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In Vitro Studies on Cigarette Smoke-Mediated Damage to Human Alpha-1-Proteinase Inhibitor.

Mark Dennis Evans
Louisiana State University and Agricultural & Mechanical College

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In vitro studies on cigarette smoke-mediated damage to human alpha-1-proteinase inhibitor

Evans, Mark Dennis, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991
IN VITRO STUDIES ON CIGARETTE SMOKE-MEDIATED DAMAGE TO HUMAN ALPHA-1-PROTEINASE INHIBITOR

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biochemistry

by

Mark Dennis Evans
B.Sc., Brunel University, 1985
December 1991
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To Charles, Eunice

& Roger Evans,

and Leonora Daffon.
This dissertation, divided into four chapters, is concerned with the interaction of water soluble cigarette smoke particulate phase components with human alpha-1-proteinase inhibitor. Chapter 1 is a comprehensive introduction covering material from the literature related to the studies in this dissertation. Chapter 2 deals with both the basic features of the "aqueous cigarette tar extract" and the assay of alpha-1-proteinase inhibitor activity. Chapter 3 covers mechanistic studies on the nature of the species in aqueous cigarette tar extract that damage alpha-1-proteinase inhibitor. Chapter 4 presents studies on the structural damage to alpha-1-proteinase inhibitor by aqueous cigarette tar extract. Much of the material in this dissertation has been published or is being prepared for publication in the scientific literature.
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ABSTRACT

The effect, in vitro, of water soluble components of the particulate phase of cigarette smoke on the elastase inhibitory capacity (EIC) of human alpha-1-proteinase inhibitor (α1PI) is studied. Basic features of aqueous cigarette tar extract-mediated damage to α1PI are presented, as are mechanistic studies addressing the identities of damaging species and the nature of damage to α1PI.

Aqueous cigarette tar extract (ACT) causes a slow loss of EIC, and retains this α1PI damaging ability for hours. The damage to α1PI by ACT is dependent on its hydrogen peroxide content. Hydroxyl radicals or hypervalent metal species in the homogeneous phase of ACT do not damage α1PI, which is compatible with their high reactivity and poor selectivity. Hydrogen peroxide accounts for the major portion of the damage to ACT-exposed α1PI, probably by direct oxidation of critical methionyl residues. Free methionine, but not methionine sulfoxide, protects against damage to α1PI by ACT. Also, ACT-exposed α1PI contains appreciable methionine sulfoxide. The α1PI exposed to ACT also undergoes non-oxidative modification to produce a more anionic protein, but such modification does not contribute to the loss of EIC.

Hydrogen peroxide, at concentrations relevant to those produced in ACT, causes a comparable rate of loss of EIC and appreciable formation of
methionine sulfoxide in $\alpha_1$PI. However, hydrogen peroxide does not reproduce the non-oxidative modifications of ACT-exposed $\alpha_1$PI. Although exposures of $\alpha_1$PI to catechol/hydroquinone shows some similarities to ACT, it is generally a poorer model compared to hydrogen peroxide.

Sulfhydryls and ascorbate protect $\alpha_1$PI in ACT due to various factors, such as direct reaction with hydrogen peroxide. Chelators do not protect $\alpha_1$PI completely from ACT-mediated damage, despite many potential roles for metals in the damaging mechanism. The small protection that is afforded by the chelators is probably related only to the inhibition of production, or the degradation of, hydrogen peroxide.
CHAPTER 1

INTRODUCTION

Inhalation of smoke from smoldering plant matter has been practiced for centuries. Tobacco is the plant most commonly associated with smoking, although a host of plant materials have been and continue to be smoked. During his voyages to the New World, Christopher Columbus observed the native Americans indulging in the modern-day habits of smoking and chewing tobacco. The use of tobacco became popular in Europe in the 16th century, aided by the promotional efforts of Sir Walter Raleigh. Also during this time, Seville in Spain became a center for an early tobacco industry, where quality cigars were produced. It was probably in Seville that the name "cigarette" first originated from the use of cigar scraps to make a poor man's substitute for relatively expensive cigars. However, the modern concept of a cigarette as tobacco rolled up in paper probably started in Central America, subsequently spreading to the Southwest United States in the early 1800s. During the 19th century the popularity of cigarettes grew, and the invention of the first cigarette-making machine in 1881 by James Bonsack of Virginia meant that the cigarette industry could develop into the multi-million dollar industry it is today. The profits to be reaped from tobacco were recognized by the
British Crown during the 17th and 18th centuries. The resulting erosion of tobacco growers' profits in the Americas, by the declaration that tobacco was a royal monopoly, undoubtedly contributed to the ultimate loss of Britain's American colonies in 1776. The financial benefits gained by industry and government from the use of tobacco, however, have been outweighed by the detrimental health effects suffered by many tobacco consumers. The harmful effects of tobacco use are the basis for the studies in this dissertation.

1.1 The Physical and Chemical Nature of Cigarette Smoke.

Throughout this dissertation, the terms "smoke" and "cigarette smoke" refer exclusively to smoke derived from burning tobacco. Cigarette smoke is a dynamic system composed of an electrically neutral aerosol of liquid particles suspended in a mixture of primarily atmospheric gases. The average diameter of the particles in fresh smoke is about 0.15 - 0.16 μm. After 1 s of aging the diameter of the particles can be as large as 0.4 μm, due to particle coalescence (1,2). Smoke, with its particle concentration of approximately 1.3 x 10^{10}/cm^3 (3), is a highly concentrated aerosol, more so than many other types of environmental pollutant. Since the average cigarette "puff volume" is 35 cm^3, a smoker inhales about 4.6 x 10^{11} particles per puff.
1.1.1 Sidestream smoke.

Sidestream smoke is the smoke that issues from the smoldering coal end of the cigarette. Numerous components of mainstream smoke, many of which are toxic, are present at substantially greater concentrations in sidestream smoke (4). Thus, passive smokers also are exposed to a significant burden of toxicants by inhalation of environmental tobacco smoke (4).

1.1.2 Mainstream smoke.

Mainstream cigarette smoke exits from the butt-end of a cigarette as air is drawn over the firecone during puffing. The enormous complexity of compounds in cigarette smoke results from a combination of distillation, pyrolysis and combustion of tobacco material at temperatures up to 900 °C in an oxygen deficient, hydrogen rich atmosphere. Dube and Green (3) have presented data showing that there are 3800 identified compounds in tobacco smoke. The most studied portion of cigarette smoke (gas and particulate phases) comprises < 20% w/w of the whole smoke, the rest is nitrogen, oxygen and carbon dioxide (3,5).

In many cases both mainstream and sidestream smoke are subdivided into gas and particulate phases for both chemical analyses and biological studies. The gas (or vapor) phase of cigarette smoke is defined as that portion of the cigarette smoke that passes through a glass fiber filter rated to remove 99.9% of all particles ≥ 0.1 μm in diameter, i.e., a
Cambridge filter (6). The smoke phase collected on the filter is the particulate phase. Also, a "semivolatile phase" has been proposed by Williamson et al. (7), which is composed of material that is volatilized at 180-190 °C from particulate matter on a Cambridge filter.

The Cambridge filter is probably the most widely used device for collecting cigarette smoke components. Cambridge filters effectively separate the gas phase from the particulate phase components, but there are components common to both phases. Components of the gas phase that are hydrophilic can deposit on the filter as it becomes wetted with the collection of particulate matter (6). Generally, the collection of cigarette smoke on a Cambridge filter at room temperature does not result in the formation of artifacts (3). However, artifacts arise as a necessary consequence of the aging of smoke and these could be important in a biological context. Such artifacts have been of particular interest to the Pryor research group, as is evident from the results presented in this dissertation and earlier work (8-11).

1.1.3 Gas phase smoke.

Table 1 shows some of the major classes of compounds in gas phase cigarette smoke along with the approximate quantity of the largest representative. Some of the compounds which are of interest from the point of view of smoke toxicology are also noted.
### Table 1. Major components of gas phase cigarette smoke.

<table>
<thead>
<tr>
<th>Class</th>
<th>Largest Representative, μg/cig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Various C compounds</td>
<td>Carbon monoxide, 10000-23000</td>
</tr>
<tr>
<td>Various N compounds</td>
<td>Nitric oxide, 50-600</td>
</tr>
<tr>
<td></td>
<td>Ammonia, 50-170</td>
</tr>
<tr>
<td></td>
<td>Nitrosamines, 0.0005-0.2</td>
</tr>
<tr>
<td>Alkanes</td>
<td>Methane, 800</td>
</tr>
<tr>
<td>Alkenes</td>
<td>Isoprene, 400</td>
</tr>
<tr>
<td>Alkynes</td>
<td>Acetylene, 25</td>
</tr>
<tr>
<td>Aromatic Hydrocarbons</td>
<td>Toluene, 80</td>
</tr>
<tr>
<td></td>
<td>Benzene, 20-50</td>
</tr>
<tr>
<td>Halogenated Hydrocarbons</td>
<td>Methyl chloride, 160</td>
</tr>
<tr>
<td></td>
<td>Vinyl chloride, 0.0013-0.016</td>
</tr>
<tr>
<td>Alcohols</td>
<td>Methanol, 180</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Acetaldehyde, 500-1200</td>
</tr>
<tr>
<td></td>
<td>Acrolein, 50-100</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde, 50-100</td>
</tr>
<tr>
<td>Ketones</td>
<td>Acetone, 350</td>
</tr>
<tr>
<td>Nitriles</td>
<td>Hydrogen cyanide, 150-300</td>
</tr>
<tr>
<td></td>
<td>Acetonitrile, 140</td>
</tr>
</tbody>
</table>

*a* - Data from Refs 5 and 12.
These include ciliatoxic agents, *e.g.* HCN, acrolein and acetaldehyde, and carcinogens, *e.g.* benzene, formaldehyde and vinyl chloride.

