chains (figure 2.1). The microheterogeneity of α_1-AT observed in isoelectric focussing (IEF) analysis of serum at pH 4-5 results mostly from differences in these carbohydrates. The two parental α_1-AT genes are codominantly expressed. Expression is controlled at the transcriptional level through sequences flanking, and within, the three 5′ non-coding exons (figure 2.2). Hepatocytes are the major source

of α_1-AT, but the gene is also expressed in mononuclear phagocytes and neutrophils, and possibly megakaryocytes, islet cells and intestinal epithelial cells (Crystal, 1989; Perlmutter *et al.*, 1989; Mornex *et al.*, 1986).

α_1-AT, has been crystallised in its active form (Elliott *et al.*, 1996; Elliott *et al.*, 1998 and Ryu *et al.*, 1996) and it crystallises after proteolytic cleavage at the reactive site (Loebermann *et al.*, 1984). Analysis of the crystal structure indicates that the single polypeptide chain is organised into well-defined secondary structural elements: three β sheets and eight α helixes. α_1-AT contains one cysteine residue, as indicated by both protein and DNA analysis. No disulfide bridge is present in the protein, although the thiol group can form a disulfide bond with other proteins, such as the IgA heavy chain and the thiol group of immunoglobulin κ light chain (Cox, 1995).

α_1-AT shows considerable genetic variability, having more than 70 genetic variants (PI types), many of which have been sequenced. Most variants are associated with quantitatively and qualitatively normal α_1-AT. Further variation can be revealed at the DNA level where a number of restriction enzymes reveal polymorphisms.

α_1-AT is modified during its passage through the endoplasmic reticulum. Some of this modification is reflected in the micro-heterogeneity observed in acid starch gel and agarose electrophoresis and in polyacrylamide isoelectric focussing, as is typical for glycoproteins near their isoelectric point. Eight bands were originally noted, numbered from 1 (anodal) to 8 (cathodal); bands 4 and 6 contain 40 and 35 percent, respectively, of the total α_1-AT and have isoelectric points of 4.52 and 4.59 (Cox,

1995). Much of the heterogeneity is due to differences in the type of carbohydrate side chain. Three carbohydrate side chains per molecule are attached at asparagine residues 46, 83, and 247. The carbohydrate chains may be biantennary or triantennary, terminating in two or three N-acetylneuraminic acid residues. The electrophoretic mobility of α_1-AT is sequentially shifted cathodally by incubation with neuraminidase as N-acetylneuraminic acid residues are removed.

The gene coding for α_1-AT, 12.2kb in length, includes a 1434-bp coding region. The gene contains six introns; exons 1A through 1C, the 5' portion of exons 2, and the 3' portion of exon 5 are non-coding regions (figure 2.3). The largest intron (between exons 1C and 2) is 5.3kb in length and contains a 143-amino acid open reading frame, an *Alu* sequence, and a pseudo-transcription-initiation region. The open reading frame does not appear to be an actual protein-coding region (Cox, 1995).

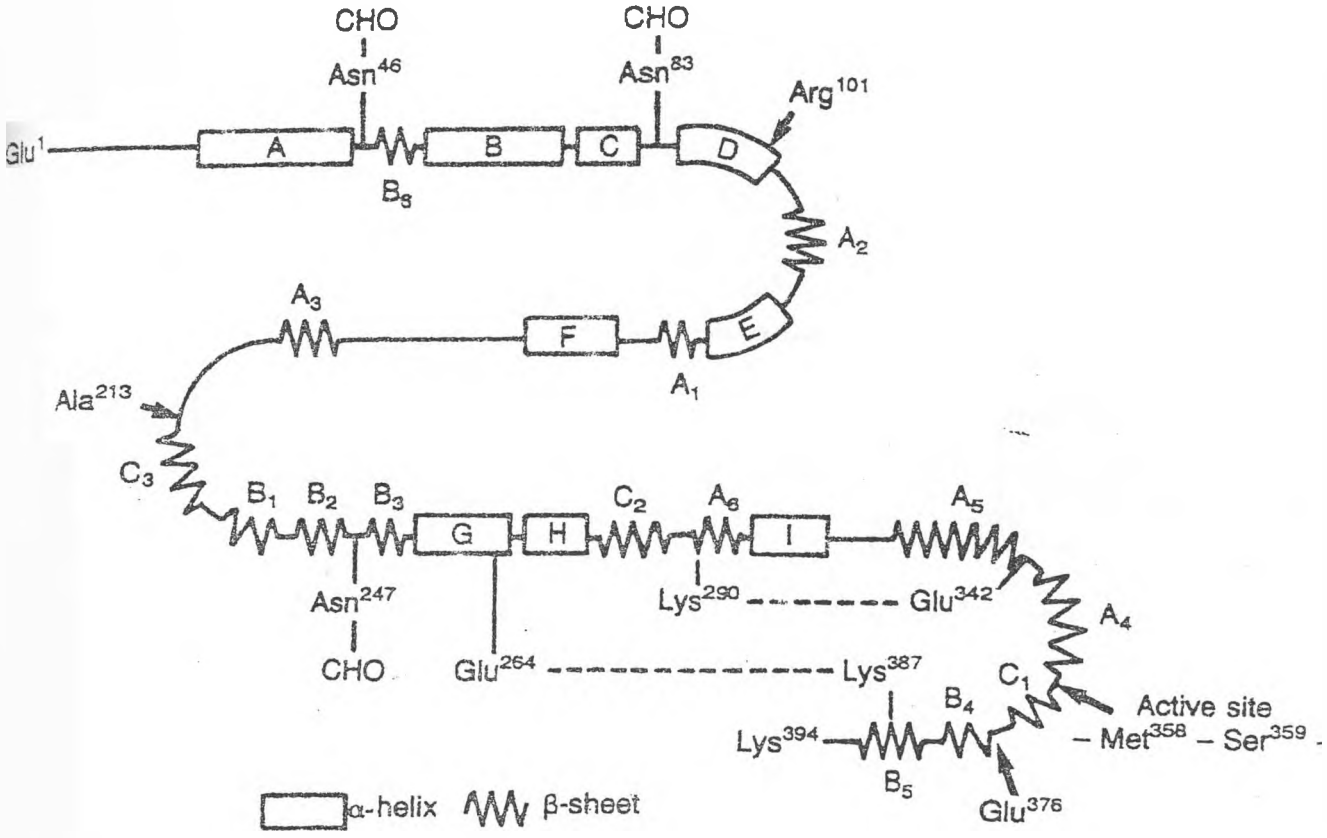

Figure 2.1: The normal α_1-AT protein shown in its linear form, a 394-amino acid single chain with three asparaginyl-linked complex carbohydrate side chains at residues 46, 83 and 247. During its synthesis, the molecule folds into a spheroid shape determined by nine α-helices (A→I) and three β-pleated sheets made up of parallel and antiparallel strands (sheet A, strands 1-6; sheet B, strands 1-6; sheet C, strands 1-3). Two internal salt links (Glu^{342}---Lys^{290}; Glu^{264}---Lys^{387}) help stabilise the molecule and play an important role in the pathogenesis of common deficiency states. The three carbohydrate side chains are on the surface of one half of the spheroid while the Met^{358}---Ser^{387} inhibitory site that combines with NE is localised at the opposite end (from Crystal, 1990).

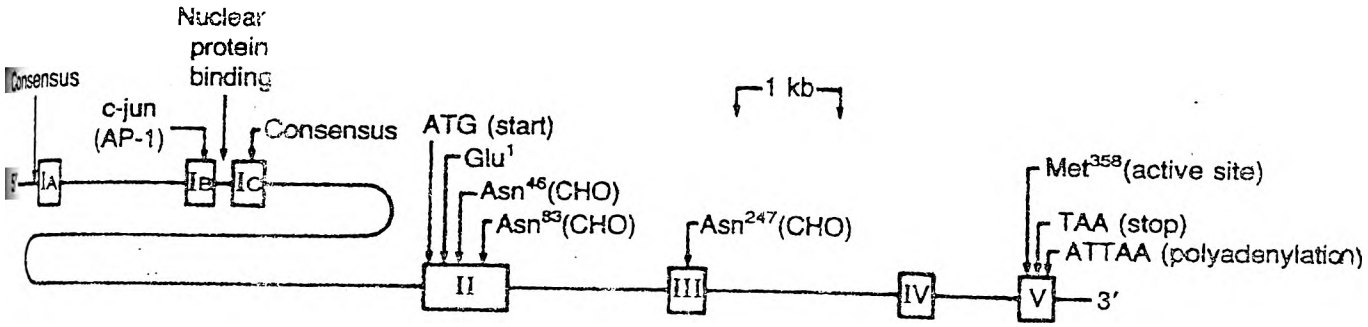

Figure 2.2: The normal α_1-AT gene spans 12.2 kb and consists of three noncoding exons (1A, 1B, 1C) and four coding exons (II-V). Exons I_A-I_C are used by a variable degree by α_1-AT synthesising cells, with most α_1-AT transcripts starting in the middle of exon I_C. 5′ to exon I_A and in the middle of I_C are typical cis-acting consensus promoter sequences. In the region between I_B are two sequences capable of binding the c-jun protein (AP-1). The start codon (ATG) is in exon II followed by sequences coding for a 24-residue signal peptide. Sequences for the 394-residue mature protein start in exon II (Glu^1) and end in exon V at the TAA stop signal. Also identified are the locations of sequences coding for the carbohydrate (CHO) attachment sites (Asn 46, 83, 247), the Met^{358} at the active inhibitor site, and the mRNA polyadenylation signal (from Crystal, 1990).

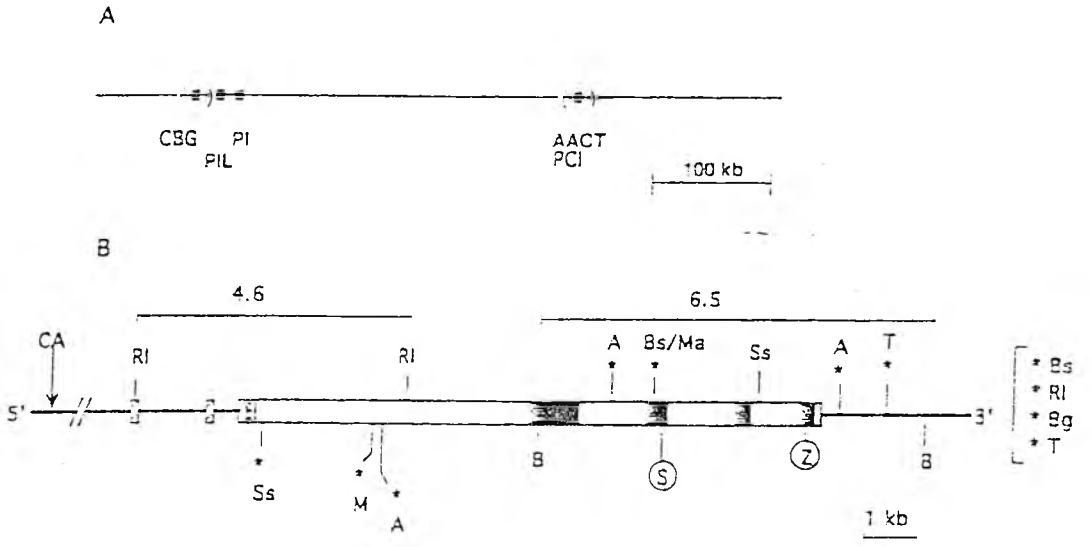

Figure 2.3: (A) PI and other loci in the serpin superfamily cluster on chromosome 14, as determined by pulsed field gel electrophoresis. PIL = PI-like, CBG =corticosteroid binding globulin, PCI = protein C inhibitor, AACT = α_1-antichymotrypsin. (B) α_1-AT gene and flanking regions. Coding regions are solid rectangles, introns are open rectangles, and untranslated regions are dotted rectangles. Crosshatched regions are exons of macrophage DNA. Asterisks indicate site of polymorphisms for the following restriction enzymes (those in the square brackets at the right indicate polymorphisms in the 3' homologous region) : A = *Avu* II; B = *Bam* HI; Bg = *Bgl* II; BS = *Bst* EII; M = *Msp* I; Ma = *Mae* III; T = *Taq* I; RI = *Eco* RI; Ss = *Sst* I; S and Z circled = site of mutations in PI*S and PI*Z, respectively. Genomic probes 4.6 and 6.5, are indicated. The arrow marks the position of the CA repeat (from Cox, 1995).

DNA and protein sequencing studies have indicated homology not only between some of the protease inhibitors, but also between inhibitors and other plasma proteins, as well as with chicken ovalbumin. Human α_1-AT and α_1-antichymotrypsin share 56% homology in their coding nucleotide sequences and 42% homology in their amino acids. Human α_1-AT and protein C inhibitor share 42% amino acid identity. An unexpectedly high degree of homology has been observed between α_1-AT and two non-inhibitor human plasma proteins: thyroxine-binding globulin, located on the X chromosome (58%) (Flink *et al*, 1986) and corticosteroid-binding globulin, located on chromosome 14 (53%) (Underhill&Hammond, 1989). There is 28% homology between the amino acid sequences of antithrombin III and α_1-AT (Kurachi *et al.*, 1981), and 27% homology between C1 inhibitor and α_1-AT (Tosi *et al.*, 1986); the position and number of introns is very different however, probably indicating a relatively ancient divergence from a common ancestral gene several hundred million years ago.

2.1.2 Physiology of α_1-AT

Alpha-1 antitrypsin inhibits a broad spectrum of serine proteases. Because of its efficiency of inhibition, broad substrate specificity and ready access to tissues, α_1-AT plays an important role in defending tissues from proteolysis. α_1-AT inhibits most serine proteases tested to date, including pancreatic and neutrophil elastase, neutrophil cathepsin G, pancreatic trypsin and chymotrypsin, collagenase from skin and synovia, acrosin, kallikrein, urokinase and renin (Cox, 1995). Since α_1-AT increases during the acute-phase response and is also a trypsin inhibitor, and since trypsin inhibitors are

known to have antibacterial activity, α_1-AT may play some role in resistance to infection. The association of α_1-AT deficiency with a spectrum of inflammatory diseases suggests that α_1-AT is important in the inflammatory response.

The two major sites of α_1-AT gene expression are hepatocytes and the mononuclear phagocyte family of cells (Permutter *et al.*, 1985). On average, hepatocytes contain approximately two-hundred fold more α_1-AT mRNA transcripts per cell than do mononuclear phagocytes, including blood monocytes and alveolar macrophages, suggesting that the liver is the major site of α_1-AT biosynthesis (Perlino *et al.*, 1987).

The path of α_1-AT biosynthesis is typical of a secretory glycoprotein (figure 2.4). The α_1-AT is translated on ribosomes bound to the endoplasmic reticulum (ER). As the α_1-AT polypeptide is synthesised, it proceeds through the membrane of the ER to the lumen, a process thought to be directed by the 24 amino acid N-terminal signal peptide on the newly synthesised α_1-AT molecule. Within the cisterna of the rough ER, the three carbohydrate side chains are added as the protein begins to fold into its three-dimensional configuration. Like other N-linked oligosaccharides, the carbohydrate side chains of α_1-AT are initially added in the form of a branched oligosaccharide, containing nine mannose residues, among other components. Once linked to the α_1-AT molecule, the so-called "high mannose" side chains are trimmed and the protein is translocated to the Golgi apparatus, where the side chain processing of the oligosaccharides is completed, to form the final "complex" type of side chains found on mature α_1-AT (Pfeffer&Rothman, 1987).

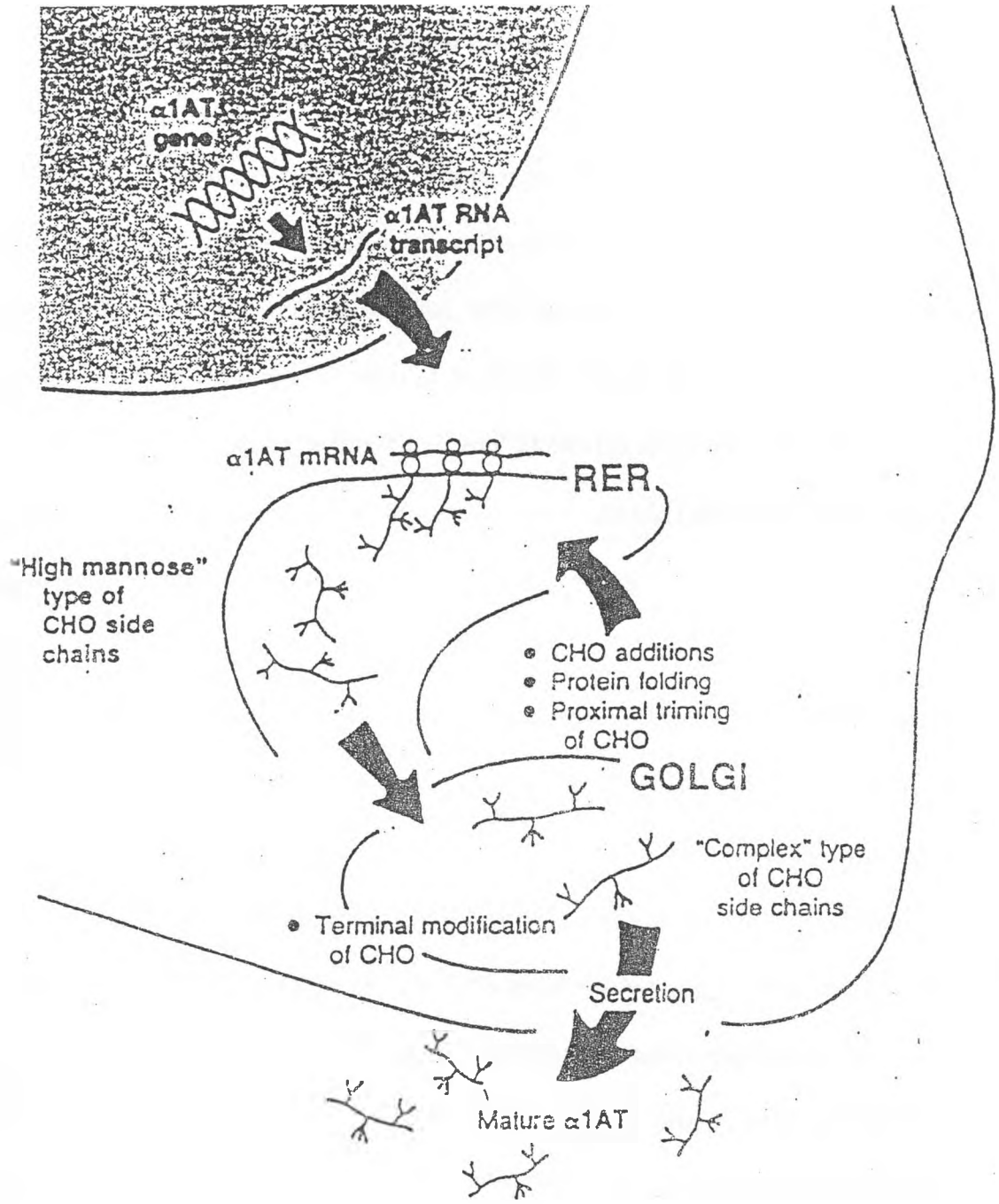

Figure 2.4: Processes involved in the normal biosynthesis of α_1-AT. In the nucleus, the mRNA precursor is transcribed from the gene. After splicing and translocation, the α_1-AT is translated on the rough ER. The newly synthesised polypeptide chain is secreted into the cisterna of the rough ER, the signal peptide is cleaved, and the carbohydrate side chains are added as the protein folds. The initial "high mannose" carbohydrate side chains undergo proximal trimming, and the protein is then translocated to the Golgi apparatus. Within the Golgi, terminal modification of the carbohydrate side chains yields a mature 550-kDa protein (from Brantly *et al.*, 1988)

2.1.3 Genetic basis for α_1-antitrypsin deficiency

The α_1-AT locus is pleomorphic, with approximately 75 alleles identified. The α_1-AT alleles are conveniently categorised as "normal" and "at risk", (table 2.1). By convention, a normal allele is one that, when inherited in a homozygous fashion, is associated with serum α_1-AT levels of 20-53 μM. The at-risk alleles relevant to a risk for lung and liver disease include "deficient" alleles (an allele that when inherited in a homozygous form is associated with serum levels < 20 μM) and "null" alleles (no α_1-AT in serum attributable to that allele), (Crystal, 1990).

The PI (protease inhibitor) variants, initially identified primarily by the method of acid starch gel electrophoresis, were named in order of their mobility: F (fast), M (medium), S (slow), and Z (the most cathodal). There are many inherited variants of α_1-AT (figure 2.5). PI*M, which can be further classified into subtypes, is the most common allele in all populations. The PI*S allele reaches polymorphic frequencies in many populations, as does the PI*Z allele, which produces a deficiency of α_1-AT. In addition to common variants of α_1-AT, more than 60 rare variants of α_1-AT have been identified. The amino acid sequence has been identified for a number of types of α_1-AT, either directly or by DNA sequence analysis. An alanine-valine substitution at amino acid position 213, noted by amino acid sequencing was further identified by DNA studies of both Z and M1. The alanine substitution is found in about 34% of PI*M1 alleles and differentiates two subtypes of M1: M1 (Ala^{213}) and M1 (Val^{213}). The PI*Z allele has the Ala^{213} substitution. However, PI types M2, M3, and S all have valine at position 213 (Nukiwa *et al.*, 1987).

Table 2.1: Sequence differences among the "normal" and the "at risk" alpha-1 antitrypsin alleles.

Category	Allele	Base allele	Mutation site (exon)	Sequence compared to base allele
Normal	M1(Ala^{213})			
	M1(Val^{213})	M1(Ala^{213})	III	Ala^{213} GCG → Val GTG
	M3	M1(Val^{213})	V	Glu^{376} GAA → Asp GAC
	M2	M3	II	Arg^{101} CGT → His CAT
	M4	M1(Val^{213})	II	Arg^{101} CGT → His CAT
	$B_{alhambra}$	Unknown	Unknown	Lys→ Asp
	F	M1(Val^{213})	III	Arg^{223} CGT → Cys TGT
	$S_{aint\ Albans}$	M1(Val^{213})	V	Asp^{341} GAC → Asn AAC
			III	Asp^{256} GAT → Asp GAC
	V_{munich}	M1(Val^{213})	II	Asp^{2} GAT → Ala GCT
	X	M1(Val^{213})	III	Glu^{204} → Lys
	$X_{christchurch}$	Unknown	V	Glu^{363} → Lys
At risk	Z	M1(Ala^{213})	V	Glu^{342} GAG → Lys AAG
	S	M1(Val^{213})	III	Glu^{264} GAA → Val GTA
	$M_{heerlen}$	M1(Ala^{213})	V	Pro^{269} CCC → Leu CTC
	M_{malton}	M2	II	Phe^{52} TTC → delete TTC
	$M_{mineral\ springs}$	M1(Ala^{213})	II	Gly^{67} GGG → Glu GAG
	$M_{procida}$	M1(Val^{213})	II	Leu^{41} CTG → Pro CCG
	$M_{nichinan}$	Unknown	II	Phe^{52} TTC → delete TTC
				Gly^{148} GGG → Arg AGG
	I	M1(Val^{213})	II	Arg^{39} CGC → Cys TGC
	P_{lowell}	M1(Val^{213})	III	Asp^{256} GAT → Val GTT
	$W_{bethesda}$	M1(Ala^{213})	V	Ala^{336} GCT → Thr ACT
	$Null_{granite\ falls}$	M1(Ala^{213})	II	Tyr^{160} TAC → delete C→5' shift→ stop TAG
	$Null_{Bellingham}$	M1(Val^{213})	III	Lys^{217} AAG → $stop^{217}$ TAG
	$Null_{matawa}$	M1(Val^{213})	V	Leu^{353} TTA → Insert T → Phe^{353} TTT → 3' shift → $stop^{376}$ TAG
	$Null_{isola\ di\ procida}$	Unknown	II-V	Delete 10kb including exons II-V
	$Null_{hong\ kong}$	M2	IV	Leu^{318} CTC → delete TC → 5' shift → $stop^{334}$ TTA
	$Null_{Bolton}$	Unknown	V	Pro^{362} CCC → delete C → 5' shift → $stop^{373}$ TAA
	$Null_{devon}$	Unknown	II	Gly^{115} → Ser
	$Null_{ludwigshafen}$	M2	II	Ile^{92} ATC → Asn AAC

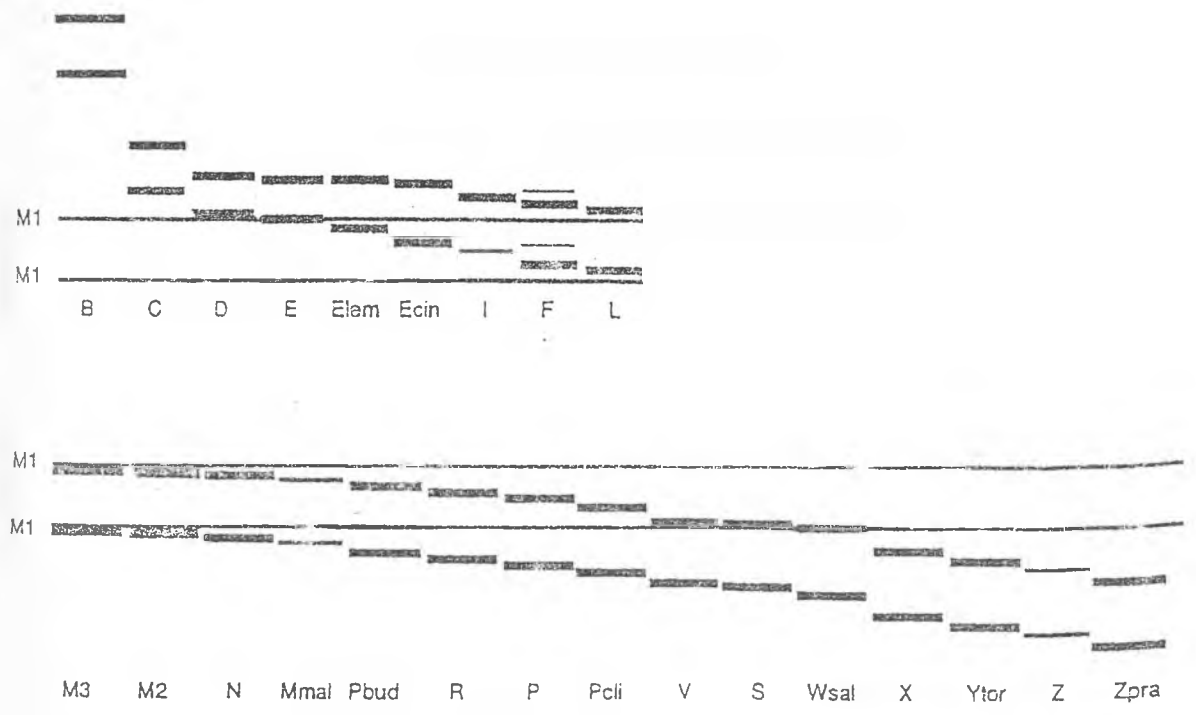

Figure 2.5: Diagram of selected anodal (top row) and cathodal (bottom row) PI variants as revealed by isoelectric focussing in polyacrylamide gels. The solid lines indicate the positions of the two major bands of M1. Anode at top. (From Cox, 1995)

The distribution of the PI alleles has now been determined for many populations. In all populations, PI*M is the most common allele. PI*M1 is distributed in all populations with the highest frequency in all populations studied, with PI*M2 the next most frequent, while PI*M3 is found to be relatively uncommon. The additional subtype allele PI*M4 has been described in several populations at a frequency of 0.002 to 0.050 (Cox, 1995). In many studies, no differentiation was made between PI types M3 and M4, since isoelectric focussing does not easily separate these alleles. The PI*S allele is rare or absent in black and oriental populations, but show the highest frequency in Spain and Portugal, a lower frequency in France, and low generally in other parts of Europe. The frequency of PI*Z is highest in Scandinavian countries, and the allele is present throughout white populations including those of the Middle East, and is absent from oriental and black populations, except in those populations known to have a white admixture, as in the United States (Cox, 1995).

The most common of the deficiency alleles is PI*Z, and most individuals with α_1-AT deficiency are of PI type ZZ. The estimated frequency of the PI*Z allele in North American white populations is 0.0122, corresponding to a frequency of PI ZZ homozygotes of 1 in 6 700 (Cox, 1995).

The Z variant differs from the M_1 (Ala^{213}) by the substitution of lysine for glutamic acid at position 342. This mutation reduces the stability of the molecule in its monomeric form and predisposes to a novel protein-protein interaction between the reactive centre loop of one molecule and the A β sheet of a second (Lomas *et al.*, 1992). This "loop-sheet" polymerisation interferes with the secretion of the molecules

from the hepatocytes and macrophages and results in decreased plasma levels of 10%-15% of normal.

The S variant differs from the M_1 (Val^{213}) by the substitution of valine for glutamic acid at the 264 position (Brantly *et al.*, 1988). This mutation also leads to the production of polymers (Elliot *et al.*, 1996). This effect reduces plasma α_1-AT concentrations to 60% of normal.

The S and Z variants have been associated with emphysema (Brantly *et al.*, 1988), liver disease (Brantly *et al.*, 1988), panniculitis (Pinto *et al.*, 1993) and asthma (Colp *et al.*, 1993). Elliot *et al.* (1998) provided the first demonstration of Z α_1-AT polymers in the lungs of patients with emphysema. The inactivated α_1-AT is unable to play any role in the anti-proteinase screen, and this serves to exacerbate lung disease associated with plasma deficiency of the Z mutation of α_1-AT (Elliot *et al.*, 1998).

However, several studies suggest that individuals with certain α_1-AT phenotypes may be predisposed to developing asthma. Gaillard *et al.* (1992) reported an increased prevalence of the non-deficient M2M2 α_1-AT phenotype in white asthmatics in South Africa, while an increased prevalence of the Z allele of α_1-AT was found to be associated with severe asthma in Swedish children (Cox, 1994; Walls, 1995). Interestingly, a heterozygote advantage has been suggested to account for the high incidence of the S and Z α_1-AT alleles in the European population. These alleles have been implicated in protection against pulmonary tuberculosis (Carrell, 1984).

2.1.4 Loop-sheet polymers and α_1-antitrypsin deficiency

The Z mutation of α_1-AT is located at residue P_{17} (17 residues proximal to the P_1 reactive centre) at the head of strand 5 of the A β-sheet and the base of the mobile reactive centre loop. It had been predicted that the mutation would act to open the A sheet, thereby favouring spontaneous loop into β-sheet polymerisation (Lomas, 1996). Functional studies showed that Z α_1-AT was more unstable and was able to polymerise at 37 °C under physiological conditions (Lomas *et al.*, 1992). The rate is accelerated by raising the temperature to 41 °C, and can be blocked by exogenous reactive loop peptides that compete with the loop for A-sheet annealing. The role of this phenomenon *in vivo* was clarified by the demonstration that polymers with identical microscopic appearance could be isolated from the liver of a Z α_1-AT homozygote (Lomas, 1996).

Although many α_1-AT deficiency variants have been described, only two other mutants of α_1-AT have similarly been associated with plasma deficiency and hepatic inclusions: α_1-antitrypsin Siiyama (Ser53Phe) and α_1-antitrypsin Mmalton (52Phe deleted). α_1-Antitrypsin Siiyama was first described in 1993 in Japan, and although rare it is the commonest form of α_1-AT deficiency in that country (Seyama *et al.*, 1995). The abnormal α_1-AT isolated from the plasma of a Siiyama homozygote was all in the form of long chains of inactive loop-sheet polymers (figure 2.6a).

The other mutation resulting in severe deficiency, α_1-AT Mmalton, has been reported sporadically in the UK and Canada (Frazier *et al.*, 1989; Curiel *et al.*, 1989). Once again, the isolation of α_1-AT from the plasma of an Mmalton null heterozygote yielded both polymeric and monomeric antitrypsin. The polymers were shorter than those of Siiyama α_1-AT, but showed the same characteristic resistance to unfolding in 8M urea (Lomas *et al.*, 1995).

The temperature and concentration dependence of polymerisation may account, along with genetic factors, for the heterogeneity in clinical features. As α_1-AT is an acute-phase protein, its concentration will rise during episodes of inflammation. At these times, the formation of polymers is likely to overwhelm the degradative pathway, thereby exacerbating hepatic inclusions and the associated hepatocellular damage (Lomas, 1996).

Crystals of recombinant protein were grown and the structure of α_1-AT solved to 2.9 Å. This structure shows the mobile loop trapped in an extended conformation that fits comfortably into the binding pocket of the proteinases trypsin and chymotrypsin (Elliot *et al.*, 1996). This conformation of the loop is in the form of a β-pleated strand, which also provides an explanation for the ready formation of loop-sheet polymers, as this strand configuration fits easily into the A β-pleated sheet of α_1-AT (figure 2.6b) to form a dimer that can extend as trimers, tetramers and so on to as many as 20 molecules in length (figure 2.6c). These filaments then tangle within the endoplasmic reticulum of the hepatocyte to form the insoluble inclusions that are characteristic of Z α_1-AT deficiency (Lomas, 1996).

The reactive loop's ready adoption of a β-strand conformation also accounts for the intimate association of another serpin, α_1-antichymotrypsin, with another β-pleated structure that of amyloid plaques of Alzheimer's disease (Ma *et al.*, 1994). The phenomenon of loop-sheet polymerisation is not restricted to α_1-AT and has now been reported to account for the deficiency of other mutants of the serpins associated with angioedema (C1-inhibitor), (Aulak *et al.*, 1993; Eldering *et al.*, 1995), emphysema (antichymotrypsin), (Faber *et al.*, 1993) and thrombosis (antithrombin), (Bruce *et al.*, 1994).

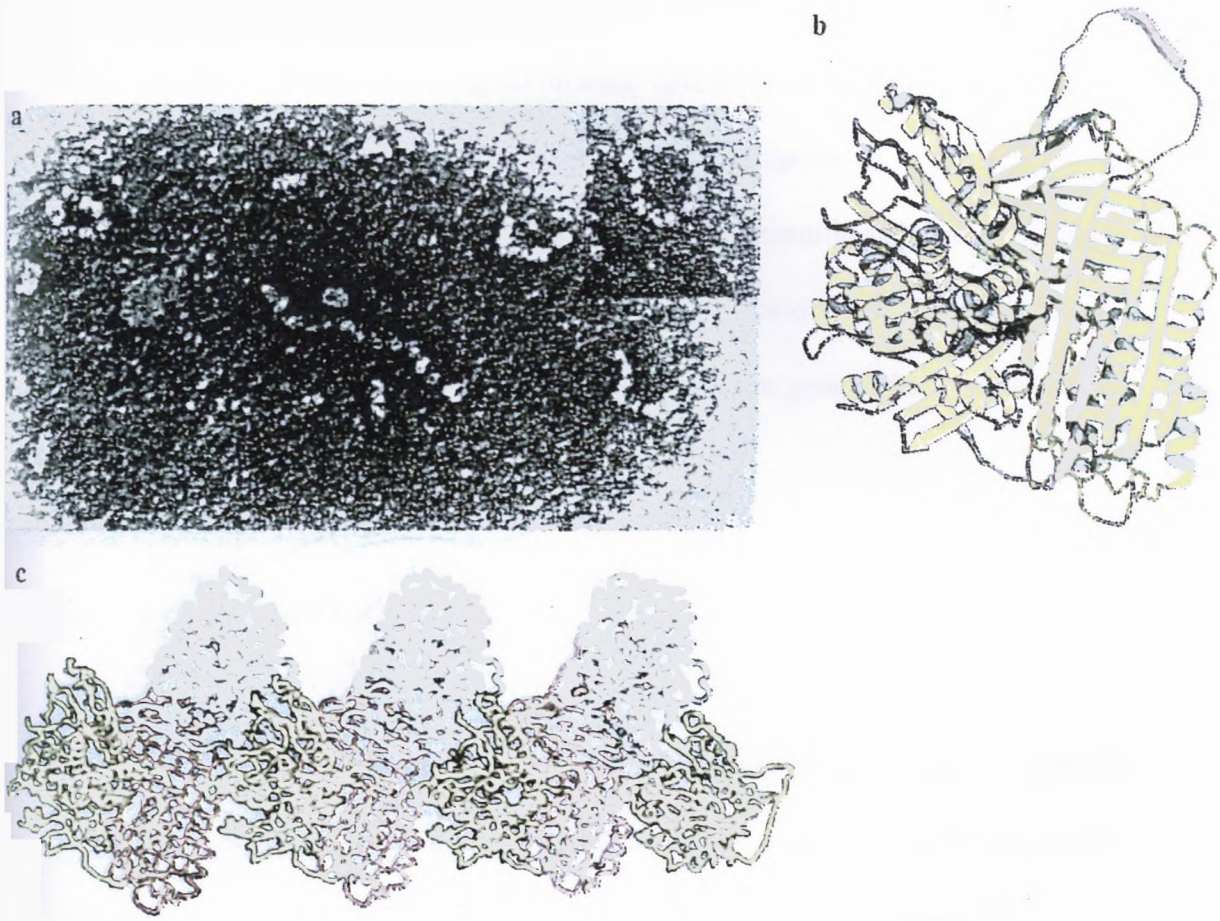

Figure 2.6: (a) Loop-sheet polymers of α_1-AT isolated from the plasma of a Siiyama α_1-AT homozygote. This point mutation favours the formation of long chains of polymers as well as circlets (inset). These polymers tangle in the liver to form insoluble inclusions that predispose to liver damage; **(b)** Crystal structure of α_1-AT provides strong support for the loop-sheet linkage being between the β-pleated reactive centre loop of one molecule (red) and the A β-pleated sheet (yellow) of the next. These dimers extend to form long chains shown in **(c)** as alternating yellow, blue and red α_1-AT molecules (from Lomas, 1996).

2.1.5 Amplification of DNA: The Polymerase Chain Reaction

The development of the polymerase chain reaction (PCR) technique for nucleic acid amplification has had a major impact on many diverse areas of both basic and clinical research. Since its inception (Saiki *et al.*, 1989), reports on a wide variety of applications for PCR have received much attention in scientific and medical literature. This technology has been shown to have a vast application to the diagnosis of human disease including such diverse areas as infectious disease, genetic disorders and cancer (Arends & Bird, 1992; Gunther *et al.*, 1993).

Basic Principle of PCR

PCR is a chemical method of exponentially increasing the concentration of a specific nucleic acid sequence relative to that of other nucleic acid sequences in the reaction mixture (Bloch, 1991). The principle of the method is shown in figure 2.7.

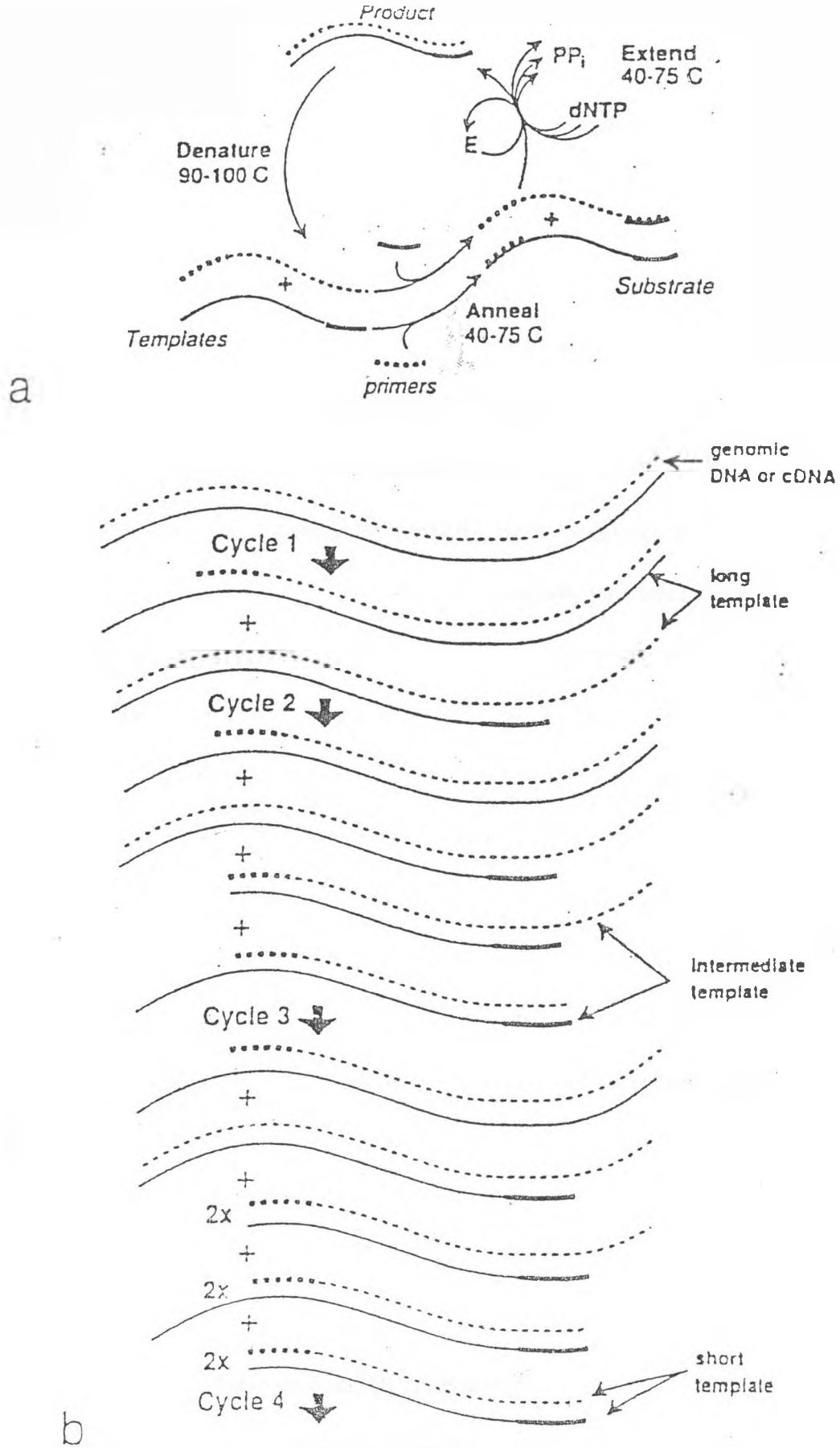

Figure 2.7: Schematic diagram of (a) a single PCR cycle and (b) the generation of the short template over the first few amplification cycles (from Bloch, 1991).

2.2 Materials and Methods

2.2.1 Materials

The following materials were obtained from Promega (Madison, WI): Agarose, acrylamide, dNTPs, Taq DNA polymerase, Hae III restriction endonuclease and proteinase K; the oligonucleotide primers were synthesised by Genosys Biotechnologies Inc. (Woodlands, TX); the bis-acrylamide, ammonium persulfate and TEMED were from BioRad (Hercules, CA); the Ampholines were supplied by Pharmacia (Uppsala, Sweden); the double-distilled water was from SABAX (Johannesburg); PCR tubes were supplied by Laboratory and Scientific Equipment Co. (Johannesburg); the Coomassie Brilliant Blue and the mineral oil were from Sigma Chemical Co. (St. Louis, MO); the Whatman filter paper was from Whatman International (England); the DNA molecular weight markers were supplied by BioVentures Inc (Murfreesboro, TN); the 2ml eppendorf tubes were supplied by Treff Laboratories (Switzerland); all other reagents used were of analytical grade Merck (Darmstadt, Germany).