1.1.4 *Particulate phase smoke.*

There are distinctions between total particulate matter (TPM; the abbreviations used throughout this dissertation are listed in Appendix A), "tar" and condensate. The TPM is the particulate material collected on a Cambridge filter using the "puff-protocol" smoking method. The puff-protocol is based on patterns noted for smokers and is a standard method of smoking cigarettes for analytical purposes. The protocol consists of puffs of $35 \pm 0.5$ mL drawn over a period of $2 \pm 0.2$ s at intervals of $60 \pm 1$ s (13). "Tar" is defined by the Federal Trade Commission as the weight of TPM minus the weight of water and nicotine (13). Cigarette smoke condensate is whole smoke collected in a low temperature trap, or bubbled through an aqueous buffer. The term "condensate" is frequently used incorrectly in the literature to refer to TPM or tar.

The particulate phase is much more chemically complex than the gas phase. Table 2 shows a typical composition for the major groups of compounds in the particulate phase. The particulate phase has been studied closely with respect to its co-carcinogenic and carcinogenic properties (discussed in section 1.2).
**Table 2.** Major groups of chemical compounds in the particulate phase of cigarette smoke.a.

<table>
<thead>
<tr>
<th>Class</th>
<th>Quantity, mg/cig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humectants</td>
<td>3.0</td>
</tr>
<tr>
<td>Nicotine/nornicotine</td>
<td>1.6</td>
</tr>
<tr>
<td>Leaf pigment</td>
<td>1.5</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>1.5</td>
</tr>
<tr>
<td>Carboxylic acids</td>
<td>1.2</td>
</tr>
<tr>
<td>Waxes</td>
<td>1.12</td>
</tr>
<tr>
<td>Phenols</td>
<td>0.6</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>0.5</td>
</tr>
<tr>
<td>Amides</td>
<td>0.1</td>
</tr>
<tr>
<td>Metals</td>
<td>0.1</td>
</tr>
<tr>
<td>Aliphatic amines</td>
<td>0.065</td>
</tr>
<tr>
<td>Polyaromatic hydrocarbons</td>
<td>0.005</td>
</tr>
<tr>
<td>Naphthalenes</td>
<td>0.004</td>
</tr>
<tr>
<td>Nitro compounds</td>
<td>0.004</td>
</tr>
<tr>
<td>Nitrosamines</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

a - Data from (5).
1.1.5 Free radical chemistry of gas phase and particulate phase cigarette smoke.

1.1.5.1 Gas phase smoke. Early studies of the radicals in cigarette smoke used electron spin resonance spectroscopy (ESR) at liquid oxygen temperatures (14). Spin trapping studies, indicating that alkoxyl radicals are a major radical species in cigarette smoke, were performed as early as 1971 (15). The results from more thorough spin trapping studies of gas phase smoke radicals suggest that carbon centered radicals generated from tobacco combustion are the precursors for alkoxyl radicals (16-18). Also, the fact that the concentration of radicals increases as the smoke ages, and that this closely parallels the formation of nitrogen dioxide, offers an explanation for the apparent longevity of gas phase smoke radicals (17,18). It has been suggested that nitrogen dioxide formation is related to a steady-state production of radicals in gas phase smoke (17,18).

The free radical chemistry of gas phase smoke has been modelled using a mixture of NO/air/isoprene (18,19). This gas phase smoke model produces both the carbon and oxygen centered radicals detected in the studies of authentic smoke and involves two other gas phase radicals, namely nitric oxide and nitrogen dioxide. However, the mechanisms proposed for the formation of the radicals in this model do not necessarily exclude other mechanisms for radical production in gas phase smoke (19).
Reactions pertinent to radical chemistry of gas phase smoke are shown in equations 1 to 7 (17-19).

\[
\begin{align*}
2 \text{NO} + \text{O}_2 &\rightarrow 2 \text{NO}_2 \\
\text{NO}_2 + \underset{\text{C=C}}{\text{C}} &\rightarrow \text{O}_2N\underset{\text{C=C}}{\text{C}} \\
\text{R}^* + \text{O}_2 &\rightarrow \text{RO}_2^* \\
\text{RO}_2^* + \text{NO} &\rightarrow \text{RO}^* + \text{NO}_2 \\
\text{RO}_2^* + \text{NO}_2 &\rightarrow \text{RO}_2\text{NO}_2 \\
\text{RO}^* + \text{NO}_x &\rightarrow \text{RO-NO}_x \\
\text{R}^* + \text{NO}_x &\rightarrow \text{R-NO}_x
\end{align*}
\]

\(\text{NO}_x = \text{NO} \text{ or } \text{NO}_2.\)

1.1.5.2 Particulate phase smoke. Studies in the late 1950s and early 1960s on the so-called "cigarette tar radical" suggested that it consisted of an odd-electron delocalized over a polynuclear aromatic hydrocarbon structure (14,20). Later, in the early 1980s, Pryor and his coworkers completely revised this previously unchallenged concept of the tar radical (17,18,21). The studies reported by Pryor's group involve a direct observation of the radicals by ESR and suggest that the principal "tar radical" consists of hydroquinone/semiquinone/quinone units held in a "tarry matrix" (18,21). Since cigarette smoke is rich in polyphenols it is reasonable that semiquinones are present, either alone or as part of a polymeric framework. Data from ESR and a series of chemical tests
revealed that the tar radical contains mainly o-semiquinone groups and shares similarities with melanin, another polymer rich in o-semiquinones.

The addition of base to alcoholic extracts of particulate phase smoke results in the appearance of strong p-benzosemiquinone radical signals (17). The relative abundance of hydroquinone in cigarette smoke is compatible with this observation.

Spin-trapping studies have been applied to aqueous buffer extracts of the particulate phase (22). Three spin adducts resulting from hydroxyl, alkyl and carbon dioxide radical anions are detectable, and their formation is dependent on hydroxyl radical production in the extracts. The hydroxyl radicals are thought to arise from metal-assisted decompositions of hydrogen peroxide. The hydrogen peroxide in turn is generated by autoxidation of polyphenols in the extracts (22-25). Thus, the abundant polyphenols in cigarette smoke could be the ultimate source for all the free radical species detected in the particulate phase.

1.2 Cigarette Smoking as a Major Health Threat.

A number of disease states are causally related to tobacco smoking. The three major pathological conditions associated with smoking are cardiovascular disease, cancer and chronic obstructive pulmonary disease (26). These are probably the major causes of morbidity and mortality in the developed world (27). Smoking is also associated with retardation of
fetal growth, increased neonatal mortality, peptic ulcer disease and allergic immune reactions (26). Non-smokers, due to passive smoking, are also at risk to the possible detrimental health effects of environmental tobacco smoke.

Cigarette smoke is thought to contribute to cardiovascular disease by increasing carboxyhemoglobin levels in smokers’ blood (28). Chronic exposure to carbon monoxide, at levels which are likely to be experienced by smokers, causes arterial wall injury and alterations in lipid metabolism leading to hypercholesterolemia (28). Chronic exposure to carbon monoxide also leads to myocardial degeneration due to stress placed on the heart as more blood is pumped to overcome oxygen deficiency (28).

Free radicals in smoke, formed either directly or through aging of smoke components, have been proposed to be involved in some cardiovascular disease events. Smoke radicals may modify low density lipoprotein and transform macrophages into lipid laden foam cells (29). Also, smoke components can alter the plasma antioxidant status to increase lipid peroxidation which could then become an important risk factor in the initial events of atherosclerotic plaque formation (30).

The relationship between tobacco smoking and cancer has been appreciated for at least 40 years. The development of lung cancer is known to be causally related to cigarette smoking; an increased incidence of lung tumors correlates with smoke dosage (31). The advent of "lower
tar" cigarettes has reduced the incidence of smoking-related lung cancer, however, the frequency of lung cancer among smokers is still greater than that observed for non-smokers (31). Cigarette smoking is also causally related to oral cancer, cancer of the larynx, esophagus, bladder, kidney and pancreas (31).

The carcinogenic activity of cigarette smoke resides primarily in the particulate phase. Most of the compounds involved in the induction and maintenance of malignancy are contained in the neutral portion of particulate phase material fractionated by a series of extractions using acidic, neutral or basic solvents (12). The potent carcinogenic fractions contain mainly polynuclear aromatic hydrocarbons, which show tumor initiation in skin tests in the presence of co-carcinogenic fractions from the particulate phase (12). The major co-carcinogen and phenolic compound in cigarette smoke is catechol. Cigarette smoke also assists in the metabolic activation of some of these carcinogens by increasing aryl hydrocarbon hydroxylase activities in the lung and other tissues (32).

Cigarette smoke contains organ-specific carcinogens, such as primary amines (bladder carcinogens), thus explaining the ability of smoking to induce cancers at sites other than the lung. The involvement of active oxygen species in the carcinogenic/DNA damaging activity of cigarette smoke has been proposed on the basis of studies in vitro and in vivo (25,33-36).
1.3 The Pulmonary Absorption and Deposition of Cigarette Smoke.

The deposition and clearance of cigarette smoke material in the lung is of prime importance with regard to its toxicity. Many of the volatile, water-soluble components of cigarette smoke (i.e., gas phase components) are absorbed in the oropharyngeal region of the respiratory tract (37). However, this does not mean that gas phase smoke has no role in pulmonary injury (38,39).

Particle size is the major determinant of deposition patterns in the lung. Since cigarette smoke particulates are small and have low volatility they can penetrate deep into the pulmonary region of the respiratory tract (40-43). The three major mechanisms for particulate deposition, viz., sedimentation, diffusion and inertial impaction, operate in the pulmonary region. Impaction is the primary deposition mechanism in the nasopharyngeal and tracheobronchial region. The sites of development of bronchocarcinoma and smoking induced emphysema are located at bifurcations of the terminal bronchioles (44-46), which are also the primary sites for the deposition of cigarette smoke particulates (44-46).

Different mechanisms for the clearance of foreign material from the lungs operate depending on the region of the respiratory tract involved. The particulate burden to distal parts of the lung is increased during cigarette smoking since the protective mechanisms in the nasopharyngeal
region (nose hairs and impaction in nasal mucus) are bypassed. However, particles can impact in mucus in the oropharyngeal and tracheobronchial regions. The mucus lining forms part of a "mucociliary escalator", in which the mucus is propelled upward along with entrapped particles to the oropharyngeal region where the mucus is removed by expectoration and swallowing.

Approximately 30-40% of cigarette smoke particulate matter is deposited in the alveolar region where the mucociliary escalator does not function (40). Alveolar clearance involves dissolution of the particles and engulfment by alveolar macrophages (AM), which then exit the lungs via the mucociliary escalator. Unfortunately, particulate clearance mechanisms are impaired in chronic smokers, thus exacerbating the toxic effects of cigarette smoke (40,47).

1.4 Protein Inhibitors of Proteinases.

There are at least four ways of controlling extracellular proteolysis in vivo: a) regulation of proteinase synthesis; b) control of proteinase release; c) zymogen activation; d) inhibition by antiproteinases. Antiproteinases, a class of protein found in plants, animals and microorganisms, function as protein inhibitors of proteolysis. By presenting a scissile peptide bond in an idealized structural environment, an antiproteinase can prevent a proteinase from hydrolyzing its normal substrate. Although the exact function of
some antiproteinases is uncertain this is not the case for mammalian blood plasma antiproteinases which constitute about 10% of the total plasma protein (48).

1.4.1 Alpha-1-proteinase inhibitor and the Serpins.

There are several families of antiproteinase which are categorized according to the mechanistic class of proteinase they inhibit. One family of proteinase inhibitors is called the serpin (serine proteinase inhibitor) superfamily (49,50). Members of the serpin superfamily have common functional domains and tertiary structural features; they are single chain glycoproteins stabilized by internal Glu--Lys salt bridges and form 1:1 complexes with their targets (51). One of the most familiar serpins is bovine pancreatic trypsin inhibitor (BPTI, Kunitz inhibitor), a favorite subject of protein biophysical studies.

The susceptible peptide bond directed toward the proteinase target is termed the "reactive site" (52). By convention, the reactive site residues are numbered leading away from the scissile bond so that \( P_1 \) and \( P'_1 \) correspond to the first residues in the N- and C- terminal directions, respectively (53). The specificity of the inhibitor is determined by the nature of the \( P_1 \) residue, e.g. if \( P_1 \) is Arg or Lys, then the inhibitor is an antitrypsin, however if \( P_1 \) is Tyr or Phe, then the protein is an antichymotrypsin.
The kinetic parameter most commonly used to describe proteinase-antiproteinase interactions is the association rate constant \( (k_{\text{assoc}}) \), which has values measuring less than the diffusion controlled rate. The higher the \( k_{\text{assoc}} \) for a particular proteinase the more likely that proteinase is to be a physiological target (54). The corresponding dissociation rate constant \( (k_{\text{dissoc}}) \) for decomposition of the proteinase-antiproteinase complex is usually very low \( (< 10^{-6}.s^{-1}) \) for effective inhibitors. Another parameter, applied to the in vivo situation, is the delay time of inhibition \( [d(t)] \), i.e., the (> 97%) time required for a given amount of antiproteinase to completely inhibit an equivalent amount of proteinase (54). Calculation of \( d(t) \) is dependent on knowledge of the \( k_{\text{assoc}} \) and of the physiological concentration of the antiproteinase. So, for an antiproteinase to be an effective inhibitor, reaction with the target must be fast and essentially irreversible (54).

Alpha-1-proteinase inhibitor (\( \alpha_1 \)PI), also known as alpha-1-antitrypsin, is the major serum antiproteinase in humans; it also acts as an antiproteinase in various tissues, notably in the lung (48). The antiproteinase function of \( \alpha_1 \)PI was first detected by its ability to inhibit trypsin. However, neutrophil elastase (NE) is the primary target for \( \alpha_1 \)PI; the \( k_{\text{assoc}} \) value for \( \alpha_1 \)PI interaction with human NE is \( 6.5 \times 10^7 \text{M}^{-1}.\text{s}^{-1} \) (55). \( \alpha_1 \)PI has been a subject of intensive investigation, partly because it is the major serum antiproteinase and also because the genetically determined deficiencies of \( \alpha_1 \)PI are associated with a number of diseases.
1.4.1.1 *Alpha,Pl* protein structure and biosynthesis. Figure 1 is a schematic representation of the major structural features of *α,PI*. *α,PI* is a single glycosylated polypeptide chain with 394 amino acids and has a molecular weight of 52 ± 1 kDa (51). *α,PI* contains no intrachain disulfide bonds, but has a single cysteinyl residue which exists as a free thiol or in a disulfide linkage with cysteine or glutathione (56). The folded protein is globular with dimensions of 6.7 x 3.2 nm; N-linked carbohydrate chains are located externally at one end of the molecule with the reactive site at the other end (57). The carbohydrate chains have a common core composition, with "antennae" of identical composition attached to this core to give to bi- or tri-antennary structures (48,58,59). The carbohydrate composition of *α,PI* varies depending on the isoform of *α,PI* (51). The folded molecule contains 30% α-helix (9 α-helices) and 40% β-sheet (3 β-pleated sheets) (57). There are two intramolecular salt bridges, both of which stabilize the protein structure and are involved in the common deficiency mutations (57). The N-terminal residue is glutamate and the C-terminal residue is lysine (48). *α,PI* is negatively charged at physiological pH (pI ca. 4.5). Also, Lys and Arg residues are concentrated at the reactive site end of *α,PI* and the acidic residues and carbohydrate chains at the opposite end, thus the molecule possess a notable dipole moment (57).
Figure 1. A schematic diagram of the major structural elements of the human $\alpha_1$PI protein. $\square$, $\alpha$-helix; $\langle\rangle$, $\beta$-sheet; CHO, carbohydrate; ---, salt bridge. (Reproduced and modified from Ref. 60 with permission of the American Society for Clinical Investigation).
The reactive site sequence is shown below (61), with the susceptible peptide bond (Met$^\text{358}$--Ser$^\text{359}$) presented to the target as part of an exposed loop:

\[
P_4 \quad P_3 \quad P_2 \quad P_1 \quad P'_1 \quad P'_2 \quad P'_3 \quad P'_4
\]

Ala Ile Pro Met$^\text{358}$ Ser$^\text{359}$ Ile Pro Pro

This reactive site loop structure places the native $\alpha_1$PI molecule in a stressed conformation, as shown by a 6.7 nm separation between Met$^\text{358}$ and Ser$^\text{359}$ in reactive site cleaved $\alpha_1$PI (57). The presence of a hydroxylated amino acid at $P'_1$ in $\alpha_1$PI is characteristic of serpins, suggesting an important function (50). The $P'_1$ residue is not directly involved in interaction of the serpin with the target proteinase but ensures correct folding of the antiproteinase into the conformationally active state (62).

There are approximately 75 known normal alleles of the $\alpha_1$PI gene (60), and within the general population there are at least twenty isoforms of $\alpha_1$PI (48). Classification of these forms is based on electrophoretic mobility, a system called the "Pi system". The commonest genotype of $\alpha_1$PI is Pi$^\text{M}$ and so Pi$^\text{MM}$ represents the normal homozygous phenotype (48). In addition to the genetic diversity, the serum of a Pi$^\text{MM}$ individual contains five isoforms of $\alpha_1$PI due to post-translational modifications (63). These
five isoforms arise due to differing structural arrangements of the three carbohydrate side-chains and differing polypeptide chain length (63). The carbohydrate side-chains play no role in the proteinase inhibitory action of α1PI, but their removal increases the thermal lability of the protein, diminishes excretion of α1PI by cells and reduces the plasma half-life of the protein (64-66).

Alpha1PI is synthesized primarily in the hepatocytes (48,63); this synthesis in turn regulates the serum levels of α1PI and hence of the lung (63). Neutrophils (PMN) and macrophages synthesize small amounts of α1PI (51). Despite the importance of α1PI and its substantial increase in concentration in the serum during systemic inflammatory responses, the mechanisms involved in controlling up-regulation of the α1PI gene are unknown (51).

1.4.1.2 Alpha1PI inhibitory mechanism. The generally accepted mechanism of action of antiproteinases is as follows:

\[ E + I \rightleftharpoons EI \rightleftharpoons C \rightleftharpoons EI' \rightleftharpoons E + I' \]  

where, E is the proteinase target; I is the antiproteinase; C is a stable proteinase-antiproteinase complex and I' is the cleaved antiproteinase (52). In "C" the P₁-P'₁ bond is intact, although the carbonyl group of the scissile peptide bond shows some tetrahedral character (52,67). This tetrahedral distortion is induced by interaction of the reactive site with the oxyanion hole and the O' of the proteinase active site seryl residue (52).
Members of the $\alpha_1$PI family have an inhibitory mechanism different from other serpins in that the cleaved antiproteinase is unable to combine with the proteinase (48,68,69):

$$E + I \xrightleftharpoons[k_{-1}]{k_1} EI \xrightarrow[k_{-2}]{k_2} C \xrightarrow[k_{-3}]{k_3} EI^* \xrightarrow[k_{-4}]{k_4} E + I^*$$ (9)

The $k_{-\text{sec}}$ is always less than expected for a diffusion controlled process, suggesting that the inhibition reaction is more complex than a simple bimolecular encounter (70). A proposed reaction profile for serpin-proteinase interaction shows docking of the two partners is a two-step process to give the stable EI complex (C) lying in a deeper free energy trough compared to the corresponding proteinase-substrate complex (68). Consequently, the free energy of activation required for hydrolysis of the reactive site peptide bond is large, and thus a "good" inhibitor is not cleaved. The structural elements of the reactive site loop that make $\alpha_1$PI an inhibitor and not just a good substrate are still unknown. Since $k_1$ and $k_2$ in equation 9 have finite rates, $\alpha_1$PI is strictly a reversible inhibitor. But the values of $k_1$ and $k_2$ are so low that $\alpha_1$PI is usually termed a "pseudo-irreversible inhibitor" (68,71,72). In a physiological context the back reactions are of no importance since proteinase-antiproteinase complexes are cleared much faster from the circulation than the rate of complex dissociation (68).
The complex between $\alpha_1$PI and its target proteinase is stable enough to imply a covalent linkage between the two, although a complex containing an acyl-enzyme intermediate or a "relaxed" inhibitor is considered unlikely (68,69). Usually NE remains attached to $\alpha_1$PI in a highly stable complex (48). Cleavage of the reactive site peptide bond of $\alpha_1$PI yields a 47.8 kDa N-terminal fragment and a 36 residue, 4.2 kDa C-terminal fragment that remains tenaciously bound to the larger fragment under physiological conditions (48). A number of human serpins modified by reactive site cleavage exhibit conformational changes resulting in a large increase in conformational stability (73). The increased stability of $\alpha_1$PI after cleavage may be due to the completion of a 5 strand $\beta$-sheet using the reorganized reactive site loop in the cleaved inhibitor (57,69,74).