2.2.2 Subjects

Thirty-three white asthmatic patients, 30 black asthmatic patients, 54 white controls and 36 black controls were used. The asthmatic patients were recruited from the Asthma and Respiratory Clinics of Hillbrow and Johannesburg Hospitals and from Krugersdorp Hospital (Paediatrics). The controls were either blood donors who

presented themselves at the South African Blood Transfusion Services in Johannesburg or outpatients at the Johannesburg Hospital Paediatric Nephrology Clinic. The patient with the novel polymorphism and the fifty controls used for the polymorphism study was from the Respiratory Medicine Unit, Cambridge Institute for Medical Research, Cambridge, United Kingdom. None of the controls had a history of atopy or any disease process associated with the respiratory system. All individuals who participated in the study were 4-45 years of age, and each subject gave informed consent (or in the case of minors, consent was obtained from parents) to participate in the study. The Committee for Research on Human Subjects of the University of the Witwatersrand approved this protocol.

2.2.3 Asthma and Atopy

Each patient had a history of bronchial asthma as defined by the American Thoracic Society, namely a history of transitory or prolonged episodes of dyspnea, coughing and wheezing, but was otherwise healthy (American Thoracic Society Statement, 1962). The diagnosis was confirmed in each patient by the finding of at least one of the following: a forced expiratory volume in one second (FEV) < 70% of the predicted value with reversibility of 20% or more following inhalation of a β_2-adrenergic bronchodilator, or a fall in peak expiratory flow rate of at least 20% during a standardised exercise test. Atopy was defined as a positive skin prick test at least 2mm greater than a negative control and a positive specific IgE titre (>0.35kU/L) or a high concentration of total serum IgE greater than published normal values for children, or greater than 400kU/L in adults. Asthma was classified as mild, moderate

or severe using the classification of Scheffer (1991).

2.2.4 DNA extraction

The polymerase chain reaction (PCR) requires a source of nucleic acid (DNA or RNA) for amplification. The technique is highly tolerant of impurities in the DNA sample that is being amplified, and the degree of nucleic acid purification necessary before PCR-amplification depends on the complexity and chemistry of the sample matrix, as well as on the target sequence. It is usually necessary to deproteinase a biological sample to ensure the removal of extraneous proteases, nucleases and phosphatases that might destroy reactants that are crucial for the amplification reaction (Bloch, 1991).

Conventional DNA extraction methods usually employ detergents to solubilise cell components and proteolytic enzymatic degradation of DNA-bound proteins (Maniatis *et al.*, 1982). This step is followed by extraction with organic solvents such as phenol and precipitation of the nucleic acid with ethanol (Maniatis *et al.*, 1982). Although this standard technique is PCR-compatible, a variety of faster, simpler methods have emerged. The method developed by Higuchi (1989), and which was used in the present study, allows for the efficient release of DNA from nucleated blood cells. It obviates the use of organic extraction and ethanol precipitation, and instead allows for the direct addition of cells or nuclei to a PCR-compatible solution that contains nonionic detergents (which lyse cell membranes by removing integral proteins) and proteinase K (which can be inactivated by heat).

This method efficiently releases DNA from ± $3x10^5$ nucleated cells, and the DNA can then be amplified by PCR with little or no inhibition (Higuchi, 1989). Porphyrins, which are derived from heme present in blood, may be the most potent cell components inhibitory to PCR and in order to separate porphyrins from nuclear DNA, this extraction method lyses cell membranes and pellets nuclei and cell debris. A number of pelleting and washing steps remove haemoglobin that is released from blood cells, as well as RNA and cytoplasmic DNA. The resulting pellet, containing nuclear DNA, is finally resuspended in proteinase K/detergent solution. The final yield is approximately 20µg of nuclear DNA/1ml of blood (Appendix I).

The integrity of the extracted DNA was determined by electrophoresis in 0.7% (w/v) agarose gels in 1xTBE buffer (0.089M Tris-borate, 0.002M EDTA, pH 8.2) at 100V for 20-30 min (Appendix II).

2.2.5 α_1-AT phenotype study

All blood specimens were collected into EDTA-containing tubes. Plasma from each sample was separated on the day of collection and stored at -60°C until analysed. α_1-AT phenotypes were identified by isoelectric focussing in polyacrylamide gels. Polyacrylamide gel electrophoresis is one of the most useful methods for the analysis of protein mixtures. Isoelectric focussing (IEF) is used for the fractionation of molecules that only differ in net charge (Andrews, 1987). The technique is performed in non-sieving media (such as polyacrylamide gels) and is capable of very high resolution, especially when shallow, immobilised pH gradients are used (Andrews,

1987). In addition, macromolecules that differ in their isoelectric point by as little as 0.001 pH units can be separated efficiently (Robard *et al.*, 1974).

The technique is based on a moving boundary, and amphoteric substances (e.g. amino acids and peptides) are separated in an electric field which has both pH and voltage gradients (Davis, 1986). The anode region has a lower pH than the cathode area and a suitable pH is maintained between the electrodes - the pH range is chosen such that the molecules being separated have their isoelectric points within that range (Davis, 1986). Substances are positively charged at pH regions below their isoelectric point and they migrate toward the cathode, but as they migrate, the pH increases and the substances become zwitterions with no net charge so that migration stops (Davis, 1986). Similarly, substances which at first occur at pHs above their isoelectric point is reached, at which stage migration ceases (Davis, 1986). As a result, amphoteric substances are focussed into narrow stationary bands (Davis, 1986). IEF is a principal technique for the analysis and characterisation of proteins, peptides, glycoproteins, lipoproteins, phosphoproteins, cell membrane proteins, isoenzyme patterns and studies with nucleic acids and glycosaminoglycans (Andrews, 1987). IEF is thus used in clinical, forensic and genetics laboratories for the separation and identification of serum proteins (Davis, 1986).

Detection of proteins after electrophoresis is most commonly done by staining with the organic dye Coomassie Brilliant Blue, the principle being that electromorphs take up a general protein stain (Constans *et al.*, 1980).

This detection method is common due to the fact that it produces little background staining, because the colloidal form of the dye does not enter the gel (Nivinskas & Cole, 1996). The α_1-AT phenotypes were identified using the following modification of the IEF method by Constans *et al.* (1980). A 0.5mm thick gel was prepared by dissolving 0.97g acrylamide, 0.03g bis-acrylamide and 2.67g sucrose in 19.3 mL of water. One millilitre of Ampholines, pH 4.2-4.9 and 30 µL of TEMED were added and the solution was degassed for 3 min. Thereafter 1 mL of a freshly prepared 10% ammonium persulfate stock solution was added and the gel was poured into a 124x257x0.5mm LKB Multiphor gel mould. The gel was allowed to set for 30 min at room temperature, and thereafter refrigerated (4°C) for 15 min. Electrode strips (17mm Whatman or LKB paper wick 1850-911) were soaked with 0.1 M sodium hydroxide (0.4 g/100 mL distilled water) for the cathode (the negative electrode) and 0.04 M phosphoric acid (0.5 mL/100 mL distilled water) for the anode (the positive electrode). The gel was pre-focussed for 30 min at 800 V, 10 mA, 10 W. The samples were applied on paper inserts (17 mm Whatman) to the cathode end of the gel and were focussed for 45 min at 500 V, 8 mA, 8 W. The inserts were then removed and the gel focussed for a further 1 hr 45 min at 1200 V, 14 mA, 14 W. Thereafter, the bands were sharpened for 45 min at 2500 V, 14 mA, 8 W. After migration, the gel was stained in a 0.1% solution of Coomassie Brilliant Blue (45% methanol, 10% glacial acetic acid) for 1 hr at 37°C and rinsed in distilled water. Following destaining (45% methanol, 10% glacial acetic acid), the phenotypes of selected α_1-AT variants observed in human sera of asthmatic patients and control subjects were read (figure 2.8).

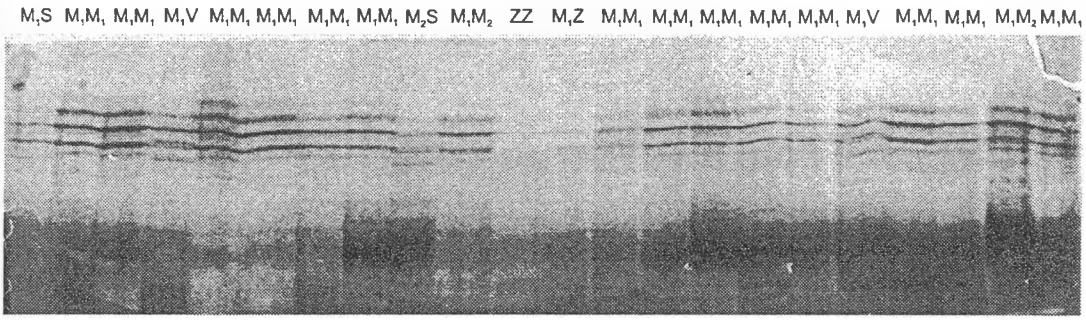

Figure 2.8: Selected α_1AT variant observed in human sera using isoelectric focussing in ultra- thin polyacrylamide gels. The anode is at the top.

2.2.6 Restriction fragment length polymorphism

Total genomic DNA was extracted from EDTA-containing whole blood according to the Higuchi method (1989), modified as previously described. The polymerase chain reaction (PCR) was used to amplify exon II of the α_1-AT gene in ten asthmatic patients and fifty control individuals. The following primers were used:

P1: 5′ -AGT TCG CCT TCA GCC TAT AC- 3′

P2: 5′ -GAA GCT AGT GGA TAA GTT- 3′

The amplified sequence is 296 bp long. Amplification (by PCR) was performed in a reaction volume of 63 µL, containing 1 µg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatine) and 1 µM each appropriate amplification primer (Appendix II).

The reaction mixture containing all the components, except the Taq DNA polymerase, was overlaid with 50 µL mineral oil and heated to 94°C for 10 min in a Hybaid DNA thermal cycler (Hybaid, UK). Following a cooling step, which reduced the sample temperature to 50°C, 1 unit of Taq DNA polymerase was added. The samples were then processed through 40 cycles of: denaturation at 94°C for 20 secs, annealing at 50°C for 20 secs and extension at 74°C for 30 secs. The final step was one of extension at 74°C for 10 min.

Restriction analysis of the amplified fragments of exon II of the α_1-AT gene was performed as follows:

20 µL of the PCR reaction mixture was digested with 2 units of Hae III (1U/µl) and incubated overnight at 37°C. Resolution of the restricted products was performed by electrophoresis in 4% agarose gels run at 70V for 2 hours. Following electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL) in 1xTBE buffer (0.089 M Tris-HCL, 0.002 M EDTA, pH 8.2), viewed under UV illumination (302nm) and photographed with a Polaroid CU-5 Land camera.

Direct sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the sequencing products were separated and analysed in an ABI 310 DNA sequencer (Appendix IV).

2.2.7 PCR of α_1-AT exons II-V

Amplification (by PCR) was performed in a reaction volume of 63 µL, containing 1 µg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatin) and 1 µM each appropriate amplification primer (Appendix II). Reaction conditions included 30 cycles of a denaturation step at 94°C for 1 minute, annealing at 60°C for 1 minute (exon II) or 52 °C for 1 minute (exon III, IV, V) and extension at 72°C for 2 minutes. PCR products were electrophoresed on 2% agarose gels at 80V for 60 minutes. Products were visualised by UV transillumination.

2.2.8 Direct DNA cycle sequencing of PCR products

PCR products were pre-treated with 10U/µl Exonuclease I and 2U/µl Shrimp Alkaline Phosphatase (Amersham Life Science, Cleveland, Ohio, USA) and incubated at 37°C for 15 minutes. Pre-treatment enzymes were inactivated by incubation at 80°C for 15 minutes. PCR products were sequenced using a Thermosequenase Radiolabelled Terminator Cycle Sequencing Kit (USB, Cleveland, Ohio, USA) and α-P^{33} ddNTP's (USB, Cleveland, Ohio, USA). Cycle sequencing was performed on a Hybaid DNA thermal cycler (Hybaid, UK) with 30 cycles of 95°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes. Reactions were stopped with 4µl stop solution. Sequencing products were denatured at 96°C for 10 minutes and electrophoresed at 60W for 2-4 hours with 1X glycerol tolerant buffer (GTB) on denaturing 8% w/v polyacrylamide-urea gels. Gels were fixed, dried and autoradiographed (Appendix III).

2.2.9 Elastase inhibitory assay

The serum elastase inhibitory capacity (EIC) was determined according to the method described by Gaillard *et al* (1987) using porcine pancreatic elastase and succinyl-p-nitroanilide as a chromogenic substrate. The absorbance was measured at 405 nm.

2.3 Results

During the course of this study, routine sequencing revealed an interesting and unique polymorphism (figure 2.9) in an asthmatic individual from the United Kingdom. This sequence has a polymorphic Hae III recognition site. The fragment will either be cut into two (142 bp + 115 bp) or three (142 bp + 133 bp + 115 bp) fragments following digestion with the enzyme Hae III (figure 2.10). The novel polymorphism identified in exon II (471) **C**TG→**T**TG was found in the heterozygous state (figure 2.10) with the common normal M3 base allele in a Caucasian female with normal α_1-AT plasma level (1.1 mg/ml; normal range 0.9-1.8 mg/ml) and asthma from the United Kingdom. This polymorphism was not detected a total of fifty controls matched for age and sex from the United Kingdom.

A five year old Caucasian boy presented with an inability to exercise due to severe wheezing and breathlessness. He also complained of early morning wakening and episodes of nocturnal wheezing. His family doctor had started budesonide 100 μg twice daily without significant benefit and the patient had required several courses of oral prednisolone. On examination the chest was hyper-inflated. The chest radiograph was normal, but pulmonary function testing revealed a baseline FEV1 of 1.76 L that fell to 1.44 L (18% reduction) after exercise. A diagnosis of asthma was made and he responded well to treatment with inhaled fluticasone 250 μg and salmeterol 25 μg both twice daily. The child's mother but not his father was also noted to have had asthma but no other clinical details were available.

The child's serum IgE was significantly elevated at 804 KU/L (normal range 0-71). He had a Rast class 2 to house dust (0.93 KU/L; rast class 0<0.35) and dog dander (0.85 KU/L), a class 5 rast to both Timothy grass (>100 KU/L) and mixed South African trees (Olive, Willow, Pine, Eucalyptus and Acacia); 77.7 KU/L, a rast class 4 to mixed South African weeds (includes Marguerite, dandelion, plantain, goosefoot and golden rod); 21.1 KU/L, and a rast class 6 to Bermuda grass; >100 KU/L.

Baseline α_1-AT plasma levels were 1.31 g/l and 1.44 g/l for the child and mother respectively (normal range 0.9-1.8 g/l). Isoelectric focussing revealed an unusual faster band, which was seen to migrate towards the anode for both the child and mother. The new variant was characterised by direct genomic sequencing and was found to have a point mutation **C→A** corresponding to amino acid position 15 of the N-terminus resulting in the substitution of a Histidine for an Asparagine on an M1 (Val^{213}) base allele. The elastase inhibitory capacities for the child and mother with the $M1E_{(JOHANNESBURG)}$ phenotype (figure 2.11) were 43.97 KU/g and 50.38 KU/g respectively. Five other non-related asthmatic patients showed an identical IEF migration pattern to the $M1E_{(JOHANNESBURG)}$ phenotype discussed (figure 2.12). Direct sequencing confirmed the mutation being present in these patients but not controls and again the mutation did not affect baseline plasma levels or the elastase inhibitory capacities in these asthmatic patients.

Isoelectric focussing identified another new variant, the $M1N_{(JOHANNESBURG)}$ (figure 2.13) in four non-related Caucasian asthmatic individuals, one of whom suffered with severe allergies. The new variant although not associated with any deficiency was

characterised by direct genomic sequencing and was found to have two mutations that were in linkage with each other. The first is a CA**G**→CA**C** mutation corresponding to amino acid position 97 in exon II resulting in the substitution of a Glutamine for a Histidine on an M1 (Val ^{213}) base allele (figure 2.14). In addition to this mutation, sequencing of exon II in these patients also revealed a base change as a result of an insertion of a **G** at position 95 (figure 2.15).

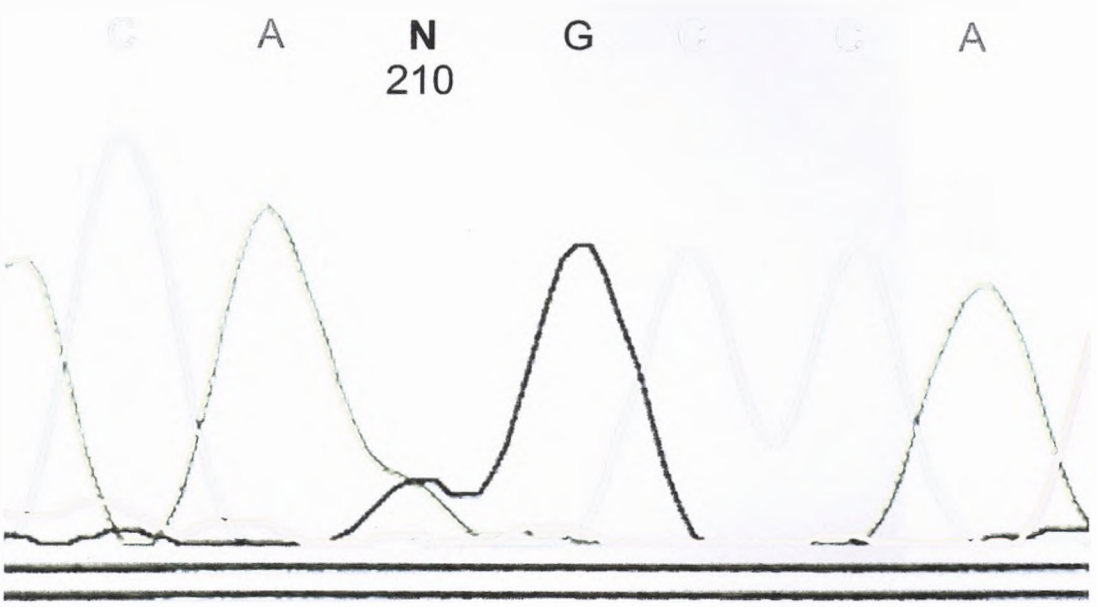

Figure 2.9: Sequence of polymorphism identified in exon II
(471) CTG ⟶ TTG

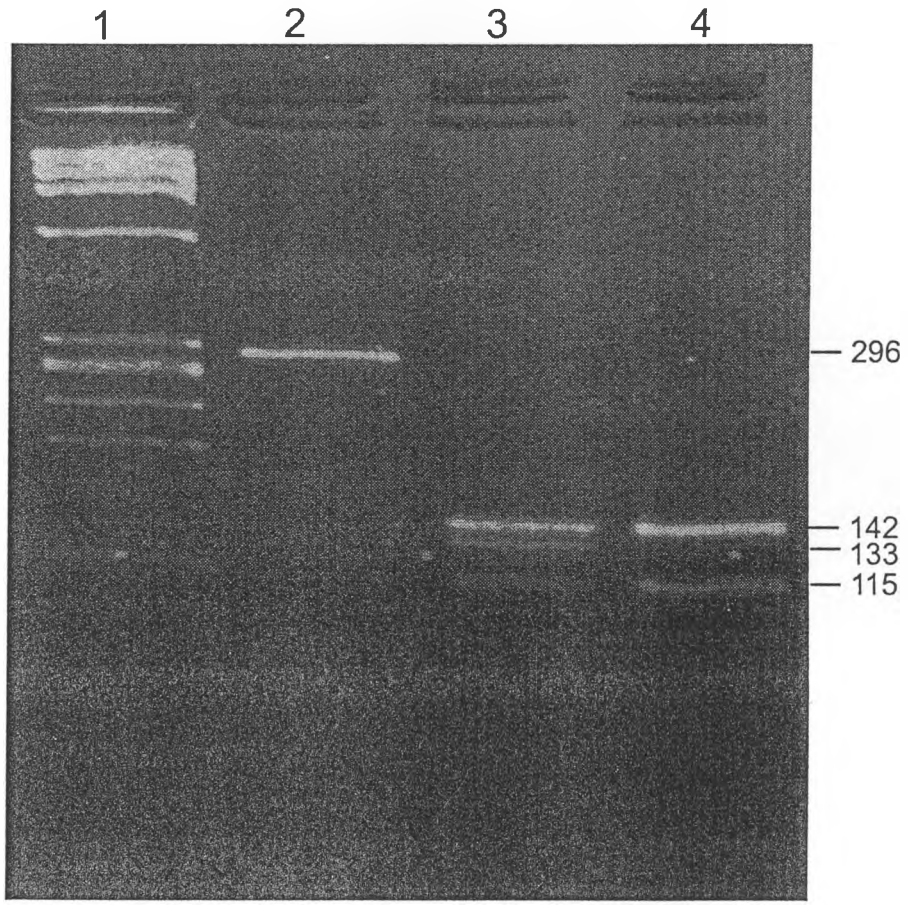

Figure 2.10: Hae III restriction of exon II of the α_1AT gene showing a homozygous individual (lane4), a heterozygous individual (lane3), lane 2, an unrestricted PCR product, lane 1, molecular weight marker.

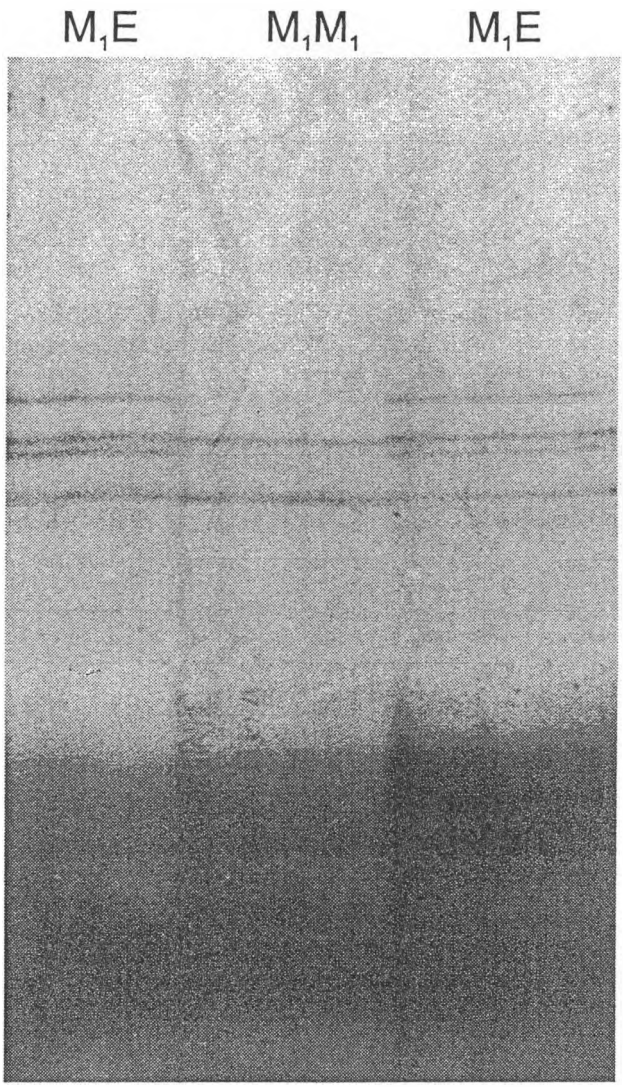

Figure 2.11: The $M_1E_{(Johannesburg)}$ α_1AT variant observed using isoelectric focussing in ultra thin polyacrylamide gels. The anode is at the top.