Smaller proteinase inhibitors, such as BPTI, can undergo reactive site cleavage, but this leads to a destabilization of the tertiary structure (73). These smaller inhibitors can also undergo reactive site peptide bond resynthesis by proteinases (73). A comparison of the solution structure of the native and cleaved forms of $\alpha_1$PI shows no evidence of gross "global" conformational changes (74). Thus, the local changes induced by cleavage result in relatively minor gross structural changes in the $\alpha_1$PI.

The cleavage of serpins is accompanied by a complete loss of inhibitory activity and such cleavage in vivo usually arises from abortive interactions with proteinases. The reactive site peptide bond in $\alpha_1$PI is also
susceptible to cleavage by proteinases that are not inhibited by $\alpha_1$PI (48).
This proteolytic action may be a physiological mechanism to control
proteolytic activity by inactivating the inhibitor.

1.4.1.3 The catabolism of $\alpha_1$PI-proteinase complexes. Uncomplexed $\alpha_1$PI
has a plasma half-life in humans of 4-5 days (60). The unglycosylated or
asialoglycoprotein has a somewhat shorter half-life, measured in terms of a
few hours (60,65).

Serpin-proteinase complexes are cleared from the circulation via
receptors on hepatocytes (75,76). This clearance is independent of the
identity of the serpin (76,77). Structural features of the serpin revealed
due to conformational changes induced by interaction with the proteinase
target are probably the basis for recognition of serpin-proteinase complexes
by receptors (77,78).

The $\alpha_1$PI-NE complex and reactive site cleaved-$\alpha_1$PI are not
biologically inert entities destined simply to be degraded. There are
receptors for the $\alpha_1$PI-NE complex on AM and other cells. These receptors
not only serve as a binding site for catabolism of the complexes but may
also be associated with signal transduction mechanisms for increasing the
expression of the $\alpha_1$PI gene (79). The cell surface receptor recognizes a
pentapeptide sequence which is revealed when $\alpha_1$PI forms a complex with
the target or when $\alpha_1$PI is proteolytically modified (80,81). While the
native $a_1$PI inhibits PMN migration (82). $a_1$PI-NE complexes and reactive site-cleaved-$a_1$PI are chemotactic agents for PMN (83,84).

1.5 Lung Proteinases, Antiproteinases and Connective Tissues.

1.5.1 Antiproteinases.

Alpha $a_1$PI is present at similar concentrations in epithelial lining fluid (ELF) throughout the lung, and constitutes > 90% of the anti-NE activity in the ELF of the lower respiratory tract (51,85,86). The highest concentration of $a_1$PI is detected in the blood plasma. The concentrations in the alveolar interstitial spaces and alveolar ELF are about 50-70% and 10%, respectively, of the plasma concentration (51). Although $a_1$PI represents the major lung antiproteinase, there are other antiproteinases present. Table 3 provides details of the major antiproteinases relevant to the lung.

1.5.2 Proteinases.

Neutrophils and AM are the major sources of lung extracellular proteinases. These proteinases are generally of the serine- and metalloproteinase mechanistic class (87). The PMN are undoubtedly the major source of lung extracellular proteinases, capable of releasing NE, cathepsin G, collagenase and gelatinase (87-91). All of the PMN proteinases are stored in an inactive form in three types of granule within the PMN cytoplasm (87). Release of the proteinases into the extracellular
### Table 3. Major antiproteinases relevant to the lung.

<table>
<thead>
<tr>
<th>Antiproteinase</th>
<th>MW (kDa)</th>
<th>Proteinase Target</th>
<th>Plasma conc. (μM)</th>
<th>ELF conc. (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1PI</td>
<td>52</td>
<td>NE, Cathepsin G</td>
<td>20 - 53</td>
<td>2 - 6</td>
</tr>
<tr>
<td>SLPI</td>
<td>12</td>
<td>NE, Cathepsin G</td>
<td>0.004-</td>
<td>1.0 - 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>α1AChy</td>
<td>68</td>
<td>Cathepsin G</td>
<td>6 - 8</td>
<td>0.5 - 1.0</td>
</tr>
<tr>
<td>TIMP</td>
<td>29</td>
<td>Collagenases</td>
<td>0.03</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2M</td>
<td>720</td>
<td>All classes of proteinase</td>
<td>2.5 - 5.0</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*a - Reproduced and modified with permission from Ref. 51.

*b - Abbreviations: α1AChy, alpha-1-antichymotrypsin; α2M, alpha-2-macroglobulin; ELF, epithelial lining fluid; NE, neutrophil elastase; SLPI, secretory leukoproteinase inhibitor; TIMP, tissue inhibitor of metalloproteinases.
medium takes place by three main modes: a) leakage from phagocytic vacuoles during particle engulfment; b) degranulation in response to an inflammatory stimulus, and c) cell death (87).

Neutrophil elastase is the most important proteinase both with regard to \( \alpha_1 \text{PI} \) and the destruction of the lung connective tissue matrix during pathological conditions (87). However, NE is essential for the antimicrobial function of PMN. The NE content of human PMN varies between individuals. Individuals with genetically determined higher levels of NE in the PMN are at a greater risk for developing lung disorders such as emphysema (92,93).

Human neutrophil elastase (HNE) is a cationic, globular, 30 kDa glycoprotein consisting of a single polypeptide chain with 220 amino acid residues, four disulfide bridges and two Asn-linked carbohydrate chains (91,94-96). Human NE derives its name from its ability to digest elastin, but it will hydrolyze appropriate peptide bonds exposed on other extracellular proteins (87). The substrate binding pocket of HNE is hemispherical, relatively hydrophobic and constricted at the base by bulky residues (96). The binding pocket is suited to accommodate residues with small aliphatic side chains; HNE preferentially hydrolyses peptide bonds with Val in the \( P_1 \) position (91). Elastin is abundant in residues such as Ala and Val (96). The HNE substrate binding site can also interact with, and hydrolyze, peptide bonds with Met in the \( P_1 \) position, e.g. Met\(^{358} \) of \( \alpha_1 \text{PI} \).
However, oxidation of Met\textsuperscript{358} in \(\alpha_1\)PI to the sulfoxide results in severe steric hinderance to binding (96), which explains the 2000-fold decrease in \(k_{\text{cat}}\) for HNE interaction with native \(\alpha_1\)PI vs. \(\alpha_1\)PI containing methionine sulfoxide at position 358 (MetSO\textsuperscript{358}) (55,72,91). Alpha\(_1\)PI containing MetSO\textsuperscript{358} is much poorer at inhibiting elastolysis than is the native inhibitor (72). Also, the HNE-\(\alpha_1\)PI complex formed from \(\alpha_1\)PI containing MetSO\textsuperscript{358} is less stable than that formed from native \(\alpha_1\)PI (71,72).

The mechanism of catalysis by HNE typifies that of the serine proteinases with regard to the so-called "catalytic triad" of Ser, His and Asp (96). The His residue acts as a proton acceptor from the nucleophilic Ser in the active site, and development of positive charge on His is stabilized by the Asp (97). The development of negative charge on the carbonyl oxygen of the scissile peptide bond during nucleophilic attack of Ser-\(\text{O}^-\) is stabilized by hydrogen bonding interactions with main chain -NH groups in the "oxyanion hole" (97).

Alveolar macrophages are also a source of cathepsins and various metalloproteinases. Although elastinolytic activity \textit{in vitro} has recently been described for AM, their role in extracellular proteolysis is poorly defined as compared to PMN (87,98-101).
1.5.3 Connective tissues.

The pulmonary interstitium is that region of lung tissue that lies between the lung epithelial cell layer and the endothelial cell layer of the capillaries. The pulmonary interstitium is composed of basement membranes associated with the respective epi- and endothelial cell layers and also various cell types and a continuous fibrous protein network. The structural integrity of the interstitium is provided by several connective tissue matrix components, the major elements being collagen, elastic fibers, fibronectin, proteoglycans and basement membranes. All of the various connective tissue components are integrated into a large structure encompassing the lung. This integrated structure is dynamic since many of the components are continually synthesized and degraded.

1.5.3.1 Elastic fibers. [The information concerning elastic fibers is derived from Ref. 102]. Elastic fibers are present in most organs of the body, where reversible extensibility or deformability are required, particularly the lungs and arteries. These fibers are composed of two structural elements; elastin and microfibrils. Elastin is an insoluble, hydrophobic, cross-linked macromolecule which forms a three-dimensional network and gives elastic fibers their elastic recoil properties. The microfibrils consist of highly glycosylated proteins. Microfibrils act as nucleation sites for the development of elastic fibers; elastin synthesis is at its most intense during the late fetal/early postnatal stages of development, resulting in lifelong
elastin deposition patterns that are complete by the end of the first decade of life. Since most of the elastic fiber framework is established early in the development of the lung, and because minimal elastin synthesis occurs in adult lung, destruction of elastic fibers is essentially an irreversible process.

Tropoelastin, the monomeric precursor of functional elastin, is synthesized in myofibroblasts as a 70 kDa protein which is secreted and readily incorporated, under cellular guidance, into growing elastic fibers. Tropoelastin consists of alternating hydrophobic and cross-linking domains, and extensive intra- and intermolecular cross-linking of tropoelastin molecules yields mature elastin. Cross-linking is initiated by oxidation of selected Lys residues to aldehydes (allysine), catalyzed by lysyl oxidase. Cross-links are formed in three main ways: a) aldol coupling of two allysines; b) Schiff’s base formation between Lys and allysine followed by reduction to lysinorleucine; c) desmosine formation from the reaction of four Lys side-chains. Elastin is also rich in Gly and there is a high proportion of small chain aliphatic amino acids such as Ala, Val, Leu and Ile. Whereas tropoelastin is sensitive to proteolytic degradation before incorporation into the elastin macromolecular structure, mature elastin is very stable.
1.6 $\alpha$,PI, Emphysema and Cigarette Smoking.

Emphysema is a chronic lung disease that is characterized by abnormal and permanent enlargement of the respiratory regions of the lung distal to the terminal bronchioles (46,103). This enlargement is frequently accompanied by connective tissue destruction (46,103). There are two major forms of emphysema: a) panacinar emphysema, which develops uniformly throughout alveoli in the distal part of the acinus; b) centriacinar emphysema, which is confined to the central region of the acinus (46,103). Centriacinar emphysema is the more commonly observed form of the disease, and is associated with cigarette smoking (46,104,105).

Emphysema develops as a result of excessive proteinase burden to the alveolar interstitium (106-109). The proteinases are derived primarily from PMN, although AM could also play a role (100,106). Individuals with an inherited deficiency of plasma $\alpha$,PI are at greater risk for developing emphysema, thus $\alpha$,PI function is suggested to be intimately associated with the pathogenesis of the disease (110). Also, since the function of $\alpha$,PI is to inhibit NE, then NE is probably the major destructive proteinase in emphysema. Support for the involvement of proteinases in emphysema is provided by the detection of emphysematous lesions in the lungs of experimental animals after intratracheal instillation of proteinases, such as porcine pancreatic elastase and NE (46,106). These various observations are unified in a common concept known as the "proteinase-antiproteinase
theory of emphysema", which proposes that emphysema develops as a result of unrestricted proteolytic activity in the lower respiratory tract (108,109,111). This theory explains the pathogenesis of emphysema resulting from cigarette smoking or inherited deficiencies of α1PI.

1.6.1 Inherited emphysema.

In order for emphysema to develop, the levels of circulating α1PI need to be below a critical level (60). Normal serum α1PI concentrations can lie between 20 and 53 μM; concentrations below 11 μM place an individual in the "at risk" category for the development of emphysema (60). There are two common inherited α1PI variants that can place a person at such a risk, these are the "Z" and "S" variants. Greater than 90% of all cases of serum α1PI deficiency are due to Z homozygotes, who have serum α1PI concentrations in the range 3-7 μM; the SZ heterozygote is mildly at risk in the 8-19 μM range (60,63,107). The protein derived from the Z gene has a Lys substituted for Glu342, which results in the disruption of a salt-bridge, and consequent incorrect protein folding (60,63,112,113). Although the Z variant is synthesized normally, only about 15% of the protein is secreted into the plasma (60,114). A consequence of the poor secretion of the Z variant is intrahepatocyte accumulation of the incorrectly folded protein which can lead to liver disease in some individuals (60,63,112,113). The S variant has a Val substituted for Glu264, disrupting
another salt-bridge, which leads to excessive intracellular degradation of newly synthesized $\alpha_1$PI (60,63,113).

For the Z homozygote, emphysema manifests itself as a slow onset of dyspnea appearing noticeably between ages 50-60; death due to respiratory failure usually occurs by age 60-70 years (60,113,114). In the case of Z homozygotes who smoke, the disease progression is usually shifted 15-20 years earlier (60,114). However, there are many instances of asymptomatic individuals with inherited or acquired $\alpha_1$PI deficiencies (60,108).

A variety of therapeutic strategies have been, and are being, developed to enhance antiproteinase activity in the lower respiratory tract of persons at risk for the development of emphysema (60). The more successful approaches have involved administration of $\alpha_1$PI directly to the lung in aerosol form (60). Also, a promising recent advance is the introduction and expression of the $\alpha_1$PI gene in the lung epithelium of rats (115).

1.6.2 Smoking-induced emphysema.

Emphysema is predominantly a smoking related disease, which would be eliminated as a major public health problem if cigarette smoking was abandoned (46,105). A variety of factors are proposed to be involved in the pathogenesis of smoking-induced emphysema, including decreased antiproteinase activity in the lower respiratory tract of smokers (116).
Oxidative damage to α1PI has received the most attention as the cause for the depletion of antiproteinase activity in smokers' lungs (45,109,116,117), however, evidence that oxidative damage to α1PI occurs in vivo is sparse (118,119). Cigarette smoke and inflammatory cells are the two main sources of oxidants that could damage α1PI.

In vitro studies using α1PI exposed to "clean" oxidation systems, such as N-chlorosuccinimide, chloramine-T or myeloperoxidase/Cl-/H2O2 have shown that two methionyl residues (Met368 and Met351) are oxidized to the sulfoxides (120,121). Also, in vitro studies using activated PMN and AM have shown that reactive oxygen species derived from these cell types can damage α1PI and the damage is dependent on myeloperoxidase (MPO) activity in the case of PMN (122-126). In vivo studies have demonstrated that MPO can decrease antielastase activity in lung lining fluid (127), and MPO activity is higher in PMN from smokers' as compared to nonsmokers' lungs (128).

Several studies using human plasma, lung fluid and purified α1PI exposed to cigarette smoke have demonstrated damage to α1PI (129-136). In some of these reports, the damage was presumed to be oxidative, perhaps involving free radical species (129,133,135,137,138), although, non-oxidative damage to α1PI by cigarette smoke is also probable (131,138). The relative importance in vivo of oxidative damage to α1PI by cellular vs. cigarette smoke-derived oxidants is unknown at present.
Methionine sulfoxide (MetSO) has been detected in α1PI recovered from healthy smokers' lung fluids, with no significant changes in other amino acids (119). The α1PI from smokers' lungs contained 4 MetSO/α1PI, and there were no changes in the molecular weight or electrophoretic mobility of the protein (119). In a different study, α1PI from healthy smokers' lung fluids was found to be present in the native molecular weight form (139). The α1PI had both normal and more cationic electrophoretic mobility and no α1PI with reversible oxidative modification was detectable (139). In contrast, α1PI from the lung fluids of smokers with emphysema has molecular weights suggestive of native, proteolyzed and complexed forms of the inhibitor and these studies also implied oxidized α1PI could be present (140).

The importance of oxidative damage to methionyl residues, particularly Met\textsuperscript{358}, as a primary mechanism for loss of α1PI activity is illustrated by two sets of data. Firstly, a Val\textsuperscript{358} α1PI mutant exposed to N-chlorosuccinimide, MPO, activated PMN and gas phase cigarette smoke is resistant to loss of activity over relatively short exposure times (138). Secondly, α1PI exposed to "clean" oxidant systems such as hydrogen peroxide, chloramine T or the MPO system can be reactivated by treatment with MetSO-containing peptide reductase (141,142). Methionine sulfoxide-containing peptide reductase is a ubiquitous enzyme which reduces peptide bound MetSO to Met using dithiothreitol or
thioredoxin/thioredoxin reductase/NADPH (143). However, MetSO-containing peptide reductase only partially reactivates $\alpha_1$PI exposed to cigarette smoke in vitro, or $\alpha_1$PI recovered from smokers' lungs (139,142). It has been proposed that MetSO-containing-peptide reductase may be a repair enzyme for MetSO$_3$$^+$-$\alpha_1$PI in vivo, but this is questionable (142,144). Unlike $\alpha_1$PI, HNE is less sensitive to oxidants such as chloramine-T, cell-derived oxidants and smoke (124,145,146), such that antiproteinase activity is expected to be decreased the most by oxidative/smoke exposures in vivo (45).

Besides oxidative damage, proteolytic inactivation of $\alpha_1$PI by proteinases released from inflammatory cells could also play a role in localized deficiencies in $\alpha_1$PI activity (89,147-149). Metalloproteinases released from PMN and AM, such as collagenase and gelatinase, are the prime candidates for proteinases that could inactivate $\alpha_1$PI (89,147-151). Both oxidative and proteolytic damage to $\alpha_1$PI may play a role in the localized depression of $\alpha_1$PI activity in lung connective tissue disorders such as smoking induced emphysema, but the relative importance of each mechanism is unknown.

There are significantly increased numbers of AM and PMN in smokers' lungs (111,152-156). Also, the increased PMN population in smokers' lungs shows a longer residence time in the lung vasculature (155,157). These observations have important implications for the
proteinase-antiproteinase theory, since PMN and AM are vectors for proteinases and oxidants (109,111). Both the AM and PMN are likely to have higher phagocytic activity, with consequent proteinase release and generation of reactive oxygen species, due to the increased burden of particulates in the lungs of smokers. The AM recovered from smokers' lungs are metabolically activated to produce greater amounts of oxidants compared to those from nonsmokers' lungs (158-161). At least for PMN,cellularly produced oxidants do not directly influence elastin degradation; the proteinases are the primary degradative agents (162).

Various, equally valid, mechanisms have been proposed for the increased content of PMN in smokers' lungs. Cigarette smoke stimulates AM to secrete PMN chemotaxins, such as leukotriene B₄ and interleukin-8 (163). Additionally, complement component C3 is modified by cigarette smoke in vitro: studies in vivo indicate that the alternative complement pathway is activated in the lung after smoke exposure, liberating the C5a fragment of complement component C5, which is highly chemotactic for PMN (164-167). Additionally, some cigarette smoke components are chemoattractants, as are some degradation products of elastin and collagen (109,111).

Although most studies of smoking induced emphysema have concentrated on factors related to elastin degradation, it is also probable that smoking could hinder repair of damaged elastic fibers (109,116). In
vitro studies have shown that aqueous solutions of gas phase smoke inhibit elastin cross-linking reactions by a substrate directed mechanism (168). Also, it has been demonstrated in vivo that activities of lysyl oxidase increase after elastase-induced tissue injury (169), but in conjunction with cigarette smoke such increases have not been observed (170). Thus, cigarette smoke generally exacerbates the effects of elastase-induced emphysema in experimental animals (171).