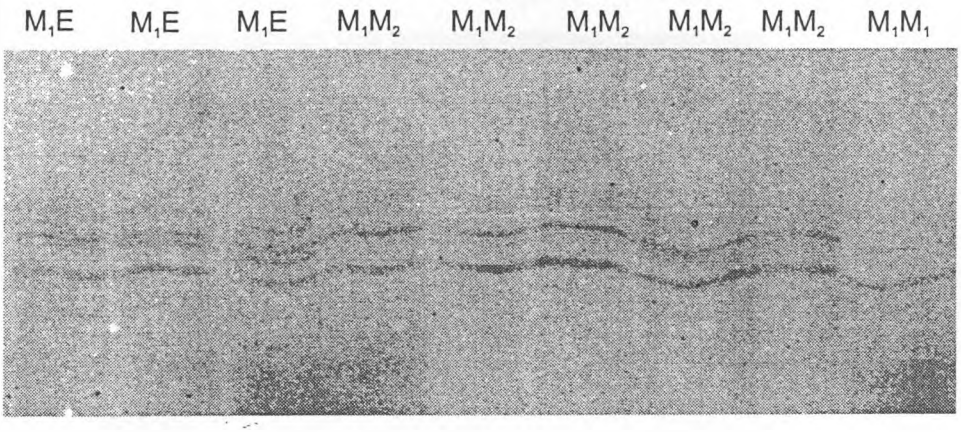

Figure 2.12: Selected α_1-AT variants observed in human sera using isoelectric focussing in ultrathin polyacrylamide gels. The anode is at the top.

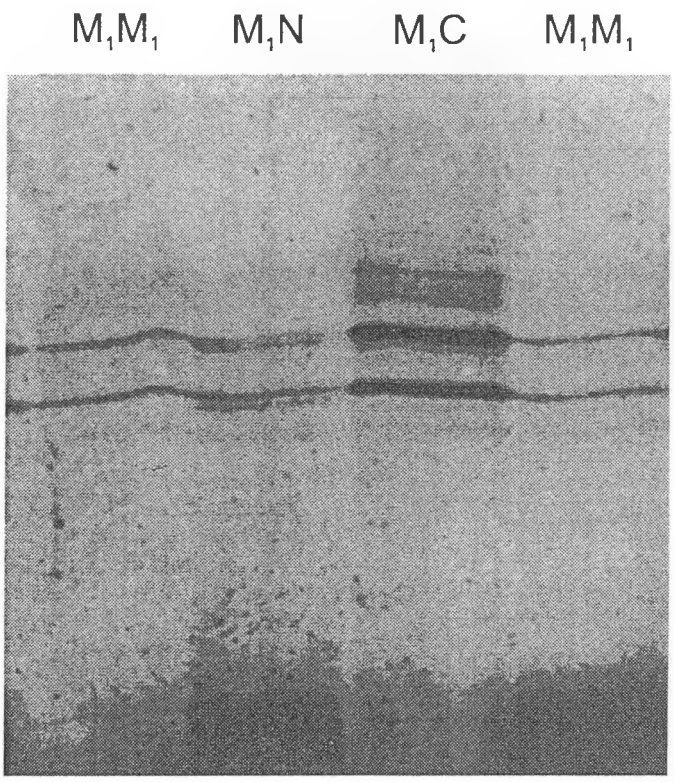

Figure 2.13: The $M_1N_{(Johannesburg)}$ α_1AT variant observed using isoelectric focussing in ultra thin polyacrylamide gels. The anode is at the top.

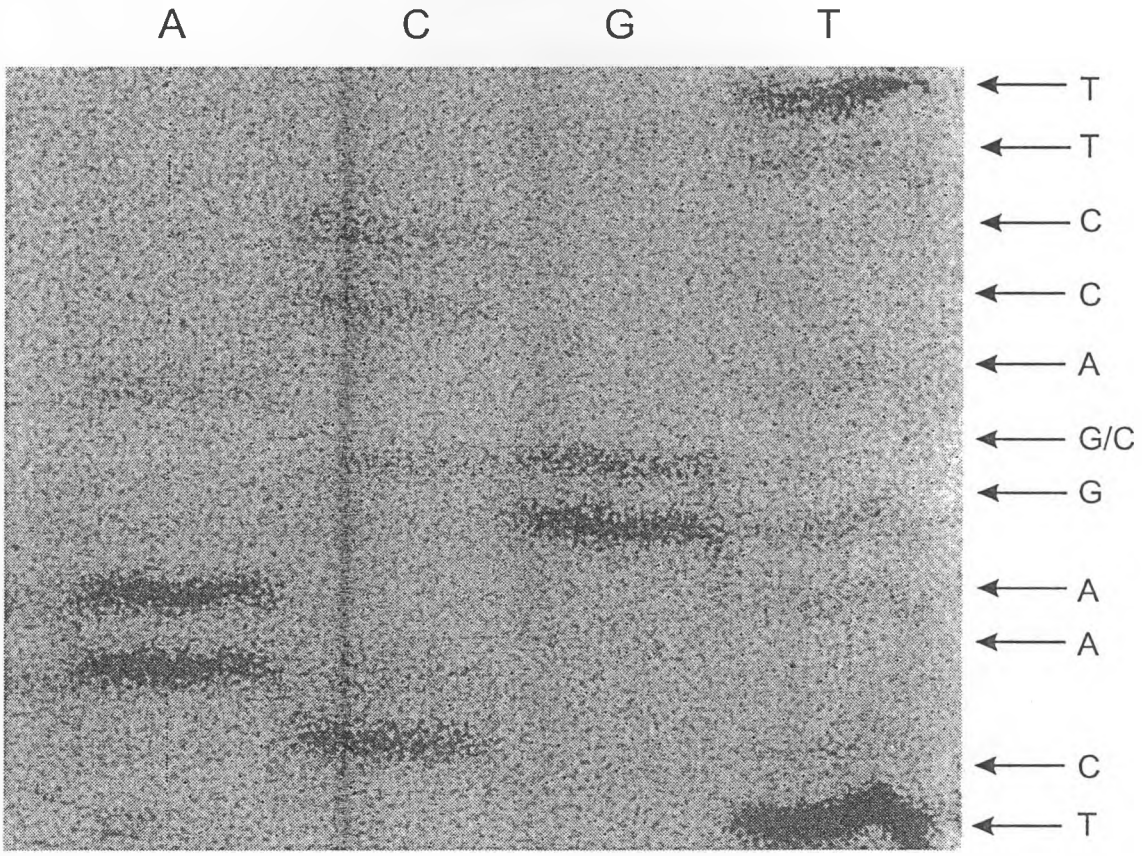

Figure 2.14: DNA Sequence showing the CAG→CAC mutation of the $M_1N_{(Johannesburg)}$ variant of α_1 AT.

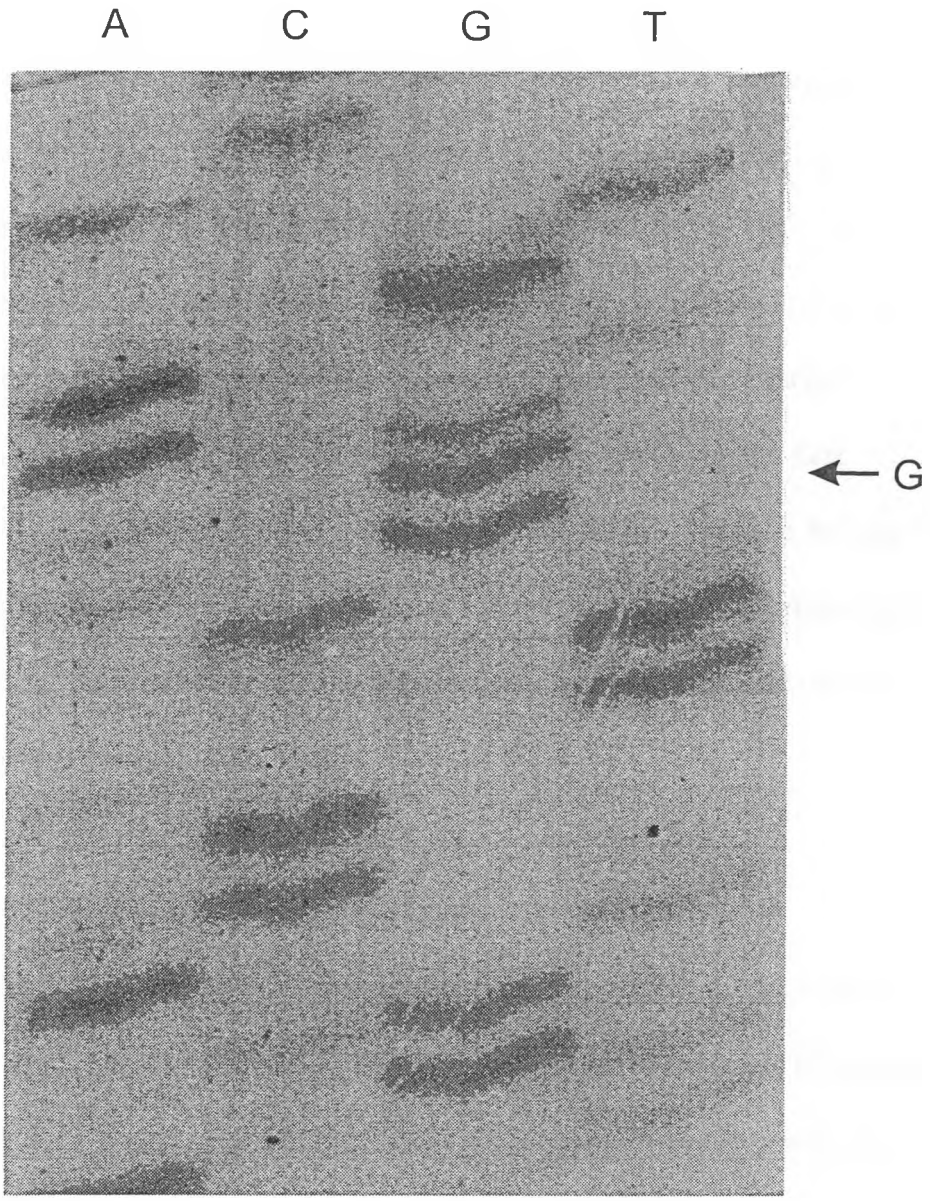

Figure 2.15: Sequence showing the insertion of the extra **G** linked to the CA**G** → CA**C** mutation of the $M_1N_{(Johannesburg)}$ variant of α_1 AT

2.4 Discussion

The differences between α_1-AT phenotypes involve simple amino acid substitutions. The two most common M1 phenotypes differ by having either valine or alanine at position 213. The normal M2 subtype differs from M1(Val^{213}) by two substitutions: histidine for arginine at the 101 position and aspartic acid for glutamic acid at the 376 position (Crystal *et al.*, 1989). Two important deficiency variants, Z and S, are associated with deficient plasma α_1-AT levels, which, in the case of the former, predisposes to emphysema. The Z molecule differs from the M1(Ala^{213}) by the substitution of lysine for glutamic acid at the 342 position, whereas the S molecule differs from the M1(Val^{213}) by the substitution of valine for glutamic acid at the 264 position (Brantly *et al.*, 1988).

The Z allele is associated with markedly reduced plasma levels of α_1-AT, directly as a result of the $Glu^{342} \rightarrow Lys^{342}$ substitution, which appears to interfere with the salt bridge to Lys^{290} causing a change in the configuration of the molecule (Loebermann *et al.*, 1987). It is also clear that the Z molecule is qualitatively deficient, being unable to maintain a complex with elastase (Ogushi *et al.*, 1987). The precise mechanism of this abnormality is not understood, but it may also relate to the altered three dimensional configuration of the molecule. The S allele is also associated with low plasma α_1-AT levels, apparently due to molecular instability with intracellular destruction (Curiel *et al.*, 1988). It is functionally normal and does not appear to confer a risk of emphysema even in its homozygous (SS) form (Ogushi *et al.*, 1988).

The mechanism by which the S mutation results in plasma deficiency is unclear and has been attributed to increased clearance, aberrant protein folding and intracellular degradation (Elliott *et al.*, 1997). It has been suggested that the deficiency of S antitrypsin result from a slow transition to form dimers and short chain polymers that result in a mild reduction in secretion from the hepatocyte and an increased rate of turnover in the plasma. The crystal structure of S antitrypsin shows that the S mutation breaks the Glu264→Tyr38 salt bridge which would predictably perturb the Phe52→Ser53 region at the commencement of the β helix and hence favour polymerisation as occurs more dramatically with the Siiyama and Mmalton mutants (Elliot *et al.*, 1997). Among populations of asthmatic patients, those heterozygous for the deficient α_1-AT variants may have more severe disease as manifested by worse spirometric results, poor bronchodilator response and steroid dependency (Gaillard *et al*, 1992).

Several deficiency alleles have been reported in which the plasma concentration of α_1-AT is detectable against standards and is generally in the range of about 2 to 15 percent of normal. All have an electrophoretic mobility different from that of the PI Z variant (Cox, 1995). Just like the variants that we describe. PI $Z_{AUGSBURG}$ and PI $Z_{WREXHAM}$ have the Z mutation as well as another benign amino acid difference. PI M_{MALTON} is associated with PAS-positive hepatocyte inclusions identical with those found in association with PI Z, and, like Z, the protein has a tendency to aggregate (Cox, 1995). PI M_{DUARTE}, first reported in a 48-year-old woman with severe bullous emphysema, migrates in acid starch gel and agarose similarly to PI M, and the isoelectric point is similar to that of M3, as determined by isoelectric focussing. PAS-

D globules were present in the liver, indicating that this deficient variant also has, like Z, a defect in secretion from the liver (Cox, 1995). Individuals with rare deficiency alleles have the same risk for obstructive lung diseases as PI ZZ individuals. Furthermore, these PI types will produce α_1-AT inclusions in the liver, particularly in heterozygotes, in the presence of an apparently normal M phenotype. When isoelectric focussing is used, the M_{MALTON} α_1-AT can be identified even in the presence of a normal M allele. M_{DUARTE} can be detected in a heterozygote with an S or Z allele but, depending on the degree of resolution with isoelectric focussing, may not be detectable in the presence of a normal M allele (Cox, 1995). Although the PI*F allele is not associated with a decrease of α_1-AT concentration, there is a suggestion that PI FM heterozygotes may be more susceptible to pulmonary function impairment, particularly when exposed to industrial pollutants. α_1-AT of the F type, which has an extra cysteine residue, appears to have an increased tendency to oxidation, as shown by its altered electrophoretic pattern after ageing, and this increased tendency to oxidation may make PI FM individuals susceptible to lung destruction in a polluted environment (Cox, 1995).

The mutation that underlies the $E_{(JOHANNESBURG)}$ and $N_{(JOHANNESBURG)}$ does not affect baseline plasma α_1AT levels or plasma elastase inhibitory capacity. Furthermore, the $E_{(JOHANNESBURG)}$ His→Asn mutation is in a poorly conserved region of the protein and is unlikely to affect the structure or function of α_1AT. Nevertheless, like the M1Ala^{213} and M2 alleles, α_1AT $E_{(JOHANNESBURG)}$ and $N_{(JOHANNESBURG)}$ may be in linkage disequilibrium with other key factors involved in the IgE-mediated inflammatory response.

Association between non-M1 variants of α_1AT and raised IgE levels were found in 68 white patients and 59 black patients with gastro duodenal ulcer disease (Redelinghuys, 2000). There have been a number of studies suggesting that atopy, asthma and bronchial hyper responsiveness are associated with heterozygosity for cystic fibrosis (CF) of the pancreas (Gerrard, 1985), also 36 white patients with CF was significantly associated with variants of α_1AT (Jamal, 2002). More recently 36 white HIV positive patients and 52 black HIV positive patients were associated with genetic variation of α_1AT. This also reinforces the association of the α_1AT variants and raised IgE levels as it is known that these patients are atopic due to the Th1/Th2 shift (Nicholson, 2004).

These observations together with the novel polymorphism described adds further support to the inflammatory and genetic basis of asthma. Knowledge of the molecular basis of variants and polymorphisms, both normal and pathogenic, can be useful for the identification of the residues most important for protein function.

The description of new α_1AT variants, particularly in diverse heterogeneous populations, such as in the case of South Africa, provides evidence for the environmentally driven evolution of the α_1AT gene.

CHAPTER 3

STABILISATION OF THE SERPIN-PROTEINASE COMPLEX THROUGH CONSERVED INTERACTIONS

3.1 Introduction

Alpha-1 antitrypsin (α_1-AT) is the most abundant circulating proteinase inhibitor and the archetypal member of the serine proteinase inhibitor or serpin superfamily. The serpins bind to and inhibit target proteinases to control proteolytic cascades and to protect the tissues from extraneous enzymatic attack (Carrell *et al.*, 1982). In particular α_1-antitrypsin inhibits the enzyme neutrophil elastase that is released at sites of inflammation (Beatty *et al.*, 1980).

The complex between α_1-antitrypsin and neutrophil elastase is extremely stable such that it is considered to be almost irreversible (Lomas *et al.*, 1993). This irreversibility of inhibition achieved by the serpins has made them principal inhibitors controlling both intra- and extracellular proteolytic pathways. In human plasma, antithrombin controls coagulation, C1-inhibitor controls complement activation and the inhibitors of plasmin and its activators control fibrinolysis (Huntington *et al.*, 2000). There have been many unsuccessful attempts over the past 20 years to crystallise the serpin-protease complex however, the recent crystal structure of α_1-antitrypsin bound to trypsin has allowed us to clarify the mechanism of stability between serpin and proteinase (Huntington *et al.*, 2000).

These crystallisation studies shows that α_1-antitrypsin in activated trypsin by swinging it from the upper to the lower pole of the molecule accompanied by a marked disruption of the catalytic site of the proteinase. The two molecules were linked by an ester bond between Met358 of α_1-antitrypsin and Ser195 of trypsin. Close examination of the interface between the proteinase and the inhibitor revealed two additional interactions. There was a salt bridge between Lys328 of α_1-antitrypsin and Asp194 of trypsin and hydrogen bonds between Asn314 of the inhibitor and the carbonyl oxygen's of Asp194, Trp215 and Ser214 of the proteinase (figure 3.1).

The contribution of these interactions to the stability of the complex is unknown. Moreover it is unclear whether these interactions are specific to the complex between α_1-antitrypsin and trypsin or a general mechanism that stabilises other serpin-proteinase complexes. Support for a general mechanism comes from the observation that all serine proteinases have an aspartic acid at position 194 and all the other interactions are with main chain atoms.

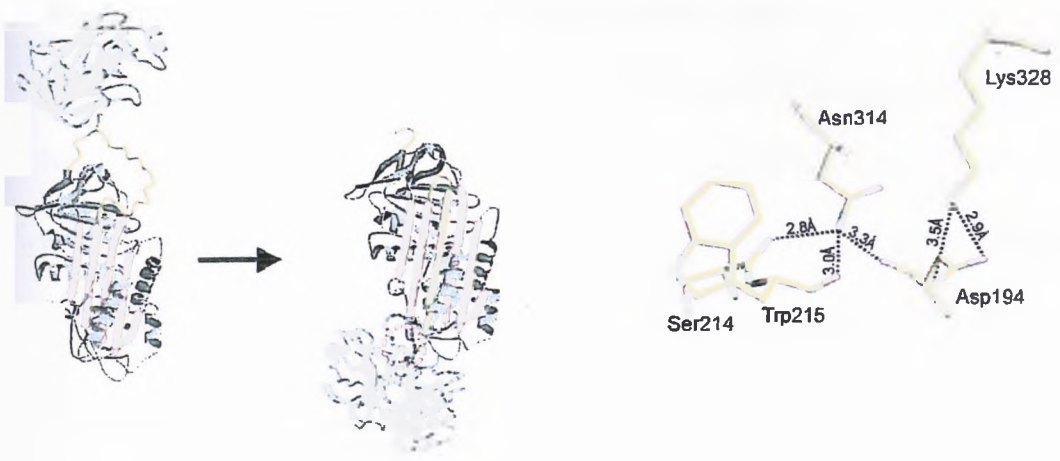

Figure 3.1: The conformational change of α_1-antitrypsin following complex formation with a target proteinase. The proteinase docks with the inhibitor to form an initial Michealis complex (left). The P_1-P_1' bond of the reactive loop is then cleaved and the proteinase is translocated to the lower pole of the molecule (right). The final complex is stabilised by an ester bond between the P_1 residue of α_1-antitrypsin (Met358) and the proteinase active site serine (Ser195). The crystal structure also showed a salt bridge between Lys328 of α_1-antitrypsin and Asp194 of trypsin (inset) and hydrogen bonds between Asn314 of the inhibitor and the carbonyl oxygen's of Asp194, Trp215 and Ser214 of the proteinase. Atomic coordinates have been deposited in the Protein Data Bank under accession number 1EZX (from Huntington *et al.*, 2000).