The validity of the proteinase-antiproteinase theory in vivo depends on the detection of decreased antielastase activity and/or increased elastinolytic activity in the lungs of smokers. Numerous studies over the years have been directed at this question and the results have aroused much controversy (108,109,111). Some investigators find the activity of $\alpha_1$PI in the lung fluid of smokers compared to nonsmokers significantly decreases (119,172), does not change (140,173-176), or increases (139). In cases where $\alpha_1$PI activity does not change it has often been suggested that increases in elastase activity are more important than decreases in the activity of antielastases (175,176). The reasons for such variations in the data can be attributed to differences in methodology and subject selection between research groups (139,177,178). Three recent studies demonstrate decreased antiproteinase (177,179) or $\alpha_1$PI (178) activity in smokers' lung lavage, supporting the proteinase-antiproteinase theory and the role of oxidative processes in emphysema (177,178). Some
investigators have demonstrated an increase in the activity of \( \alpha_1 \)PI in smokers' as compared to nonsmokers' plasma, which probably results from an inflammatory response due to chronic smoke inhalation (180,181). Other studies on the elastolytic activity of lung fluids demonstrate that activity is increased in smokers' lung fluid, mainly as a result of chronic exposure to smoke (176,182,183). Also, NE has been detected in close association with elastic fibers in emphysematous lung tissues (184). Since PMN from smokers' lungs display no increased propensity to release NE upon stimulation, the observed increases in elastolytic activity in smokers' lung fluid arise from the simple increase in the number of PMN (185).

The issue of decreased \( \alpha_1 \)PI activity in smokers' lungs is debatable, and the controversy is difficult to solve with available lung lavage procedures. The lavage methods currently used result in mixing of protein populations from different lung microenvironments. Also, the sampling of lung ELF is probably a poor measure of \( \alpha_1 \)PI and/or elastolytic activity in the lung compartment of primary interest to emphysema, i.e. the interstitium.

This dissertation is concerned with the \textit{in vitro} interaction of water soluble components of cigarette smoke particulate phase with \( \alpha_1 \)PI. The studies are aimed at examining some basic features of the damaging system and formulating a mechanism for the damage to \( \alpha_1 \)PI. Prior to these studies, there have been several reports in the literature dealing with this aspect of cigarette smoke research. Pryor \textit{et al}. have studied the
interaction action of gas phase and sidestream cigarette smoke with $\alpha_1$PI in aqueous buffer (129, 130, 186-188). Additionally, several studies have dealt with the effects of whole cigarette smoke, prepared in aqueous buffers (133, 135-137) or dimethyl sulfoxide (132, 134), on $\alpha_1$PI activity. Particulate phase material *per se* has not been studied in any depth, unlike the gas phase. Some of the earlier studies implied that damage to $\alpha_1$PI was oxidative, but no direct evidence for this has been presented.

The studies in this dissertation complement some of the studies previously presented by Pryor *et al.* concerning the effects of gas phase cigarette smoke on $\alpha_1$PI and particulate phase components on DNA (33, 34, 129, 130). It is hoped that the results presented in this dissertation will support some, and modify other, existing ideas concerning the effects of cigarette smoke components on biomolecules, particularly $\alpha_1$PI. Some speculations about the *in vivo* situation can be made from the data, although further experimentation is necessary to determine the relative importance of cigarette smoke vs. cellularly derived species as effective $\alpha_1$PI damaging agents.
BASIC FEATURES OF THE AQUEOUS CIGARETTE TAR EXTRACT SYSTEM
AND THE DETERMINATION OF $\alpha_1$PI ACTIVITY.

2.1 Basic Features of the Aqueous Cigarette Tar Extract System.

Many of the studies of cigarette smoke damage to $\alpha_1$PI in vitro have been done using organic solvent extracts of whole smoke or cigarette smoke condensate. Whereas tar is essentially composed of particulate phase components, the condensate contains both particulate and gas phase components. Components of the TPM that are extractable by an aqueous buffer are studied in this dissertation and this is not the same as whole smoke bubbled through an aqueous buffer. The fractionation of cigarette smoke into gas and particulate phases is probably the crudest fractionation procedure, and has been used traditionally to simplify the examination of the chemistry and biological effects of smoke. This type of fractionation necessarily neglects effects arising from interaction between components of the two phases. Obviously, from a biological perspective, an aqueous extraction is more realistic than an organic solvent extraction. The water extractable gas phase and particulate phase components have differing chemistries and probably damage $\alpha_1$PI by different mechanisms, as will be evident from some of the data presented in this dissertation.
2.1.1 Experimental.

2.1.1.1 Preparation of ACT. Research cigarettes were purchased from the University of Kentucky Tobacco and Health Research Institute. The cigarettes were stored at -10 °C and before use they were conditioned over saturated aqueous ammonium nitrate at ~ 22 °C (~ 60% relative humidity) for at least 24 h (A. Vaught, University of Kentucky Tobacco & Health Research Institute, personal communication, 1982). The 1R1 reference research cigarette was used for most of the studies. The 2R1F cigarette, a filter-tipped version of the 2R1 (a reference cigarette equivalent to the 1R1, but manufactured in 1974 rather than 1969) was also used. Some characteristics of the 1R1 and 2R1F cigarettes are shown in Table 4.

Cigarette smoke particulate matter was collected on a Cambridge filter (No. CM-113; Cambridge Filter Corp., Syracuse, NY) from two 1R1 cigarettes, smoked down to a 23 mm butt length. Smoking was done using the "puff protocol" (13). Smoke was drawn by hand through the Cambridge filter supported in a holder into a 50 mL glass syringe connected to a three-way stopcock. Gas phase smoke was expelled to the air. The Cambridge filter with adsorbed particulate matter was extracted for 30 min at 37 °C with 5 mL of incubation buffer. The extract was then filtered to remove fibers coming from Cambridge filter, yielding undiluted ACT. The ACT from filter tipped cigarettes was prepared in the same manner, except the filter tipped cigarettes were smoked to down to 3 mm
Table 4. Characteristics of research cigarettes used in this study.

<table>
<thead>
<tr>
<th>Cigarette</th>
<th>TPM (mg/cig.)</th>
<th>FTC\textsuperscript{b} Tar (mg/cig.)</th>
<th>Nicotine (mg/cig.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R1</td>
<td>35.4</td>
<td>30.1</td>
<td>1.48</td>
</tr>
<tr>
<td>2R1F</td>
<td>28.6</td>
<td>23.4</td>
<td>1.74</td>
</tr>
</tbody>
</table>

\textsuperscript{a} - Source, Ref 189.
\textsuperscript{b} - Federal Trade Commission.
from the filter overwrap. The term "incubation buffer" is used throughout
the text to refer to Chelex®-100-treated 0.1 M sodium phosphate, pH 7.4.
Chelex®-100 chelating resin (200-400 mesh, sodium form; Bio-Rad
Laboratories, Richmond, CA) was routinely used to deplete 0.1 M
phosphate, pH 7.4 of redox active transition metal ions. The buffer was
continuously stirred with Chelex®-100 (2.5 g resin/L buffer) for at least 12 h. Adjustment of pH was made when necessary, and stirring continued for
at least 3 h after each adjustment. Chelex®-100 was removed by vacuum
filtration, and the pH was always rechecked.

2.1.1.2 The estimation of water soluble particulate matter in ACT. Pre-
weighed vials were filled with 1 mL of either undiluted ACT or a control
solution consisting of an extract from a Cambridge filter not exposed to
smoke. The solutions were lyophilized to remove volatile ACT
components. It was assumed that volatile components were removed to
an equal extent from all of the vials. The weight of the residue from a non-
smoke-exposed Cambridge filter was subtracted from the weight of the
residue from the ACT. The material balance was termed 'non-volatile ACT
components'.

2.1.1.3 The standard protocol for incubation of α1PI with ACT. The ACT
prepared as above was, in most cases, diluted by half in the incubations
with α1PI. This "half-diluted ACT" is referred to in the text as simply
"ACT". In a few cases, however, where it is felt more appropriate, the
extent of dilution is specified as "% v/v ACT" (referring to the percentage of ACT, original 5 mL solution, diluted in incubation buffer). Since the actual weight of material in ACT was difficult to assess accurately, % v/v was considered the most suitable way to express the concentration.

The text that follows details the typical incubation conditions, but variations to the protocol are described at the appropriate places in the text. Alpha,PI used in these studies was from human serum or plasma (purity >95% by SDS-PAGE; Calbiochem Corp., San Diego, CA). Stock solutions of 250 µg/mL α,PI in incubation buffer were routinely used. The stock solution of α,PI was mixed with ACT (1:1, v/v) to give the standard incubation concentrations. The total volume of the incubation was 300 µL. The incubations were performed in the dark, in 500 µL Eppendorf vials, at 37 °C for 24 h prior to determining elastase inhibitory capacity (EIC) (see section 2.2).

2.1.1.4 Time course for EIC loss of α,PI exposed to ACT and gas phase smoke. The control for this experiment consisted of 125 µg/mL α,PI incubated in buffer alone. As a further control experiment, 125 µg/mL α,PI was incubated with an aqueous extract of a Cambridge filter. Cambridge filter either exposed or not exposed to 20 puffs of ambient air was extracted as described for a cigarette smoke exposed filter (section 2.1.1.1).
The gas phase extracts (GPE) were prepared by bubbling smoke from two 1R1 cigarettes, using the puff protocol, into a vial containing 0.75 mL of incubation buffer maintained at 37 °C. Immediately after smoking, 0.75 mL of a 250 μg/mL α,PI solution was added to give a final volume of 1.5 mL. This 1.5 mL was incubated under standard conditions. Aliquots of 20 μL were removed periodically for the EIC assay. Control incubations were prepared using 0.75 mL of incubation buffer exposed to 20 puffs of ambient air by the puff protocol. This solution was then treated as described for GPE.

Direct gas phase (DGP) exposure was performed as follows; 1.5 mL of α,PI (125 μg/mL) at 37 °C was exposed directly to the gas phase smoke from two 1R1 cigarettes smoked using the puff protocol. This 1.5 mL was then treated in the same way as noted for GPE. The control incubation consisted of 1.5 mL of 125 μg/mL α,PI solution exposed directly to 20 puffs of ambient air via the puff protocol. The ACT from filter tipped 2R1F research cigarettes was also tested for its α,PI damaging properties using the incubation conditions described for the ACT from 1R1 cigarettes.

2.1.1.5 Dose dependency of ACT damage to α,PI. For concentrations up to and including 50% v/v ACT a 250 μg/mL α,PI stock was used, diluted by half with appropriate combinations of ACT and incubation buffer to achieve the desired ACT concentration. For concentrations of ACT above
50% v/v, a 926 μg/mL α1PI stock was used (40.5 μL stock in 300 μL total to give 125 μg/mL α1PI).

2.1.1.6 The effect of aged ACT on its ability to damage α1PI. The effect of aging ACT on its ability to damage α1PI was studied in two ways. Firstly, fresh ACT or aged ACT (aged for 12 h at 37 °C, in the dark) was incubated with α1PI and the EIC determined at 2 and 24 h. Secondly, the ability of ACT to damage successive additions of α1PI was determined as follows. The EIC of an incubation containing ACT and 125 μg/mL α1PI was determined after 24 h ("first 24 h incubation"). The control was 125 μg/mL α1PI exposed to incubation buffer only. For the EIC assays 40 μL (2 x 20 μL) of incubation mixture was removed from a total of 300 μL, leaving 260 μL of incubation mixture. To this 260 μL, 2.5 μL of incubation buffer and 37.5 μL of 1 mg/mL α1PI was added to replenish the ACT incubation with fully active α1PI (125 μg/mL) for the "second 24 h incubation". The EIC was assessed after 0.75 h, to ensure 100% α1PI activity had been restored, and then again after 24 h. An additional control for the "second 24 h incubation" consisted of a mixture of 110 μL fresh ACT plus 150 μL of 250 μg/mL α1PI and 40 μL of incubation buffer. This control accounts for the fact that the second α1PI aliquot is exposed to a diluted ACT compared to the first exposure.

2.1.1.7 pH change of ACT over a 24 hour incubation period. The pH of 4.5 mL of freshly prepared ACT was measured initially and after incubation
for 24 h under the standard conditions. For the sake of correct comparison, the buffer alone was incubated under identical conditions and the pH measured.

2.1.1.8 Exposure to ACT of α,PI enclosed in dialysis tubing. Dialysis tubing (Spectra/Por 6, Wet-Form, Molecular Weight Cut-Off 1,000; Spectrum Medical Industries Inc., Los Angeles, CA) was prepared as follows: 1) the tubing was cut into convenient lengths and washed inside and out with deionized water; 2) the tubing was boiled for 10 min in 500 mL of 2% sodium bicarbonate/1 mM EDTA, and then rinsed with deionized water; 3) the tubing was boiled for 10 min in deionized water, cooled and stored in a refrigerator. The dialysis tubing was washed both inside and out with deionized water just before use. Two ACT preparations were pooled then diluted by half to give sufficient ACT to work with.

Approximately 5 cm of dialysis tubing, closed at both ends with clips and containing 400 μL of 125 μg/mL α,PI was immersed in 10 mL of either incubation buffer or ACT in 15 mL screw-cap vials. The vials were incubated under the standard conditions. The EIC was determined after 24 h.
2.1.2 Results.

2.1.2.1 The estimation of water soluble particulate matter in ACT. Using six ACT preparations the following data were obtained (mean ± s.d.):

Weight of Cambridge filter extract = 16.15 ± 0.11 mg

Weight of ACT = 20.33 ± 0.73 mg

Weight of non-volatile ACT components in 1 mL ACT

= 4.18 ± 0.74 mg

Thus, the 5 mL of undiluted ACT contains approximately 21 mg of material. Since two 1R1 cigarettes could yield approximately 71 mg of TPM, then approximately 30% was extracted into the buffer as relatively non-volatile material. This material was diluted by half in order to add α,PI solution, buffer and other components of the incubation. Therefore, each experiment contains about 2.09 mg/ml of water soluble non-volatile particulate phase components. This half-dilute preparation is the standard ACT solution for α,PI exposure and corresponds to the concentration of water soluble particulate matter from one 1R1 cigarette. These data also illustrate that the extraction procedure is reasonably reproducible, given the relatively crude preparation method.

2.1.2.2 Time course for EIC loss of α,PI exposed to ACT and gas phase cigarette smoke. Exposure of α,PI to ACT derived from two 1R1 cigarettes causes a monophasic loss of EIC that is similar in extent to that seen for the first 5 h of exposure to gas-phase extract (Fig 2). In ACT a loss of EIC
of 60-80% is observed over 24 h compared to approximately 50 and 40% loss of EIC for DGP and GPE exposures, respectively. Aqueous extracts of a Cambridge filter, whether exposed, or not, to 20 puffs of ambient air, has no effect on the EIC of \( \alpha_1 \)-PI. The native \( \alpha_1 \)-PI displayed the same EIC over the 24 h incubation period.

In certain cases the amount of EIC lost by \( \alpha_1 \)-PI after exposure to ACT is dependent on the lot number of \( \alpha_1 \)-PI used! This variability was not anticipated at the outset of these studies. The reason for this inter-lot variation in loss of EIC is unknown. However, the variation in loss of EIC after ACT exposure was relatively small. The smallest loss of EIC observed after 24 h incubation with ACT is that shown in Fig 2 (59 ± 6%). Other experiments showed greater EIC losses, e.g., a mean % EIC ± s.d. of 23 ± 7% for one particular lot-number of \( \alpha_1 \)-PI exposed to 42 different ACT preparations. Despite these relatively large variations in sensitivity to ACT, the 24 h incubation period was used for the majority of the studies. The data for the \( \alpha_1 \)-PI damaging activities of the ACT derived from the various research cigarettes are shown in Table 5.

2.1.2.3 Dose dependency of ACT damage to \( \alpha_1 \)-Pl. The ACT causes a concentration-dependent loss of EIC (Fig 3).

2.1.2.4 The effect of aged ACT on its ability to damage \( \alpha_1 \)-Pl. ACT aged for 12 h at 37 °C shows only slightly less \( \alpha_1 \)-Pl damaging ability compared to a fresh sample of ACT (Table 6). Also, ACT has the ability to damage
Figure 2. Time courses of the loss of EIC of α, ρ: ACT (■); gas phase extract (●); direct gas phase (▲). Data shown are the mean of three independent runs; error bars represent s.d. where this exceeds symbol size (Reproduced with permission from Ref. 190).
Table 5. A comparison of the effects of ACT from 1R1 and 2R1F research cigarettes on the EIC of $\alpha$-PI.

<table>
<thead>
<tr>
<th>Cigarette type</th>
<th>% EIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R1</td>
<td>23 ± 7b</td>
</tr>
<tr>
<td>2R1F</td>
<td>22 ± 3c</td>
</tr>
</tbody>
</table>

a - These data were obtained with the same lot number of $\alpha$-PI. Data are means ± s.d.

b - 42 ACT preparations.

c - 5 ACT preparations.
Figure 3. Dose dependence effect of ACT on the EIC of \( \sigma_{\text{Pl}} \). Data are mean of three independent ACT preparations; error bars represent s.d., where this exceeds symbol size (Reproduced with permission from Ref. 190).
successive additions of α₁PI, causing similar losses in EIC (Fig 4). The data in Table 7 concerning residual PPE activities and % EIC for the controls is required for full interpretation of Fig 4. The data in Table 7 should be used as follows: "control α₁PI" displays 81% PPE inhibition, and the "ACT, 0.75 h" shows that after addition to ACT of the second α₁PI aliquot control levels of PPE inhibitory ability were restored. "Dilute ACT" is meant to be compared with the 48 h point on Fig 4, to determine the true difference in EIC loss due to any depletion of damaging species with aging of ACT. The data concerning the pH change of ACT incubations are presented in Table 8, and show a consistent decrease of 0.1 pH unit over a period of 24 h.

2.1.2.5 Exposure to ACT of α₁PI enclosed in dialysis tubing. Under the conditions used for dialysis, α₁PI retains its porcine pancreatic elastase (PPE) inhibitory activity after 24 h (Table 9). Data in Table 9 are also presented for α₁PI incubated in buffer only ("native") or ACT under the standard incubation conditions.

2.1.3 Discussion.

In terms of the degree and rate of EIC loss, ACT resembles GPE more closely. However, these two methods of exposure are different in the amount of cigarette smoke material involved. Material from two 1R1 cigarettes was extracted into different volumes of buffer, 1.5 and 5 mL for the studies using the gas phase and ACT, respectively. ACT was further diluted by 50% for incubations with α₁PI, i.e. the material from two 1R1
Table 6  The effect of aged ACT on its ability to damage human α₃PI.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Incubation time (h)</th>
<th>% EIC remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>2 or 24</td>
<td>100</td>
</tr>
<tr>
<td>Fresh ACT</td>
<td>2</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>Fresh ACT</td>
<td>24</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Aged ACT*</td>
<td>2</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>Aged ACT</td>
<td>24</td>
<td>26 ± 4</td>
</tr>
</tbody>
</table>

a - Reproduced with permission from Ref. 190.
b - Data are means ± s.d. of three ACT preparations.
c - Aged at 37 °C, light protected for 12 h.
Figure 4. The effect of ACT on the activity of two successive additions of $\alpha_1$PI. Each point shown is mean of three independent ACT preparations; error bars represent s.d. (Reproduced with permission from Ref. 190).
**Table 7** Control data for use in conjunction with Fig 4.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% PPE inhibited*</th>
<th>% EIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilute ACT, 24 h</td>
<td>---</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>ACT, 0.75 h</td>
<td>86 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>Control α1,PI</td>
<td>81</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* - Data are means ± s.d. of three ACT preparations.