3.2 Materials and methods

3.2.1 Materials

The following were prepared by the media kitchen in the Laboratory of Molecular Biology (MRC Centre, Cambridge, UK) according to the method of Sambrook (Sambrook *et al.*, 1989): 2xTY (16g bacto-tryptone, 10g bacto-yeast extract and 5g NaCl per litre, pH 7.0 sterilised by autoclaving); ampicillin agar plates (15g agar, 8g NaCl, 10g bacto-tryptone, 5g yeast extract per litre, autoclaved then ampicillin added to a final concentration of 100μg/ml before pouring). The T4 ligase and all the restriction enzymes were from New England Biolabs (UK) and the oligonucleotides were synthesised by Life Technologies Corp. The pQE31 expression vector, *E.coli* strain SG13009 (PREP4) and Ni-NTA gel were from Qiagen Corp, the Hi-trap Q-Sepharose column was from Pharmacia (Uppsala, Sweden) and the chromogenic substrate S-2222 was from Chromgenix. Human neutrophil elastase, porcine trypsin and ampicillin were purchased from the Sigma Chemical Co (Poole, UK). Wizard DNA Maxipreps were from Promega (Southampton, UK); electroporation cuvettes (2mm gap) were from Equibio (Kent, UK). Dialysis membranes were from Medicell International Ltd (London, UK). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from Amersham (Buckinghamshire, UK). Both 1.5ml and 2ml micro centrifuge tubes were from Starsted (Germany) and 15ml and 50ml screw-cap tubes were from Falcon (New Jersey, USA). Vent polymerase was from New England Biolabs; *Taq* polymerase was from Promega (Southampton, UK).

3.2.2 Mutagenesis, Expression and Purification of Recombinant α_1-antitrypsin

Human α_1-antitrypsin cDNA was excised from pWombat (a gift from Dr A. Zhou) using the BamHI and Hind III restriction sites (figure 3.2) and subcloned into pQE31. The Asn314Ala and Lys328Ala mutants were then prepared by site-directed mutagenesis using a two-step PCR. A third reaction incorporated both of the above changes to give the double mutant Asn34Ala/Lys328Ala (Appendix V).

The following primers were used for the site-directed mutagenesis:

314Asn→Ala coding: 5′-AAGGTCTTCAGC**GCT**GGGGCTGACCTC-3′

non-coding: 5′- GCCCTAAGCTTATTTTTGGGTGGGATTCACCAC-3′

328Lys→Ala coding: 5′- GAGGCACCCCTG**GCG**CTCTCCAAGGCC-3′

non-coding: 5′- GCCCTAAGCTTATTTTTGGGTGGGATTCACCAC-3′

All mutations were confirmed by automated DNA sequence analysis with an ABI 310 genetic analyser (Appendix IV). The rest of the α_1-antitrypsin gene was also sequenced to ensure that there were no PCR errors.

Recombinant α_1-antitrypsin was expressed with an N-terminal MRSHHHHHH-tag which can bind Ni-NTA agarose. Plasmids containing the wildtype and mutant α_1-antitrypsin were transformed into SG13009 (PREP4) *E.coli* and the cells grown in 2 litres of 2xTY media in a shaking incubator at 37°C until the OD_{600} was between 0.6-

0.9. IPTG was then added to a final concentration of 1mM and the temperature lowered to 30°C for a further 3 hr. The cells were collected by centrifugation, resuspended in buffer A (10mM phosphate buffer, pH 8.0; 0.5M NaCl; 1mM β-mecaptoethanol), disrupted by sonication and the supernatant then loaded onto a 20mL Ni-NTA column. After washing to baseline with buffer A, the bound protein was eluted with 0-0.2M imidazole in buffer A. The fractions were collected, dialysed overnight against buffer B (10mM Tris-Cl, pH8.0; 1mM β-mecaptoethanol) and loaded onto a Hi-trap Q-sepharose column (5mL). The protein was then eluted with a NaCl gradient (0-0.5M) in buffer B. Recombinant α_1-antitrypsin eluted as the major peak at approximately 0.2M NaCl (figure 3.3). The fractions were pooled and the protein concentration determined spectrophotometrically using the extinction coefficient $A280nm^{1\%}5.3$ (Pannell *et al.*, 1974). The samples were aliquoted, snap-frozen in liquid nitrogen and stored at -70°C.

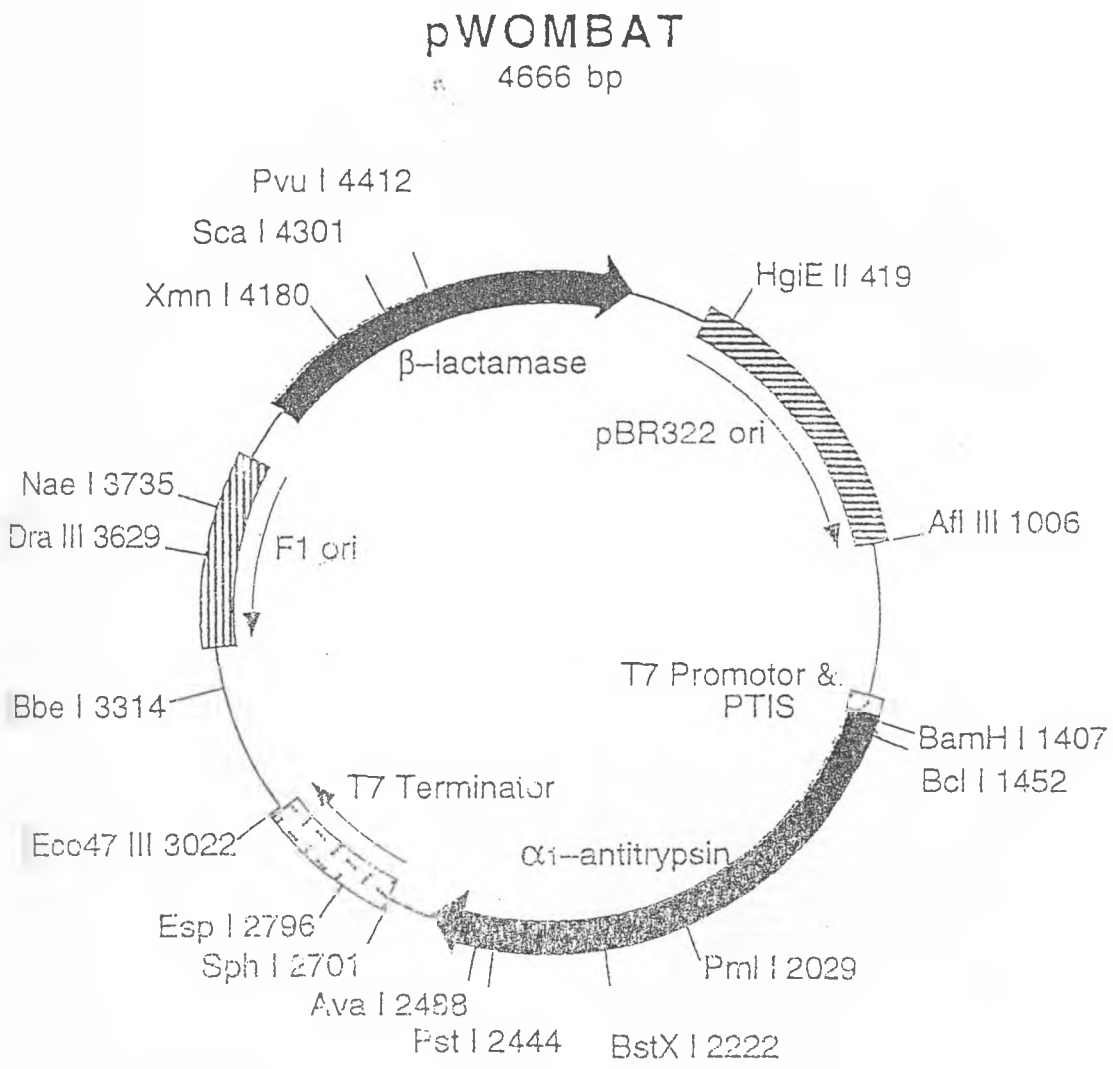

Figure 3.2: pWombat vector

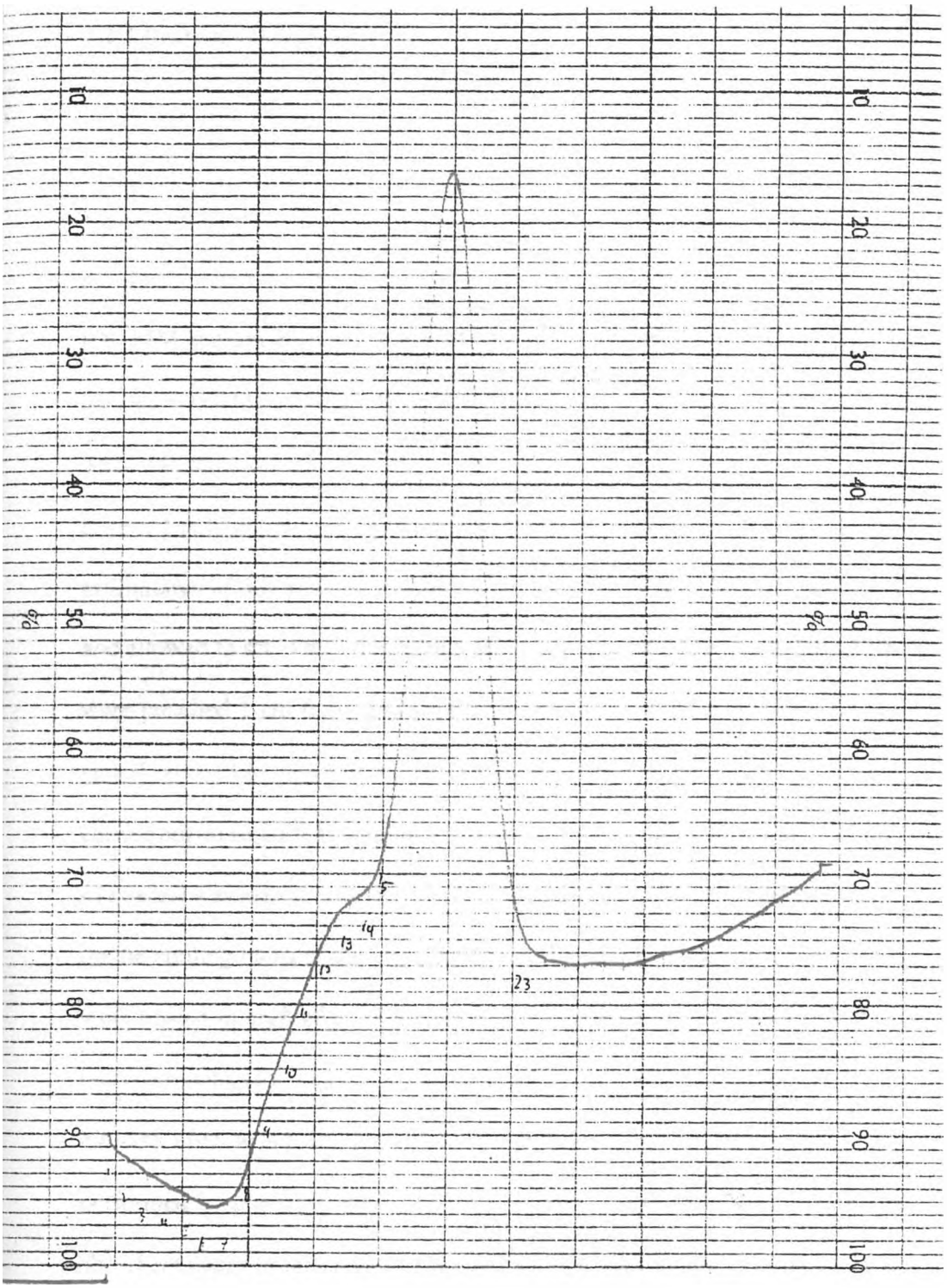

Figure 3.3: Typical elution profile of recombinant wildtype and mutant α_1-antitrypsin protein. Samples under the peak were positive for α_1-antitrypsin on rocket immuno-electrophoresis.

3.2.3 Protein characterisation

3.2.3.1 Rocket immuno-electrophoresis

Single dimension rocket immuno-electrophoresis was performed as described previously (Weeke, 1973). A solution consisting of 1% (w/v) ultra-pure agarose in 0.0315M sodium barbital, 0.0057M diethyl barbituric acid, 0.187M Tris, 0.375M glycine, 0.005M sodium azide, pH8.6 was heated in a microwave until totally melted. This was then cooled until the temperature reached approximately 56°C and the required polyclonal antibody added to a final concentration of 0.4% (w/v). Nine millimetres of this solution was rapidly poured onto a level 8 x 9 cm gel-bond plate and allowed to set. Once thoroughly set, a line of 2 mm diameter holes 0.5 cm apart were punched 1 cm from, and parallel to, the edge of the gel. The gels were then stored covered in cellulose film at 4°C until required. Rocket electrophoresis was carried out on an LKB Multiphor Electrophoresis Unit. Five microlitres of the sample of interest was loaded into each well of the rocket gel. The rocket gel was then placed on the cooling block of the electrophoresis chamber and lint wicks soaked in buffer were placed at each edge of the gel to act as charge carrying bridges between the reservoir tanks and the gel. Rocket gels were run at a current of 70 mA for 60 to 80 minutes after which the plate was squash blotted to facilitate drying. This was performed by placing 1 sheet of blotting paper over the gel and overlaying it with absorbent paper towels, a glass plate and a heavy lead weight. Pressure was applied for 15-20 minutes after which the gel was dried under a hair dryer and the 'rockets' visualised by staining with Coomassie brilliant blue and then destained (figure 3.4).

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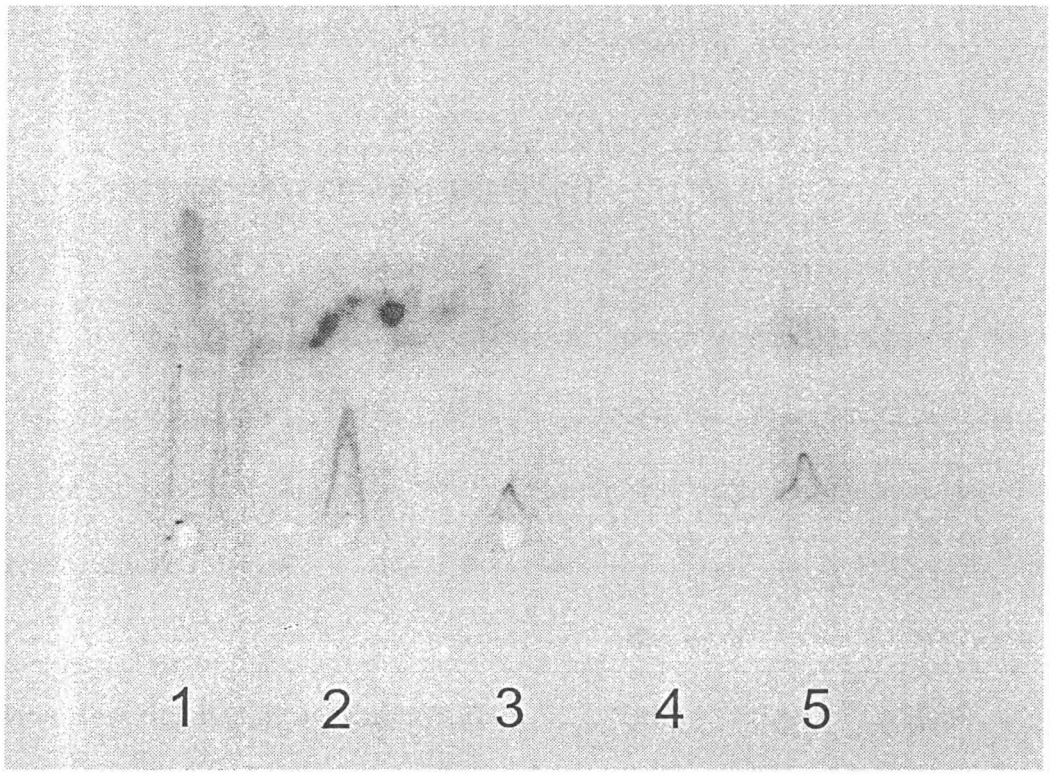

Figure 3.4: Rocket gel run at 70V for 2 hours, Lane 1, positive control (1.58mg/ml) Lane2, recombinant wildtype α_1 - antitrypsin. Lane 3, recombinant Asn 314 Ala/Lys328 Ala α_1 - antitrypsin. Lane 4, negative control. Lane5, recombinant Asn 314 Ala α_1 - antitrypsin.

3.2.3.2 Polyacrylamide gel electrophoresis (PAGE)

The protein samples were confirmed as being homogeneous, monomeric α_1-antitrypsin by SDS and non-denaturing PAGE (Appendix VI).

3.2.3.3 Transverse urea gradient gels (TUG)

Transverse urea gradient gels were carried out using a similar procedure to that of Goldenberg (Goldenberg, 1989). Gels were essentially the same composition as non-denaturing gels consisting of 7% (w/v) polyacrylamide with a 0 to 8M urea transverse linear gradient.

	0M urea	**8M urea**
Non-denaturing PAGE separation buffer	3mL	3mL
Acrylamide	2.8mL	2.8mL
Urea	0g	5.76g
Water	6.2mL	to 12mL
TEMED	12µl	12µl

10% (w/v) AMPS added as described in text

The gradient was achieved by pouring the urea gradient (3.45mL of 0M urea mixing into 3.45mL of 8M urea gel mix with 20µl of freshly prepared 10% AMPS added to each well immediately before pouring) with the glass plates at 90° (sealed sides and bottom with 1% agarose gel), then rotating back through 90° and pouring the stacker (1.5mL of non-denaturing PAGE stacking gel mix with 20 µl of freshly prepared 10% AMPS added). The glass plates were specially cut as 100 x 100 mm squares, with a central 10 x 80 mm section cut out of the top (by final orientation) of one plate to create ears (figure 3.5).

Protein samples of 150µg (for Coomassie-stained gels) or 20µg (for sliver-stained gels) were prepared by addition of an appropriate volume of 5x non-denaturing PAGE loading buffer and loaded in a well across the length of the stacking gel. The gels were run using non-denaturing PAGE discontinuous buffers in specially constructed gel tanks, at a constant current of 25mA, and fan-cooled, until the loading buffer dye front reached the bottom of the gel. Proteins were visualised by staining with Coomassie Blue and destaining or by silver staining.

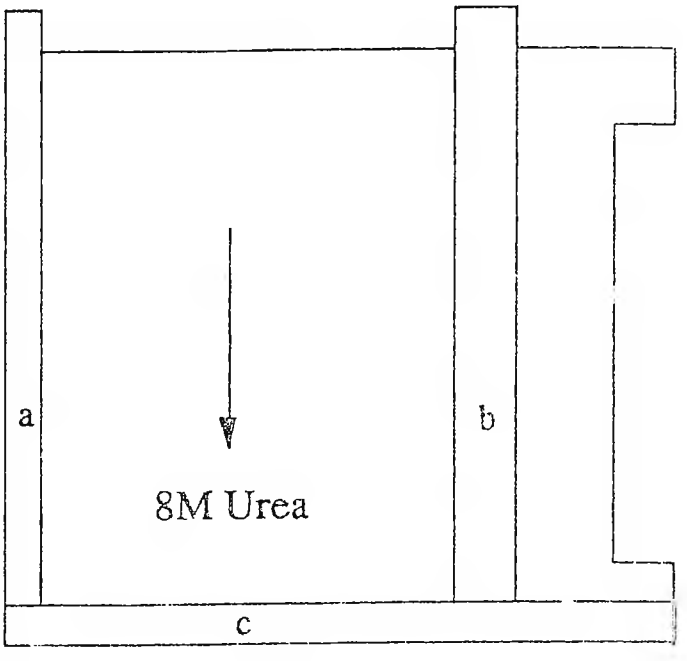

Figure 3.5: Diagram showing orientation of TUG gel plate

3.2.4 Active site titration

Active site titration's of α_1-antitrypsin were performed by incubating the increasing concentrations of the inhibitor with bovine chymotrypsin of known active site (Kezdy, 1970). Recombinant wildtype and mutant α_1-antitrypsin was diluted from stock in reaction buffer (0.03M sodium phosphate, 0.16M NaCl and 0.1% (w/v) PEG 4000, pH 7.4), to give an initial solution estimated to have an active site concentration of 0.1μM. Increasing amounts of α_1-antitrypsin (0-5pmol of estimated active site) were mixed with 5pmol of bovine chymotrypsin in reaction buffer, in a total volume of 100μl. The mixture was incubated for 10 minutes (>> 5 x $t_{1/2}$), according to previous calculations of association kinetics (Lomas, 1995), at room temperature and the residual proteolytic activity was determined by adding 900μl of reaction buffer containing the substrate succinyl-L-alanyl-L-alanyl-prolyl-L-phenylanalyl-p-nitroanilide (Succ-Ala-Ala-Pro-Phe-pNa) at a concentration of 0.16mM. The change in OD_{405} over 3 minutes was observed. Active site values were obtained by plotting residual proteolytic activity against the amount of inhibitor and extrapolating to the x-intercept (Beatty *et al.*, 1980). This value gives the amount of total α_1-antitrypsin required to inhibit 5pmols of bovine chymotrypsin. Since inhibition is effectively irreversible in these conditions and requires 1:1 stoichiometry of enzyme:inhibitor active site, the specific activity of the sample is defined as:

$$\frac{5 \times 10^{-12}}{\text{value at intercept of x-axis}} \times \frac{\text{molecular mass of inhibitor}}{\text{concentration of inhibitor in initial solution}}$$

3.2.5 SDS-PAGE assessment of the stability of the serpin-proteinase complex

Wildtype or mutant recombinant α_1-antitrypsin was incubated with varying molar ratios of human neutrophil elastase or trypsin at 37°C for up to 13 days. Aliquots were withdrawn at different time points, heated at 98°C for 5 minutes and then snap frozen and stored at -70°C until required. The stability of the serpin-proteinase complex was assessed by SDS-PAGE with the intensity of the bands being determined by densitometry.

3.2.6 Kinetic assessment of the stability of the serpin-proteinase complex

Complexes were prepared by incubating 4μM α_1-antitrypsin with trypsin in a 9:1 molar ratio in 50mM Tris, 0.15M NaCl, pH 7.4 for 60 minutes at room temperature. They were dissociated by 200-fold dilution into 0.3mM substrate (S2222) at 20 or 37°C. This quantity of substrate prevented any released enzyme re-associating with the inhibitor. Complex dissociation was monitored continuously for 60 minutes by measuring the generation of p-nitroanilide at OD_{405}. Under these conditions, less than 5% of the substrate was converted to product and less than 3% of the complex dissociated. Thus the data from the initial linear rate of complex dissociation could be fitted to the equation proposed by Jesty, 1979.

$$A_t = A_0 + V_o t + K_{off} \times [E\text{-}I]_0 \times TN \times t^2/2$$

Where A_t and A_0 are the absorbance at time t and 0 respectively, V_0 is the rate of change in OD_{405} at time 0, k_{off} is the first order rate constant for complex dissociation, $[E\text{-}I]_0$ is the starting concentration of complex and TN is the value for hydrolysis of substrate by enzyme under the conditions of the experiment, expressed as the rate of change in OD_{405} per unit of enzyme concentration. The coefficients of the second order polynomial equation were fitted parameters and were calculated from the fitted coefficient of the t term using the concentration of complex and independently measured turnover number. The α_1-antitrypsin that dissociates from the complex is in an inactive form. Thus there was no contribution from the inhibitor re-associating with enzyme during the experiment.

3.3 Results

The Asn314Ala, Lys328Ala and Asn314Ala/Lys328Ala α_1-antitrypsin mutants were prepared to assess the effect of As314 and Lys328 on the stability of the serpin-proteinase complex. The recombinant wildtype and mutant α_1-antitrypsin were confirmed to be monomeric on non-denaturing and SDS-PAGE (figure 3.6). When assessed by transverse urea gradient (TUG) PAGE, the recombinant α_1-antitrypsin had an unfolding profile characteristic of a native, metastable serpin (figure 3.7) a similar unfolding pattern was observed for the Asn314Ala, Lys328Ala and Asn314Ala/Lys328Ala α_1-antitrypsin mutants.

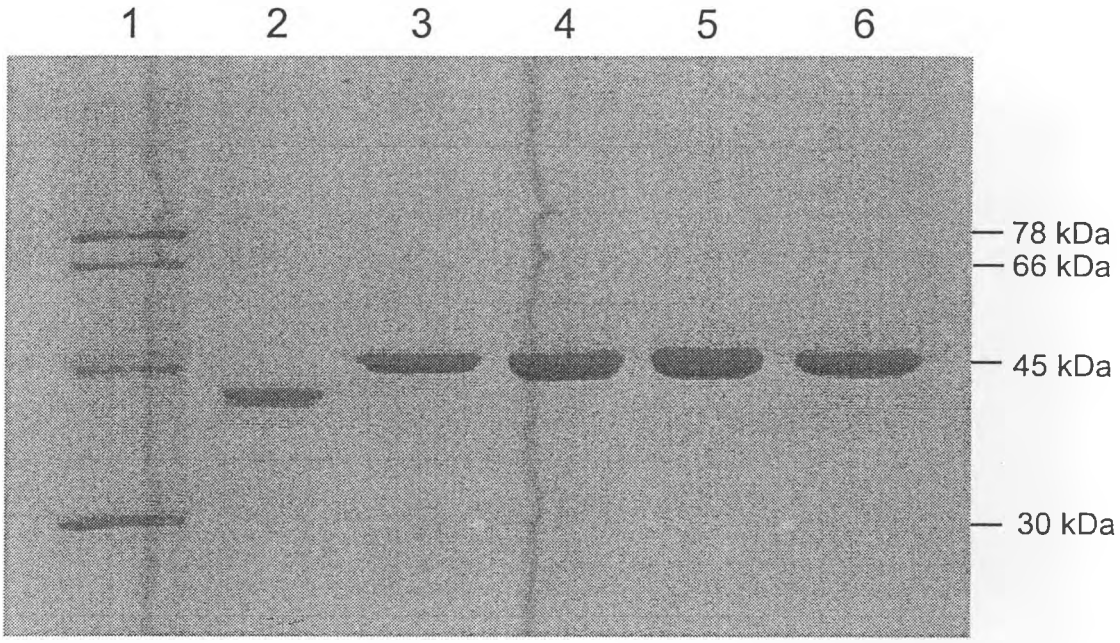

Figure 3.6: 7.5 - 15% SDS - page gel. Lane 1, molecular weight marker. Lane 2, cleaved recombinant wildtype α_1 - antitrypsin. Lane 3, recombinant wildtype α_1 - antitrypsin. Lane 4, recombinant Asn 314 Ala α_1 - antitrypsin; Lane 5, recombinant Lys 32B Ala α_1 - antitrypsin; Lane 6, recombinant Asn 314 Ala/Lys 328 Ala α_1 - antitrypsin.

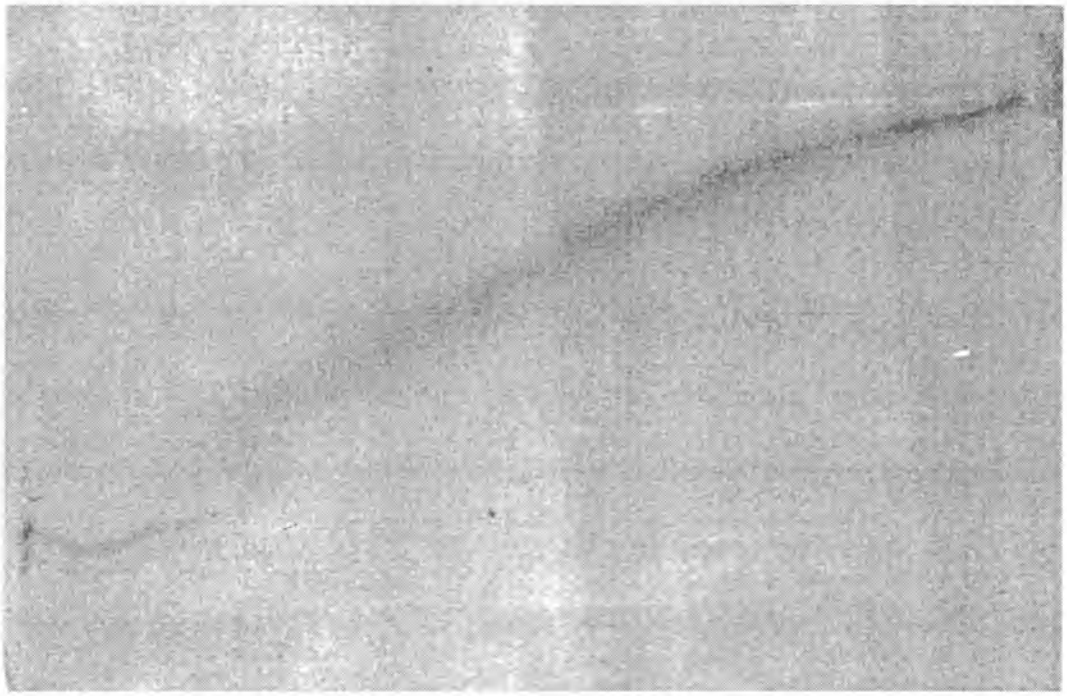

Figure 3.7: 0-8m urea, 7% (w/v acrylamide) TUG PAGE of recombinant wildtype α_1 - antitrypsin. 20ug of protein was loaded and visualised by coomassie blue staining.

The recombinant wildtype α_1-antitrypsin was 59.67% active as an inhibitor of bovine chymotrypsin, while the Asn314Ala mutant was 64.73% active, the Lys328Ala 67.56% active (figure 3.8) and the double mutant Asn314Ala/Lys328Ala was 63.93% active as an inhibitor of bovine chymotrypsin. The susceptibility of the different mutants to cleavage by Papain and V8Protease was identical to that of wildtype α_1-antitrypsin over a range of concentrations. The thermal stability assay also showed no difference between the mutant α_1-antitrypsin and the recombinant α_1-antitrypsin.

All three mutants had the same stoichiometry of inhibition and a similar association rate constant with trypsin (table 3.1). Wildtype α_1-antitrypsin formed SDS-stable complexes when incubated with increasing concentrations of both porcine trypsin and neutrophil elastase (figure 3.9). The three mutants all formed SDS-stable complexes with neutrophil elastase (figures 3.10 and 3.11) and porcine trypsin (figures 3.13 and 3.14) when assessed by SDS-PAGE but in all cases the complexes dissociated when incubated at 37°C for 13 days. This was in contrast to wildtype α_1-antitrypsin, which formed stable complexes that dissociated only slowly when incubated under identical conditions.

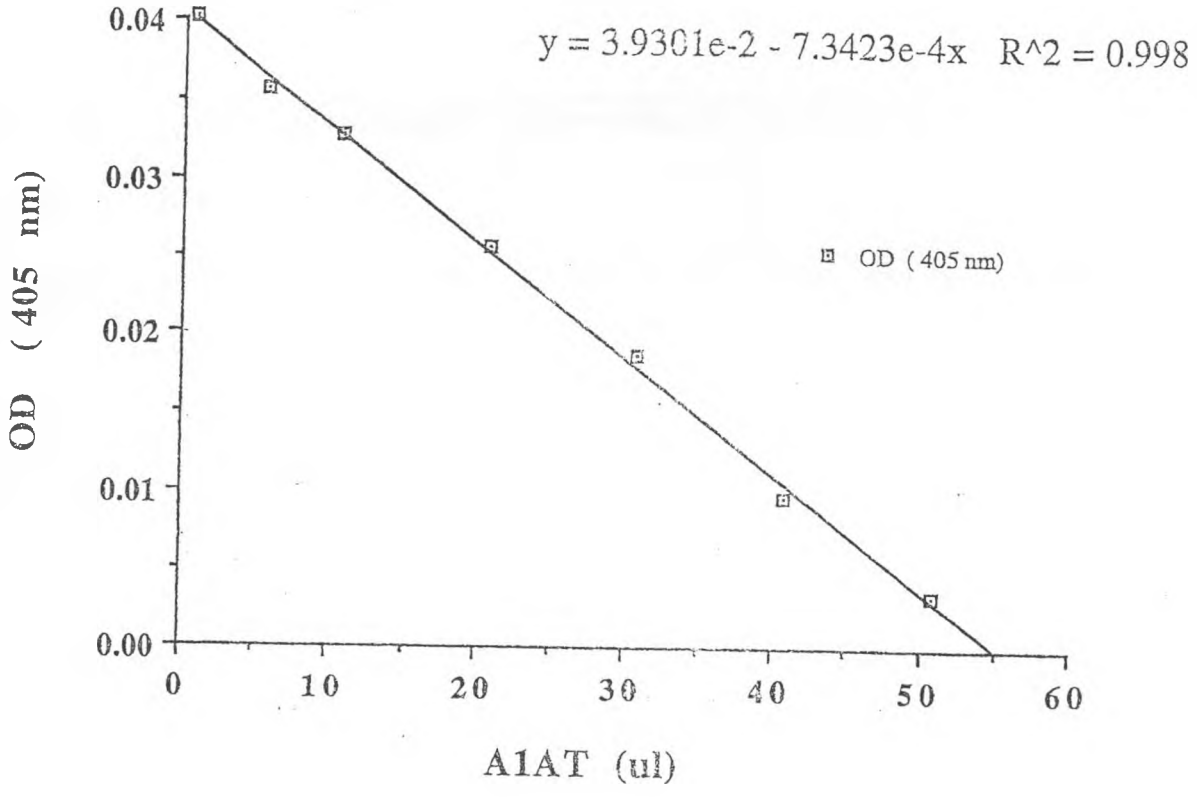

Figure 3.8: Active site titration of recombinant Asn314Ala α_1-antitrypsin. 0-5pmol of initially estimated active site of α_1-antitrypsin was reacted in 100µl reaction volume with 5pmol bovine chymotrypsin. The reaction was incubated at room temperature for 10 minutes (>> 5 x $t_{1/2}$) and residual proteinase activity was then assessed by adding the substrate Succ-A-A-P-F-pNA.

Table 3.1: The second order rate constants (k_{ass}) of α_1-antitrypsin mutants with porcine trypsin and the dissociation rate constants (k_{diss}) of complexes with porcine trypsin at 20 and 37°C.

	Wildtype	**Asn314Ala**	**Lys328Ala**	**Asn314Ala/ Lys328Ala**
Porcine trypsin k_{ass} ($M^{-1}s^{-1}$, 20°C)	$6.14+/-0.53x10^{4}$	$6.26+/-0.48x10^{4}$	$6.19+/-0.58x10^{4}$	$6.35+/-0.20x10^{4}$
Porcine trypsin k_{diss} (s^{-1}, 20°C)	$5.71\pm0.51x10^{-8}$	$2.90\pm0.52x10^{-7}$	$6.02\pm1.08x10^{-7}$	$1.63\pm0.36x10^{-6}$
Half-life (days)	140	28	13	5
Porcine trypsin k_{diss} (s^{-1}, 37°C)	$1.63\pm0.10x10^{-7}$	$1.30\pm0.11x10^{-6}$	$2.43\pm0.14x10^{-6}$	$3.79\pm0.16x10^{-6}$
Half-life (days)	49	6	3	2

The results are the mean and standard error of 3 independent experiments. The half-life of the α_1-antitrypsin-trypsin complexes is shown.

Analysis of the gels showed that the Asn314Ala and Lys328Ala α_1-antitrypsin had similar effects on complex formation with neutrophil elastase but combining the mutants in Asn314Ala/Lys328Ala α_1-antitrypsin increased the instability of the complex (figure 3.11). No proteinase inhibitor such as aprotinin was added to these reaction mixtures and thus the dissociation of the Asn314Ala/Lys328Ala α_1-antitrypsin/neutrophil elastase complex was visualised as a loss of the high molecular mass complex band accompanied by an increase in cleaved α_1-antitrypsin.

The significance of the Asn314 and Lys328 residues was underscored by assessing the interaction with porcine trypsin. Once again wildtype, Asn314Ala, Lys328Ala and Ans314Ala/Lys328Ala α_1-antitrypsin all formed an initial complex with trypsin that was stable on SDS-PAGE (figure 3.12, 3.13 and 3.14). Asn314Ala and Lys328Ala α_1-antitrypsin (figure 3.13) both caused a reduction in stability of the inhibitory complex which was most marked with the double mutant, Asn314Ala/Lys328Ala α_1-antitrypsin (figure 3.13). The serine proteinase inhibitor aprotinin was added to these reactions in 100 fold molar excess after complex formation. Thus the released trypsin from complex dissociation was unable to attack and cleave the residual native α_1-antitrypsin. In these experiments the stability of the complex was assessed by the loss of the high molecular mass complex band on SDS-PAGE and an increase in free proteinase.

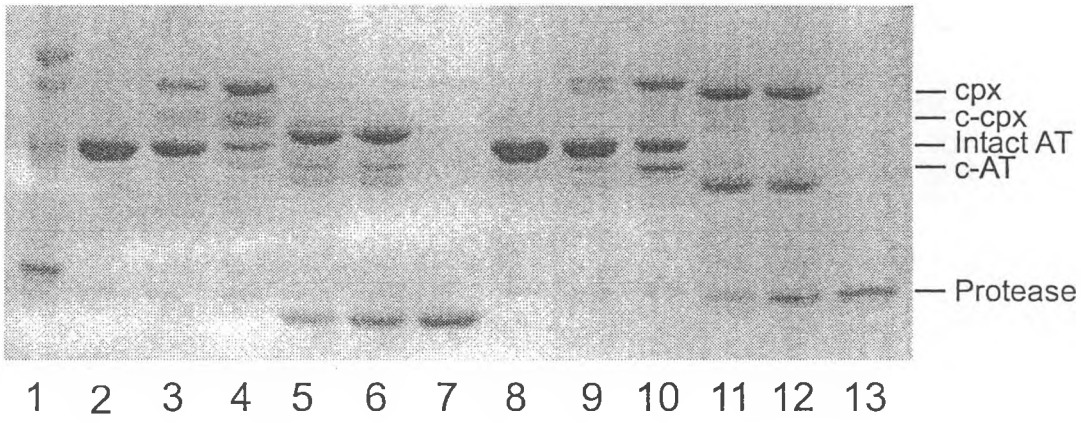

Figure 3.9: 12% w/v SDS-PAGE to assess the stability of complexes of α_1-antitrypsin with human neutrophil elastase and trypsin. Wildtype, Asn314Ala, Lys328Ala and Asn314Ala/Lys328Ala α_1-antitrypsin were added to human neutrophil elastase and porcine trypsin at varying molar ratios and then incubated at 37°C before being assessed by gel electrophoresis. Cpx indicates the α_1-antitrypsin-proteinase complex, c-cpx is cleaved complex, intact AT is monomeric unreacted α_1-antitrypsin, c-AT is reactive loop cleaved α_1-antitrypsin and protease is unreacted or dissociated enzyme. Lane1, molecular mass markers (78, 66, 45 and 30 kDa); lane2, wildtype α_1-antitrypsin; lanes 3-6, wildtype α_1-antitrypsin:trypsin at 1:0.25, 1:0.5, 1:1 and 1:2 molar ratios respectively; lane 7, porcine trypsin; lane 8, wildtype α_1-antitrypsin; lane 9-12, wildtype α_1-antitrypsin-neutrophil elastase at 1:0.25, 1:0.5, 1:1 and 1:2 molar ratios respectively; lane 13, neutrophil elastase.

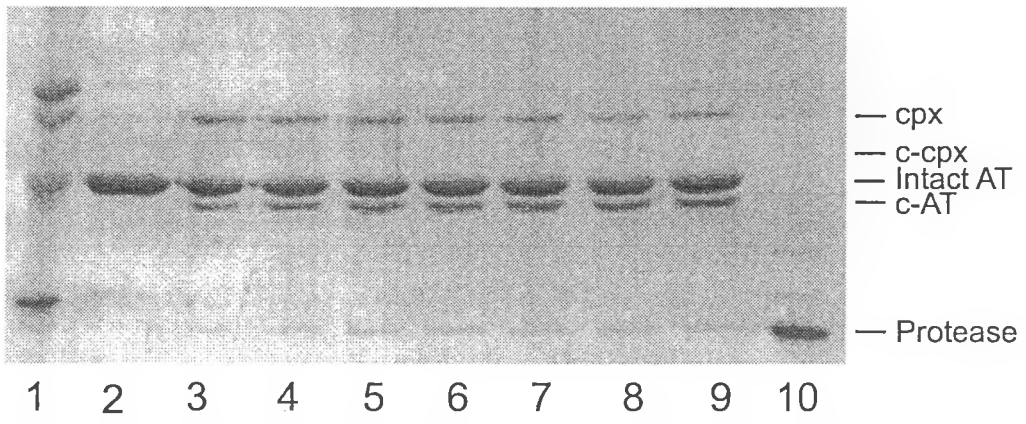

Figure 3.10: Assessment of complex formation of wildtype α_1-antitrypsin with human neutrophil elastase. α_1-Antitrypsin was incubated in 4:1 molar ratio with neutrophil elastase at 37°C and aliquots were removed at the times shown. Lane 1, molecular mass markers (78, 66, 45 and 30 kDa); lane 2, α_1-antitrypsin; lane 3, α_1-antitrypsin-neutrophil elastase complex at 30 minutes; lane 4-9, α_1-antitrypsin-neutrophil elastase complex at 1, 2, 3, 4, 5 and 6 days respectively; lane 10, neutrophil elastase.

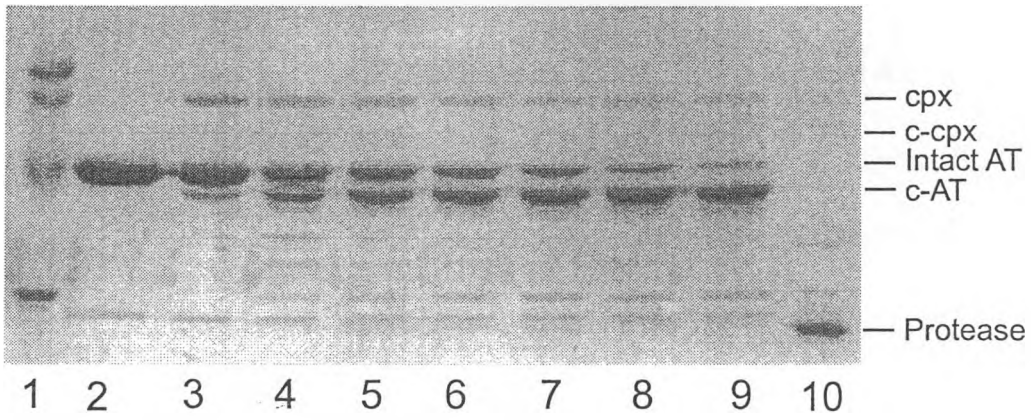

Figure 3.11: Assessment of complex formation of Asn314Ala/Lys328Ala α_1-antitrypsin with human neutrophil elastase. α_1-Antitrypsin was incubated in 4:1 molar ratio with neutrophil elastase at 37°C and aliquots were removed at the times shown. Lane 1, molecular mass markers (78, 66, 45 and 30 kDa); lane 2, α_1-antitrypsin; lane 3, α_1-antitrypsin-neutrophil elastase complex at 30 minutes; lane 4-9, α_1-antitrypsin-neutrophil elastase complex at 1, 2, 3, 4, 5 and 6 days respectively; lane 10, neutrophil elastase.

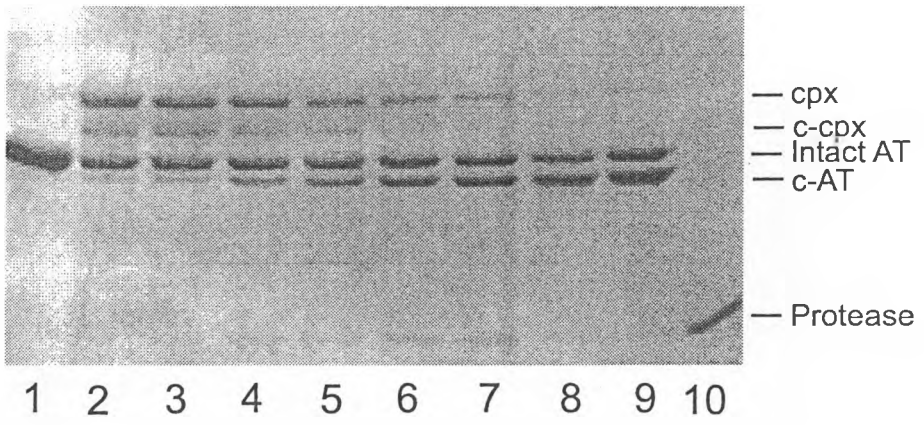

Figure 3.12: Assessment of complex formation of wildtype α_1-antitrypsin with porcine trypsin. α_1-Antitrypsin was incubated in 4:1 molar ratio with porcine trypsin at 37°C and aliquots were removed at the times shown. Aprotonin was added to a final concentration of 1 mg/ml after the 30 minute time point. Lane 1, α_1-antitrypsin; lane 2, α_1-antitrypsin-porcine trypsin complex at 30 minutes; lane 3-9, α_1-antitrypsin-porcine trypsin complex at 1, 3, 5, 7, 9, 11 and 13 days respectively; lane 10, porcine trypsin.

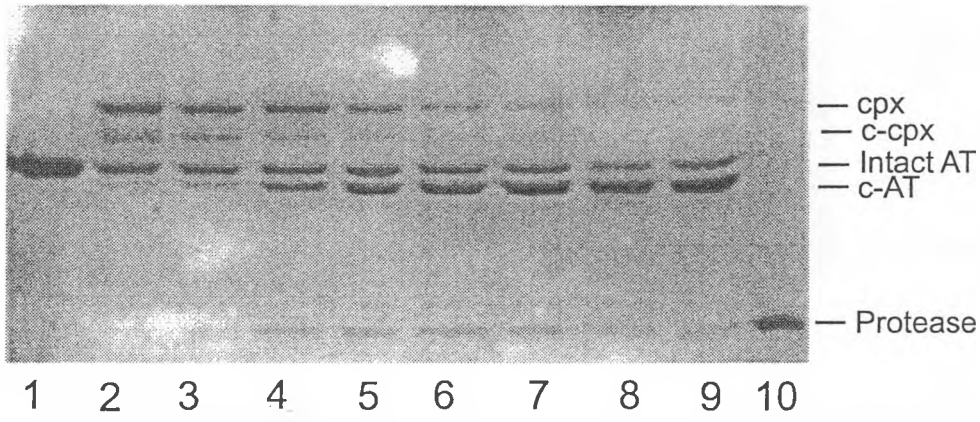

Figure 3.13: Assessment of complex formation of Lys328Ala α_1-antitrypsin with porcine trypsin. α_1-Antitrypsin was incubated in 4:1 molar ratio with porcine trypsin at 37°C and aliquots were removed at the times shown. Aprotonin was added to a final concentration of 1 mg/ml after the 30 minute time point. Lane 1, α_1-antitrypsin; lane 2, α_1-antitrypsin-porcine trypsin complex at 30 minutes; lane 3-9, α_1-antitrypsin-porcine trypsin complex at 1, 3, 5, 7, 9, 11 and 13 days respectively; lane 10, porcine trypsin.

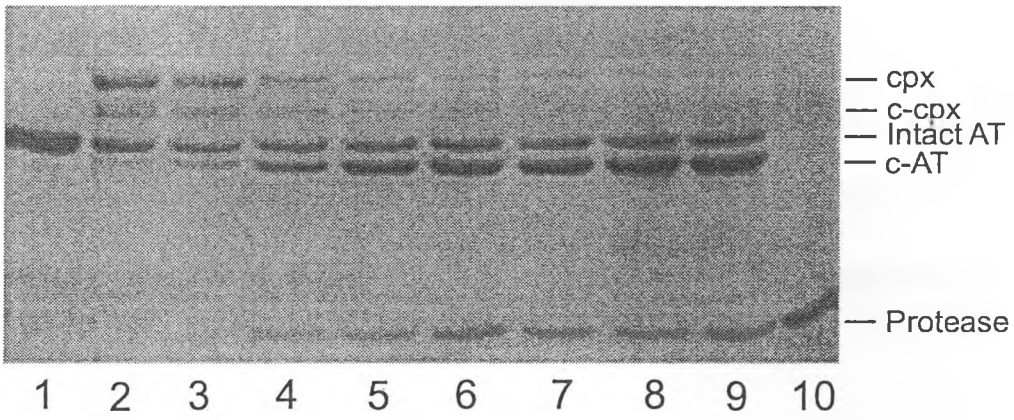

Figure 3.14: Assessment of complex formation of Asn314Ala/Lys328Ala α_1-antitrypsin with porcine trypsin. α_1-Antitrypsin was incubated in 4:1 molar ratio with porcine trypsin at 37°C and aliquots were removed at the times shown. Aprotonin was added to a final concentration of 1 mg/ml after the 30 minute time point. Lane 1, α_1-antitrypsin; lane 2, α_1-antitrypsin-porcine trypsin complex at 30 minutes; lane 3-9, α_1-antitrypsin-porcine trypsin complex at 1, 3, 5, 7, 9, 11 and 13 days respectively; lane 10, porcine trypsin.

The dissociation rate constant for the inhibitory complexes with trypsin was determined by kinetic analysis at 20 and 37°C (table 3.1 and figure 3.15). Asn314Ala and Lys328Ala α_1-antitrypsin both destabilised the inhibitory complex with trypsin and increased that rate of complex dissociation at 20°C. When mutations were combined there was an additive effect with Asn314Ala/Lys328Ala α_1-antitrypsin having a dissociation rate constant 29 fold greater than the wildtype protein. The effect of the mutants on the stability of the complex was exacerbated by raising the temperature of the reaction mix to 37°C. This increased the dissociation rate constant of all 3 mutants with trypsin but also increased the dissociation rate constant of the wildtype protein.

The dissociation rate constant of Asn314Ala/Lys328Ala α_1-antitrypsin was 23 fold greater than that of the wildtype protein at this temperature (37°C). Once again the effect of Asn314Ala and Lys328Ala α_1-antitrypsin was additive indicating that both the Asn314 and Lys328 residues independently play an important role in stabilising the complex. The significance of these data was illustrated by considering the half-life of the inhibitory complexes (table 3.1).

The Asn314Ala/Lys328Ala mutant reduced the half-life of the α_1-antitrypsin-trypsin complex from 140 days to 5 days at 20°C and from 49 days to 2 days at 37°C. The dissociation rate constants of complexes with human neutrophil elastase were not determined as the K_m of the substrate was too high.

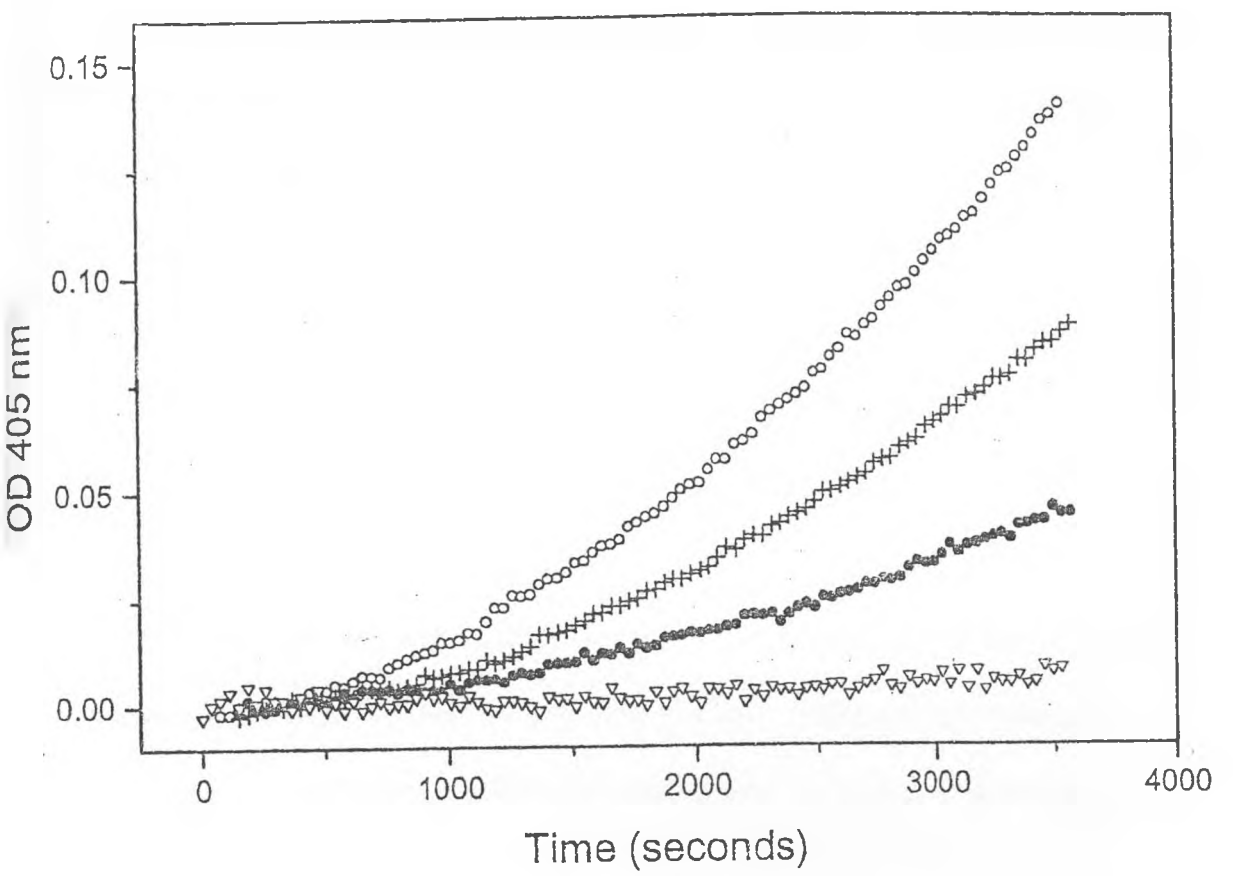

Figure 3.15: Kinetic assessment of complex dissociation. Complexes of α_1-antitrypsin and trypsin were prepared by incubating 2nM of enzyme with a 9 fold molar excess of α_1-antitrypsin at room temperature. They were dissociated by 200-fold dilution into 0.3mM substrate (S2222) AT 37°C. Inverted triangle, wildtype α_1-antitrypsin; filled circle, Asn314Ala α_1-antitrypsin; cross Lys328Ala α_1-antitrypsin; open circle, Asn314Ala/Lys328Ala α_1-antitrypsin.

3.4 Discussion

Lys328 and Asn314 are not widely conserved in the serpin superfamily (Whistock, 1996). However, these stabilising serpin-proteinase interface interactions are also likely to be important in other pairs of serpins and proteinases. Examination of the cleaved structures of α_1-antitrypsin (Loebermann *et al.*, 1984), α_1-antichymotrypsin (Baumann, *et al.*, 1991), antithrombin (Delarue *et al.*, 1990), plasminogen activator inhibitor-1 (Aertgeerts *et al.*, 1995) and horse leucocyte elastase inhibitor (Baumann *et al.*, 1992), showed that a residue that corresponds to position 328 or 314 in α_1-antitrypsin is available to hydrogen bond with the proteinase.

The amide side chain of Asn314 of α_1-antitrypsin forms hydrogen bonds with the main chain carbonyl oxygen's of Ser214, Trp215 and 194Asp in the proteinase (Huntington *et al.*, 2000). Lys328 forms a salt bridge with Asp194. In α_1-antichymotrypsin the 328 position is filled by an Ala residue that cannot form hydrogen bonds. However, the 314 position is occupied by a Ser that can hydrogen bond to the main chain carbonyl oxygen's of Ser214, Trp215 and 194Asp in the proteinase. In antithrombin the 328 position is occupied by 364Tyr that can hydrogen bond to 194Asp in the proteinase. The 314 position is filled by 347Pro, which would be unable to stabilise the inhibitory complex.

In horse leucocyte elastase inhibitor the 328 position is filled with a phenylalanine but the 314 position contains an arginine, which can readily substitute for the interactions at the 328 site. Finally plasminogen activator inhibitor-1 has 334His in the 328 position and 319Gln in the 314 position. Both can provide hydrogen bonds to stabilise the target proteinase. Taken together these observations underscore the importance of the residues that occupy positions 328 and 314 in α_1-antitrypsin and imply that this interaction may be a general mechanism in stabilisation of the serpin-proteinase complex.

The demonstration of an interaction that stabilises the serpin-proteinase inhibitory complex confirms that the enzyme must be fixed at the distal pole of the serpin in the final inhibitory complex (figure 3.1). This is in keeping with biophysical analysis (Stratikos & Gettins, 1997; Stratikos & Gettins, 1998; Wilczynska *et al.*, 1997) and the recent crystal structure of the complex (Huntington *et al.*, 2000). It strongly argues against the suggestion that in the final complex the proteinase oscillates between 2 positions characterised by partial and full loop insertion of the inhibitor (O'Malley & Cooperman, 2001).

CHAPTER 4

MAJOR FINDINGS AND CONCLUSIONS

4.1 The Role of Alpha-1-Proteinase Inhibitor in Asthma

The finding of functionally abnormal α_1-AT in asthmatic patients may be of considerable importance in light of the fact that asthma has been recognised as an inflammatory disorder. In-addition to a novel polymorphism we identified 2 new variants of α_1-AT in association with asthma. Although these new variants were not related to a deficiency state, they demonstrate the extent and complexity of genetic susceptibility to atopic asthma. Knowledge of the molecular basis of variants and polymorphisms, both normal and pathogenic, can be useful for the identification of the residues most important for protein function.

4.2 Stabilisation of the serpin-proteinase complex through conserved interactions

Making use of the recently described serpin-proteinase complex crystal structure and site directed mutagenesis we have demonstrated residues in alpha-1 antitrypsin that are important in stabilising the final serpin-proteinase complex. Structural homology suggests that this interaction is likely to be important in stabilising the inhibitory complex in other serpin-proteinase pairs.

Appendix I

PCR Protocol

Materials:

The Taq DNA polymerase and the dNTPs were from Promega (Madison, WI); the primers used were synthesised by Genosys Biotechnologies Inc (Woodlands, TX); the sterile mineral oil was supplied by Sigma Chemical Co (St Louis, MO); the sterile double-distilled water was supplied by SABAX (Johannesburg); the filtered pipette tips and the 0.5 mL PCR tubes were from Laboratory and Scientific Equipment Co (Johannesburg); all other reagents were of analytical grade (Merck, Darmstadt, Germany).

Reagent buffer

1.5 mM $MgCl_2$	0.031 g
10 mM Tris base	0.121 g
50 mM KCl	0.355 g

Dissolve in 95 mL of double-distilled water (ddH_2O), adjust the pH to 8.3 with HCl and adjust the volume to 100 mL with ddH_2O. Autoclave and store at 4°C, or aliquot and store at -20 °C (long-term storage).

The work in this project has used molecular and structural biology to understand clinical conditions. It has also continued the trend of providing insights into protein structure and conformational transitions by the study of clinically relevant pathological mutants and protein-protein interactions. Such dialogue between the clinical and the structural will help our understanding of the genetic data amassing on associations between genetic polymorphisms and disease.

It will be particularly important in understanding conditions that can be classed as conformational diseases. Moreover it is an approach that is vital to achieving the goal of designing and targeting therapeutic agents capable of modulating pathological transitions.

Standard PCR protocol

The following was the standard PCR protocol used in this study, with modifications as described in the relevant chapters.

To a 0.5 mL PCR tube, the following reagents were added:

25 μL reagent buffer

10 μL dNTP mix

1 μL (typically 100 pmol) each appropriate primer

20-25 μL (~1 μg) DNA

Each sample was overlaid with 50 μL of lightweight mineral oil and the tubes were placed in a Hybaid DNA thermal cycler. Following the "hot start" and the subsequent reduction of the sample temperature to the appropriate annealing temperature, 2 units of Taq DNA polymerase were added to each sample. The samples were then processed as described in the relevant chapters.

APPENDIX II

Agarose gel electrophoresis

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments (Maniatis *et al.*, 1985). The technique is rapid, simple and resolves mixtures of DNA fragments that cannot be separated efficiently by other sizing procedures. The technique is based on the principle that negatively charged DNA fragments migrate toward a positive pole at different rates, depending on the following parameters:

(a) the molecular size of the DNA-linear duplex DNA molecules migrate in an end-on position through the gel matrix at rates that are inversely proportional to the $\log_{10}$ of their molecular weights (Maniatis *et al.*, 1982; Stine, 1989).

(b) the agarose concentration - a DNA fragment of a particular size migrates at different rates through gels containing different agarose concentrations. The relationship between the logarithm of the electrophoretic mobility of DNA (μg) and the gel concentration (τ) is linear, and is described by the following equation:

$$\log_{\mu} = \log_{\mu 0} - K_r\tau,$$

where μ_0 is the free electrophoretic mobility and Kr is the retardation co-efficient, a constant that is related to the gel properties and the size and conformation of the migrating DNA molecules (Maniatis *et al.*, 1982).

(c) the applied current - at low voltages, linear DNA molecules migrate at a rate that is proportional to the applied voltage. As the strength of the electric field increases, the mobility of the high molecular weight DNA fragments increases differentially. Consequently, the effective separation range of agarose gels decreases as the voltage is increased-maximum resolution of DNA is obtained by running gels at no more than 5 V/cm (Maniatis *et al.*, 1982; Brown, 1989).

(d) base composition and temperature - the base composition of the DNA and the gel temperature during electrophoresis do not affect DNA migration through the gel significantly, which means that the relative electrophoretic mobilities of DNA molecules of different sizes do not change between 4°C and 30°C (Maniatis *et al.*, 1982).

The location of DNA within the gel can be determined directly by staining the DNA in the gel with a fluorescent, intercalating dye such as ethidium bromide (EtBr); as little as 1 ng of DNA can be detected by direct examination of the gel upon ultraviolet (UV) illumination (Maniatis *et al.*, 1982), typically at a wavelength of 302nm. Ethidium bromide is a phenanthridine derivative and contains a planar group that intercalates between the stacked bases of DNA (Williams, 1989). The fixed position of the planar group and its proximity to the bases causes dye that is bound to DNA to fluoresce at a greater intensity than dye that is in free solution (Williams, 1989). UV irradiation absorbed by the DNA at 260nm and transmitted to the dye, or irradiation that is absorbed at 300nm and 360nm by the bound dye, is emitted at 590nm in the red-orange region of the visible spectrum (Maniatis *et al.*, 1982; Stine, 1989).

Materials:

The agarose and low melting temperature agarose were supplied by Promega (Madison, WI); the Ficoll (Type 400) was from Sigma Chemical Co. (St Louis, MO); the sterile distilled water was from SABAX (Johannesburg); all other reagents were of analytical grade (Merck, Darmstadt, Germany).

Gel concentration (%)	Agarose (g)	5xTBE, pH8.2 (mL)	dH_2O (mL)
0.7	0.20	5	25
1	0.33	5	25
2	0.67	10	25
4	3:1*	10	90

*3 g agarose:1 g low melting temperature agarose

1. Add buffer at room temperature to a beaker that is 3-4 times the volume of the final solution.

2. Sprinkle agarose powder into the solution while it is being stirred in order to prevent the formation of lumps.

3. Boil the slurry in a microwave oven (100% power, 2-4 min), stirring at intervals, to liquefy the agarose.

4. After agarose is dissolved, cool to 50°C (higher for the 4% gels) add 1.5 µL/30 mL or 5-10 µL/100 mL EtBr (from a 10 mg/mL stock solution) to a final concentration of

0.5 μg/mL and pour the solution into a clean, sealed gel cassette with the comb in place (there should be a space of 0.5-1.0 mm between the bottom of the teeth and the base of the cassette, so that the sample wells are sealed).

5. After the gel sets at room temperature for 30 min, over with a 2-3 mm layer of running buffer and refrigerate for another 30 min.

6. Mix 10 μL of each PCR reaction with 8 μL of a 6x loading dye and load 15 μL of this mixture per well.

7. Run at 4.5 V/cm (for a gel of 12.5x20 cm) or 8.8 V/cm (for a gel of 5.5x9 cm); for the purposes of the present study, all agarose gels were run at 80 V in 0.5XTBE buffer for 45 min, (1% and 2% gels) and 1½ hour (3% and 4% gels).

Electrophoresis buffers

Buffer	**Working solution (1x)**	**Concentrated stock (per litre)**
		50x
Tris-acetate EDTA (TAE)	0.04 M Tris-acetate	242 g Tris base 57.1 mL glacial acetic acid
	0.002 M EDTA	100 mL 0.5 M EDTA, pH 8.0
		10x
Tris-borate EDTA (TBE)	0.089 M Tris-borate	108 g Tris base 55 g boric acid
	0.002 M EDTA	40 mL 0.5 M EDTA, pH 8.0

6x Gel loading dye

bromophenol blue	0.25 g
xylene cyanol FF	0.25 g
Ficoll Type 400	15.00 g

Dissolve in 100mL sterile water, aliquot and store at 4°C.

APPENDIX III

Direct sequencing of PCR products

The most commonly use method for DNA sequencing is the chain termination method developed by Sanger *et al.* (1977). This method involves the *in vitro* synthesis of a DNA strand by a polymerase, using a single-stranded DNA template (Sanger *et al.*, 1977), with modern methods, double-stranded PCR products can be sequenced directly. Synthesis is only initiated at the site at which a primer binds to the template and the synthesis is terminated upon the incorporation of a nucleotide analogue that does not allow continued DNA strand elongation (Sanger *et al.*, 1977; Sanger *et al.*, 1982; Brown, 1989). These nucleotide analogues are 2',3'-dideoxynucleoside 5'-triphospates (ddNTPs) which lack the 3'-OH (hydroxyl) group that is essential for polymerase recognition and strand elongation, so that termination occurs specifically at places where the deoxynucleotide should be incorporated (Sanger *et al.*, 1977; Sanger *et al.*, 1982). Therefore, if a primer is incubated with a template with a DNA polymerase in the presence of a mixture of a ddNTP and the four dNTPs (one of which is radioactively labelled), a mixture of fragments all having the same 5' end and ddNTP residues at their 3' ends is obtained (Sanger *et al.*, 1977; Sanger *et al.*, 1982).

When using direct sequencing, most of the dNTPs and primers used during the PCR remain intact and will interfere with normal sequencing methods, which also utilise primers and nucleotides. Two hydrolytic enzymes, shrimp alkaline phosphatase (SAP) and exonuclease I (exo I) can be used to remove the unwanted molecules. The exonuclease I removes residual single-stranded (ss) primers and any ssDNA produced

by the PCR, while the SAP removes remaining dNTPs from the PCR, which would affect the labelling step of the sequencing reaction. It is essential that high quality and good yield PCR is performed in order to obtain high quality sequence information.

Materials:

The Thermo Sequenase radiolabelled terminator cycle sequencing kit, the shrimp alkaline phosphatase, the exonuclease and the 20x glycerol tolerant buffer (GTB) were obtained from Amersham Life Science Inc. (Cleveland, Ohio); the acrylamide and the urea were from Promega (Madison, WI); the ammonium persulfate, bis-acrylamide and TEMED were supplied by BioRad (Hercules, CA); the Sigmacote was from Sigma Chemical Co. (St. Louis, MO); the 0.5 mL microcentrifuge tubes were supplied by Laboratory and Scientific Equipment Co. (Johannesburg); the sterile double-distilled water was from SABAX (Johannesburg); the 3 MM Whatman filter paper was from Whatman International (England).

Sequencing protocol

1. Template preparation

To a 0.5 mL microcentrifuge tube add:

5 μL (0.5 pmol) PCR amplification mixture

1 μL (1.0 U/μL) Exonuclease I (exo I)

1 μL (2.0 U/μL) Shrimp alkaline phosphatase (SAP)

Spin the samples briefly to collect all the reagents at the bottom of the vial and incubate at 37°C for 15 min and inactivate the exo I and SAP by heating to 80°C for 15 min (performed in a Hybaid DNA thermal cycler).

2. Termination mixes

Prepare the termination mixes on ice. Mix 2 μL of nucleotide master mix (dGTP) and 0.5 μL of [α-^{33}P] ddNTP (G, A, T, or C - one of each per sequence) to produce a termination mix for each ddNTP. Label, fill and cap four tubes ('G', 'A', 'T', 'C') with 2.5 μL of each termination mix. To prepare termination mixes for (n) reactions, mix:

	G	A	T	C
Nucleotide master mix (μL)	(2 x n)	(2 x n)	(2 x n)	(2 x n)
[α-^{33}P] ddNTP (μL)	(0.5 x n)	(0.5 x n)	(0.5 x n)	(0.5x n)
Total (μL)	**(2.5 x n)**	**(2.5 x n)**	**(2.5 x n)**	**(2.5xn)**

3. Reaction mixture

For multiple (n) reactions with different primers and/or templates, prepare a n+1 batch of reaction buffer, water, polymerase and aliquot; then add the unique primer and/or template in the appropriate concentration and volume to the aliquots.

Reaction buffer	2 µL
DNA	_ µL (50-500 ng)
Primer	_ µL (0.5-2.5 pmol) of each primer
H_2O	_ µL (to adjust total volume to 20 µL)
Thermo Sequenase polymerase (4U/µl)	2 µL (8 units polymerase-add last)
Total	**20 µL**

4. Cycling termination reactions

Transfer 4.5 µL of reaction mixture (prepared in step 3) to each termination tube ('G', 'A', 'C', 'T') from step 2. Mix well and overlay with 10-20 µL of mineral oil (if needed). Cap and place the tube in the thermal cycler (Hybaid DNA thermal cycler). The following cycling parameters were used: 95°C for 1 min, 55°C for 1 min and 72°C for 2 min for a total of 30 cycles.

5. Termination reaction

Add 4 µL of stop solution to each of the termination reactions, mix thoroughly and centrifuge briefly to separate the oil from the aqueous phase. Alternatively, remove 6 µL from each termination reaction and transfer to a fresh tube containing 3-4 µL of stop solution. Samples may be stored frozen until ready to load the sequencing gel. Immediately before loading, heat samples to 90°C for 10 min and load 3 µL/lane (all the samples should be loaded onto the gel within 5 min).

Plate pretreatment

The plates should be cleaned thoroughly with absolute ethanol and allowed to dry, following which the smaller of the two plates (i.e. the notched plate) should be siliconised (Sigmacote was used for the present study). The spacers are then placed along the edge of the large plate and the notched plate is then placed on top, such that all the edges are even. The plates are then taped together, taping the bottom edge last.

Gel preparation

Gel concentration (%)	Acrylamide/ bis-acrylamide	Urea (7 M)	20x glycerol tolerant gel buffer (GTB)	ddH_2O
8	7.2 g/0.8 g	42 g	4-5 mL*	~45 mL

*use 4 mL for faster gel migration.

Dissolve the above reagents, adjust the volume to 100 mL with ddH_2O, filter at 4°C (the colder the gel solution, the easier it is to pour) and degas for 3 min. When ready to pour, add 1 mL of 10% ammonium persulfate and 25 µL TEMED. Gels should be prepared 2-20 hrs prior to use (if kept overnight, store covered at 4°C).

The gels were pre-run at 90 W constant power for 30 min, until the gel temperature reached 48-50°C. Samples were then loaded and the gels were run for 1½-3 hrs at room temperature at 60 W (constant power) or until the bromophenol blue dye front reached the bottom of the gel. The gel temperature was maintained at 48-50°C. The running buffer used was 0.5xGTB. After electrophoresis, the gels were blotted onto 3 MM Whatman filter paper, dried on a slab gel dryer at 80°C for 1-1½ hrs and autoradiographed for 24-72 hrs (depending on the age of the [α-^{33}P] ddNTP).

Appendix IV

Automated sequencing of PCR products using the ABI Prism® BigDye™ terminator cycle sequencing ready reaction kit

The reaction kit was obtained from PE Biosystems (Cheshire, UK) and in the ready reaction format, the dye terminators, deoxynucleotide triphosphates, AmpliTaq DNA polymerase, magnesium chloride and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates, e.g. BAC clones. The dNTP mix includes dITP in place of dGTP to minimise band compressions. The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

The kit contains the sequencing enzyme AmpliTaq DNA polymerase, FS. This enzyme is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against dideoxynucleotides. This enzyme also has a second mutation in the amino terminal domain that virtually eliminates the 5′→3′ nuclease activity of AmpliTaq DNA polymerase. The enzyme has been formulated with a thermally stable inorganic pyrophosphatase to eliminate problems associated with pyrophosphorolysis.

Cycle sequencing protocols that rely on the use of AmpliTaq DNA polymerase, FS offer the following advantages over traditional sequencing methods:

(a) Less hands-on operation.

(b) No alkaline denaturation step required for double stranded DNA.

(c) Same protocol for both single- and double-stranded DNA.

(d) Less starting template needed.

(e) More reproducible results.

PE Biosystems has developed a set of dye terminators labeled with novel, high-sensitivity dyes. The dye structures contain a fluorescein donor dye linked to a dichlororhodamine (dRhodamine) acceptor dye. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor. The BigDye terminators are labeled with the following dRhodamine acceptor dyes:

Terminator	**Acceptor Dye**	**Colour of Raw Data on ABI Prism 310 Electropherograms**
A	dR6G	Green
C	DROX	Red
G	dR110	Blue
T	dTAMRA	Black

For optimum results, purify the PCR product before sequencing. In general, any method that removes dNTPs and primers should work. The recommended method is the use of the QIAquick™ PCR Purification Kit (an alternative is Shrimp Alkaline Phosphatase and Exonuclease I, but this is not as effective).

1) PCR

2) Cleaning of PCR products

3) Cycle Sequencing

i) For each PCR product add the following reagents to a separate tube:

1.0 µL Big Dye™

1.5 µL 5x Big Dye Diluent

0.5 µL Primer (10-20 pmols/µL)

3.5 µL Cleaned PCR product

3.5 µL ddH_2O

ii) Mix well and spin briefly.

iii) Place tubes in a thermal cycler. Repeat the following for 25 cycles:

96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes

Hold at 4°C until ready to purify.

iv) Spin down the contents of the tube in a microcentrifuge.

4) Purifying Extension Products

Unincorporated dye terminators must be completely removed before the samples can be analysed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with sequence interpretation.

Ethanol/Sodium acetate Precipitation:

i) For each sequencing reaction, prepare a 1.5 mL microcentrifuge tube containing the following:

2.0 μL of 3M sodium acetate (NaOAc), pH 4.6

50.0 μL of 95% ethanol (EtOH)

ii) Add 10.0 μL ddH_2O to the extension reaction tube to make a working volume of 20.0 μL.

iii) Pipette the entire contents (20.0 μL) of each extension reaction into a tube of sodium acetate/ethanol mixture. Mix thoroughly.

iv) Vortex the tubes and place on ice for 10 minutes to precipitate the extension products.

v) Spin the tubes in a microcentrifuge for 30 minutes at 13 000 g.

vi) Carefully aspirate the supernatant with a pipette and discard.

vii) Rinse the pellet with 250 μL of 70% ethanol. Add carefully. Pellet not visible.

viii) Vortex briefly.

ix) Spin for 5 minutes in a microcentrifuge at 13 000 g. Again carefully aspirate the supernatant and discard.

x) Dry the pellet on a heating block for 5 minutes at 95°C. Do not over-dry.

5) Electrophoresis on the ABI Prism 310

i) Resuspend each sample pellet in 20 μL of Template Suppression Reagent (TSR), which is supplied with the polymer.

ii) Vortex and spin the samples.

iii) Heat the samples at 95°C for 2 minutes to denature, then chill on ice.

iv) Vortex and spin the samples again. Place on ice until ready to use.

v) Refer to the ABI Prism 310 Genetic Analyser User's Manual for guidelines on loading the samples.

Appendix V

Mutagenesis, Expression and Purification of Recombinant α_1-antitrypsin

The expression and purification of recombinant proteins facilitates production and detailed characterization of virtually any protein. Although a wide variety of heterologous expression systems have been developed and are currently used to produce recombinant proteins, the purification of the proteins obtained can still be problematic. Classical purification procedures can be employed, but in most cases recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest; the use of these affinity tags simplifies the purification of the recombinant fusion proteins by employing affinity chromatography methods.

We made use of the QIAexpress® system, which is based on the remarkable selectivity and affinity of QIAGEN's exclusive, patented nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules that have been tagged with 6 consecutive histidine residues.

Site-directed mutagenesis

A PCR mutagenesis method was used to introduce mutations into the α_1-antitrypsin gene between the unique restriction sites Bam HI and Hind III. Complementary oligonucleotide primers were designed with the chosen mutation centrally in a

sequence ~20 bases long as described in chapter 3. They were otherwise identical to the wildtype α_1-antitrypsin template sequence, they ideally primed from G or C and were not prone to forming hairpins or primer dimers. PCR mutagenesis was carried out using 2 μL Vent Taq polymerase with 20μL of Vent Taq polymerase buffer, 4 μL of each primer (primer containing mutation and the C terminal primer of the PQE31 vector), 4 μL of wildtype α_1-antitrypsin in PQE31 as a template, 6 μL H_2O and 20 μL of mixed dNTP stock. The PCR reactions were performed in a MJ Research PTC-200 DNA Engine Peltier-cycled PCR block programmed to denature at 95°C for 5 minutes, and then to cycle 25 times through a denaturing step at 95°C for 45 seconds, an annealing step at 50°C for 45 seconds and then an extension step at 72°C for 1 minute. The mixture then underwent a final extension step at 72°C for 5 minutes. The PCR products were cleaned using the QIAgen PCR purification kit. A second PCR was performed using the N-terminal primer of the PQE31 vector and the previously cleaned PCR product, which was used as the second primer. The reaction contained 0.5 μL Vent Taq polymerase with 5 μL of Vent Taq polymerase buffer, 2 μL of the N-terminal primer, 0.5 μL of wildtype α_1-antitrypsin in PQE31 as a template, 3 μL H_2O, 5 μL of mixed dNTP stock and 34 μL of the previously cleaned PCR product. The cycling conditions were identical to that described above. The PCR products were run on a 1% (w/v) agarose/TBE gel (1% ethidium bromide) at 90V for 30 minutes. DNA was visualised by ultraviolet fluorescence of intercalated ethidium bromide and the PCR products were cut out and gel purified using the QIAgen gel extraction kit.

The cleaned DNA was digested at 37°C with 2 μL of each of the restriction enzymes Bam HI and Hind III. PQE31 plasmid containing wildtype α_1-antitrypsin was similarly digested and the restriction products from both digests were gel purified from a 1% (w/v) agarose/TBE/ethidium bromide gel.

The following ligation reactions were then set up:

	Control	**Insert**
Vector backbone (Bam HI/Hind III restricted)	2 μL	2 μL
Bam HI/Hind III restricted PCR product	0 μL	6 μL
Water	6 μL	0 μL
T4 Ligase	1 μL	1 μL
T4 DNA Ligase buffer	1 μL	1 μL
Total	10 μL	10 μL

These were incubated at room temperature for 2 hours. 2 μL of the ligation mixture was transformed into 50 μL *E.coli* suspension by electroporation using 2 mm cuvettes. These were then transferred to a sterile tube and incubated on a shaker at 37°C for 1 hour. After an hour 300 μL was plated on agar plates containing 100mg/mL ampicillin and allowed to grow overnight at 37°C. Single colonies were then picked and two or three colonies grown up. The DNA was prepared and then sequenced to check for the mutation of interest and the integrity of the DNA sequence (Appendix IV).

Preparation of electro-competent *E.coli*

All procedures involving cells were carried out using a strict sterile technique to prevent contamination. Five hundred millilitres of sterile 2xTY was inoculated with a loop of non-transformed SG13009 *E.coli* from an agar plate or 10 μL from pre-existing stocks and grown at 37°C until mid log-phase (OD_{600} of 0.6-0.8). The flask was cooled in iced water and the cells harvested by centrifugation at 4000 rcf for 3 minutes, the cell pellet was then resuspended in 50mL of ice-cold sterile distilled water and centrifuged again at 4000 rcf for 3 minutes. The cells were resuspended in 5mL of ice-cold sterile distilled water and once again centrifuged at 4000 rcf for 3 minutes. The cells were then resuspended in 1mL of 10% (v/v) ice cold glycerol. Aliquots of 50μL were then snap frozen in liquid nitrogen and stored at -70°C.

Electro-transformation

Forty microlitres of electro-competent cells were mixed with 1 to 2 μL of plasmid DNA and placed in a 0.2 cm-gap electroporation cuvette, which was inserted into the chamber slide of a Gene Pulsar. The electroporation parameters used were: 2.5 KV, 25 μF and 200 W, which gave a field strength of 12.5 KV/cm and a time constant of 4.2 to 4.8 ms. Immediately after electroporation the cells were resuspended in 1 mL of 2xTY, transferred into a sterile tube and incubated at 37°C for 30 minutes. One hundred microlitres of each 1 mL sample was plated onto an ampicillin plate and the rest was microfuged at 13 000 rpm for 1 minute, resuspended in 100 μL of sterile

2xTY and plated onto a second ampicillin plate. The plates were then incubated at 37°C overnight.

Protein Expression

A colony of SG13009 *E.coli* transformed with wildtype or mutant α_1-antitrypsin in a PQE31 expression vector was picked from an agar/ampicillin plate and used to inoculate 400 mL of 2xTY/40μg ampicillin which was then incubated at 37°C at 250 rpm overnight. Ten millilitres of this culture was then inoculated into each of 8 litres of 2xTY, 100μg ampicillin/litre, 1litre/shaker flask. The flasks were incubated at 37°C/250 rpm, and cell growth monitored by assessing the OD_{600} of 1mL samples relative to a 2xTY control. Cell growth was considered to have reached early log phase once the OD_{600} was between 0.6-0.9, allowing α_1-antitrypsin expression to be induced in cells without it operating as a negative selection factor. Expression was induced by the addition of IPTG to a final concentration of 1mM and the temperature lowered to 30°C for a further 3 hr. After this the cells were centrifuged down into a pellet in 500mL flat-bottomed centrifuge flasks in a Sorval RC 5B Plus centrifuge, GS3 rotor at 8 000 rpm for 15 minutes. The pellet was then snap-frozen in liquid nitrogen and stored at -70°C until it was required for protein purification as described in chapter 3.

Appendix VI

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS-) PAGE

SDS-PAGE stock solutions

Acrylamide	30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide
Separation gel buffer	1.5M Tris/HCl pH 8.8
Stacking gel buffer	0.75M Tris pH 6.5 with H_3PO_4
10x running buffer	0.25M Tris/HCl, 1.92M Glycine pH 8.8
Sample loading buffer	10mL stacking gel buffer, 2mL glycerol, 0.04% (w/v) bromophenol blue in 20mL of water, 3.0% (w/v) SDS
Stain	0.5% (w/v) Coomassie Blue R250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid in water
Destain	20% (v/v) methanol, 10% glacial acetic acid in water
AMPS	10% ammonium persulphate (w/v) in water
TEMED	NNN'N'-tetramethylethylenediamine

SDS-PAGE Gel mixes

	7.5% (w/v)	10% (w/v)	15% (w/v)	20% (w/v)	Stacking gel, 4.5% (w/v)
Buffer	3.75mL	3.75mL	3.75mL	3.75mL	1.6mL
Acrylamide	3.75mL	5mL	7.5mL	10mL	1.6mL
Water	7.3mL	6mL	3.4mL	0.9mL	10.6mL

10% (w/v) SDS	150µL	150µL	150µL	150µL	150µL
TEMED	20µL	20µL	20µL	20µL	25µL
Sucrose	___	___	2.5g	2.5g	___
10% bromophenol blue	___	___	150µL	150µL	___
10% AMPS	Added as described in text				

SDS-PAGE was carried out in a BioRad Mini Protean II system using a continuous buffer method, according to the method of Laemmli (1970). Gradient gels were poured with acrylamide gradients using a double chamber gradient forming system. The higher percentage acrylamide (w/v) solution was added to the chamber closest to the outlet port and the lower percentage acrylamide (w/v) solution to the other chamber resulting in a gel with the maximum acrylamide concentration at the bottom. The solutions in each chamber (1.75mL) were mixed constantly using a magnetic stirrer, 7.5µL of freshly prepared 10% AMPS solution was added to each chamber and the tap between the two chambers was opened. The acrylamide solutions were then allowed to flow through the tubing attached to the outlet port of the gradient former and a 21-gauge butterfly needle into the glass plates.

The gradient was formed by the gradual mixing of the lower concentration acrylamide solution into the higher concentration chamber as the solution from this chamber ran into the glass plates. In contrast non-gradient gels were poured directly by pipetting 3.5mL of acrylamide solution of the required concentration thoroughly mixed with 15µL of freshly prepared 10% AMPS between the glass plates.

In either case, after pouring, 1mL of water was overlaid and the gel left for 30 minutes at room temperature to polymerise. Once set firm the water overlaying the separation gel was poured off. One and a half millilitres of stacking gel plus 20μL of 10% AMPS was then poured over the separation gel and a well forming comb inserted. Once set the comb was removed, the wells were washed out with water and the gel placed into the running apparatus according to the manufacturers instructions. Running buffer was then added to the top and bottom reservoirs to form a continuous system.

Samples were prepared by adding an equal volume of SDS sample buffer and heating at 95°C for 2 minutes prior to loading onto the gel. The gel was run at a constant voltage of 150V at room temperature until the bromophenol blue marker reached the bottom of the gel. Protein bands were visualised by staining with Coomassie brilliant blue and then destained until the background was clear.

Non-denaturing PAGE

Non-denaturing PAGE stock solutions

Acrylamide	30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide
Separation gel buffer	1.5M Tris/HCl pH 8.9
Stacking gel buffer	0.47M Tris pH 6.9 with H_3PO_4
Anode running buffer	100mM Tris/HCl pH 7.8
Cathode running buffer	53mM Tris/HCl, 68mM Glycine pH 8.2
Sample loading buffer	10mL stacking gel buffer, 2mL glycerol, 0.04% (w/v) bromophenol blue in 20mL of water

Stain	0.5% (w/v) Coomassie Blue R250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid in water
Destain	20% (v/v) methanol, 10% glacial acetic acid in water
AMPS	10% ammonium persulphate (w/v) in water
TEMED	NNN'N'-tetramethylethylenediamine

Gel mixes

	7.5% (w/v)	15% (w/v)	Stacking gel, 5.4% (w/v)
Buffer	3.75mL	3.75mL	1.6mL
Acrylamide	3.75mL	7.5mL	2.7mL
Water	7.3mL	3.5mL	10.6mL
TEMED	11μL	11μL	25μL
Sucrose	—	1.5g	—
10%AMPS	Added as described in text		

Non-denaturing gels were run in the same BioRad Mini Protean II system as SDS gels but using a discontinuous buffer system. Most gels were poured as 7.5-15% (w/v) gradients using a double chamber gradient forming system in the same way as the SDS gels were poured. One millilitre stacking gel plus 20μL of 10% AMPS was then poured over the separation gel and a well-forming comb inserted. Once set, the comb was removed, the wells were washed out with water and the gel was placed into the running apparatus according to the manufacturers instructions. Cathodic running buffer was placed in the top reservoir and anodic running buffer in the lower reservoir.

Seven and a half microgram samples were prepared by adding the appropriate volume of 5x loading buffer. The samples were then loaded into wells and the gels run at 150V until the loading buffer dye front reached the bottom. Protein bands were visualised by staining with Coomassie brilliant blue and destained until background was clear.

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