**Table 8** The pH change of ACT incubated at 37 °C.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>pH at 0 h</th>
<th>pH at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% v/v ACT*</td>
<td>7.38 ± 0.03</td>
<td>7.26 ± 0.01</td>
</tr>
<tr>
<td>Incubation buffer</td>
<td>7.41 ± 0.00</td>
<td>7.39 ± 0.01</td>
</tr>
</tbody>
</table>

*a* - Data are means ± s.d. of three ACT preparations.

*b* - Data are means ± av.dev. of two samples.
Table 9  The effect of ACT on α,PI enclosed in a dialysis bag with a molecular weight cut-off of 1,000.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% PPE inhibited</th>
<th>% EIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis Control</td>
<td>73 ± 4\textsuperscript{a}</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis ACT</td>
<td>---</td>
<td>12 ± 1\textsuperscript{a}</td>
</tr>
<tr>
<td>Native</td>
<td>78 ± 1\textsuperscript{b}</td>
<td>100</td>
</tr>
<tr>
<td>ACT</td>
<td>---</td>
<td>20 ± 8\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are means ± s.d. of three incubations or three ACT preparations.
\textsuperscript{b} Datum is mean ± av.dev. of two separate incubations.
\textsuperscript{c} Datum is mean ± s.d. of four ACT preparations.
cigarettes was effectively diluted into 10 mL. Thus, the material from two 1R1 cigarettes in the ACT incubations is 6.7 times less than in the gas phase systems. The data in Fig 2 and the relative amounts of cigarette smoke material in the gas phase and ACT imply that cigarette smoke particulate matter releases greater amounts of damaging species and/or such damaging species are more potent than those derived from gas phase smoke.

The 1R1 cigarette and its counterpart, the 2R1, is widely considered the reference research cigarette for chemical and biological studies. However, although the 1R1 is unfiltered, the addition of a filter tip makes no substantial difference to the α,PI damaging properties of an ACT (Table 5).

The experiments concerned with the aged ACT should furnish some information about the stability/turnover of α,PI damaging species. The ACT loses little damaging capability with aging, thus indicating that the damaging species are relatively stable. Also, ACT can generate more than enough species to damage successive additions of α,PI. If damaging species accumulate in ACT with aging then the loss of EIC of α,PI exposed to aged ACT would be more rapid if the damaging reaction(s) is fast. The assessment of EIC at 2 h shows that either damaging species do not accumulate, or they do accumulate but the damaging reaction(s) is slow.
Data presented later in the dissertation (section 3.2.3.2) indicate that the latter conclusion is probably correct.

Since the pH of ACT does not change drastically over 24 h, pH change is not responsible for the EIC loss of α₁PI. Alpha₁PI is relatively resistant to pH change and loses only 35% activity when the pH is decreased from 7.5 to 5.0 (191). However, below pH 5.0 the proteinase inhibitory activity drops precipitously (191). Some or all of the EIC loss suffered by α₁PI exposed to ACT could be due to particulates either initially present or formed over time in ACT. Such particulates may adsorb α₁PI, for example, and remove it from solution to give the appearance of EIC loss due to molecular damage. While other data in the dissertation support molecular damage as the reason for EIC loss, the data from the dialysis experiment imply that EIC loss is not due to particulates initially present in ACT. However, high molecular weight polymeric material could form from species entering the dialysis bag early on in the incubations. Both fresh and aged ACT showed no visual evidence of particulates, even though smoke solutions have been noted to form polymeric material upon aging (192).

2.2 The Assay of Elastase Inhibitory Capacity.

Porcine pancreatic elastase (PPE), rather than HNE, is used frequently for determining α₁PI activity. Both enzymes are inhibited by
α₁PI, but the k_{assoc} values for this interaction are different, 6.5 \times 10^7 and 1.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1} for HNE and PPE respectively (55). The most dramatic change in k_{assoc} values is noted for α₁PI containing MetSO₃, where the k_{assoc} values are 1.0 \times 10^5 and 0 \text{ M}^{-1}\text{s}^{-1} for HNE and PPE, respectively (55).

2.2.1 Experimental

2.2.1.1 Assay protocol. The activity of PPE (Pancreatopeptidase E, E.C. 3.4.21.36; 140 U/mg; Calbiochem Corp., San Diego, CA) was measured at pH 8.0 and 25 °C according to the method of Bieth et al. (193). The reaction mixture in a final volume of 1 mL contained 0.2 M Tris-HCl, pH 8.0, 0.14 U PPE, 2.5 μg α₁PI, 1 mM N-succinyl-(L-Ala)₃-p-nitroanilide (SANA) and 0.8 % v/v 1-methyl-2-pyrrolidinone. The PPE was preincubated with native or treated α₁PI (usually a 20 μL aliquot of 125 μg/mL α₁PI) for 5 min at 25 °C and the reaction started by the addition of SANA. The increase in absorbance at 410 nm was measured against a reference consisting of buffer and SANA only. The initial linear rate of absorbance change due to uninhibited PPE was taken as 100% PPE activity; the decrease in this rate due to native α₁PI was taken as 100% EIC. The % EIC was calculated using equations 10 to 12 (overleaf). Control values for uninhibited PPE were determined under identical conditions, but with omission of α₁PI from the assay mixture.
\[
\text{% PPE active} = 100\left(\frac{\Delta A_{410} \text{ min}^{-1}, I}{\Delta A_{410} \text{ min}^{-1}, U}\right) \quad (10)
\]

\[
\text{% PPE inactive} = 100 - \text{% PPE active} \quad (11)
\]

\[
\text{% EIC} = 100\left(\frac{\text{% PPE I, } \alpha_1\text{PI}^*}{\text{% PPE I, } \alpha_1\text{PI}}\right) \quad (12)
\]

Where, "\(\Delta A_{410} \text{ min}^{-1}, I\)" is the initial rate for PPE activity in the presence of \(\alpha_1\text{PI}\); "\(\Delta A_{410} \text{ min}^{-1}, U\)" is the initial rate for PPE activity in the absence of \(\alpha_1\text{PI}\); "\(\text{% PPE I, } \alpha_1\text{PI}^*\)" is the \% PPE inactive in the presence of \(\alpha_1\text{PI}\) exposed to some treatment; "\(\text{% PPE I, } \alpha_1\text{PI}\)" is the \% PPE inactive in the presence of \(\alpha_1\text{PI}\) exposed to incubation buffer only.

2.2.1.2 Effect of ACT on PPE activity and the ability of \(\alpha_1\text{PI}\) to inhibit PPE.

Normally, 20 \(\mu\text{L}\) aliquots of sample containing \(\alpha_1\text{PI}\) and 50% v/v ACT were used in the EIC assay, thus ACT was present at \(20 \mu\text{L}/1000 \mu\text{L}\) 1% v/v in the assays. To test the effect of ACT on PPE activity, the assay was conducted as described in section 2.2.1.1, substituting the 20 \(\mu\text{L}\) aliquot of \(\alpha_1\text{PI}\) sample with 20 \(\mu\text{L}\) of 85% v/v ACT (aged for 24 h under the standard conditions). In a similar experiment 2% v/v ACT was tested for any effects on the ability of undamaged \(\alpha_1\text{PI}\) to inhibit PPE. The assay for this experiment was conducted as described in section 2.2.1.1, except that 40 \(\mu\text{L}\) of 50% v/v ACT was substituted for an equivalent volume of assay buffer.

2.2.1.3 Estimation of the \(\alpha_1\text{PI}:\text{PPE ratio in the assay mixture and its effect on EIC determination.}\) The concentration of \(\alpha_1\text{PI}\) was determined by calculation as described in section 2.2.2.3. The PPE concentration was
determined using a method described by Shotten (194). Stock solutions of PPE (100 μg/mL) were prepared in 0.1 M sodium hydroxide. Dilutions of 0.5 and 0.75 of the stock solutions were made with 0.1 M sodium hydroxide, and the absorbencies measured at 280 nm against a 0.1 M sodium hydroxide blank.

The effect of the $\alpha_1$PI:PPE ratio on EIC determination was examined by assessing the % EIC of ACT-damaged $\alpha_1$PI using a 10 or 20 μL aliquot of $\alpha_1$PI sample for the EIC assay. Incubations of $\alpha_1$PI in buffer alone or with ACT were performed under the standard conditions.

2.2.1.4 The incubation time of PPE with $\alpha_1$PI. The standard EIC assay protocol, section 2.2.1.1, includes a 5 min co-incubation of $\alpha_1$PI and PPE to achieve inhibition before the addition of SANA. In the literature, the selection of this time is dependent on various factors such as the incubation temperature and the concentration of $\alpha_1$PI in the sample. Although this 5 min time was always used, some experiments were performed to assess the effects of increasing this time on the inhibition of PPE and the EIC values. These studies simply involved incubating native or ACT-exposed $\alpha_1$PI for differing times and determining the PPE activities.

2.2.2 Results and Discussion.

2.2.2.1 Effect of ACT on PPE activity and the ability of $\alpha_1$PI to inhibit PPE. Data from four experiments gave 102 ± 1% PPE activity (mean ± s.d.) in the presence of 1.7% v/v ACT. Incubation of native $\alpha_1$PI with PPE and 2%
v/v ACT had no effect on the degree of PPE inhibited. These data imply that the 1% v/v ACT present in the EIC assay of ACT-exposed α1PI is highly unlikely to affect either PPE activity or the ability of α1PI to inhibit PPE.

2.2.2.2 The incubation time of PPE with α1PI. Since α1PI activities were always expressed relative to a control, i.e. as % EIC, the effects of various treatments on the activity of α1PI could be easily compared. The use of % EIC also compensates for any inherent variations in the native (i.e. untreated) α1PI activity. The degree of inhibition of PPE was relatively consistent within a lot number of α1PI. Five different lots of α1PI gave the following means ± s.d. (sample size in parentheses) for percent PPE inhibited (after co-incubation of α1PI with PPE for 5 min): 74 ± 2 (n = 4), 91 ± 1 (n = 4), 70 ± 4 (n = 11), 81 ± 2 (n = 8) and 87 ± 4 (n = 17).

Figure 5 shows the effects of increasing the α1PI/PPE incubation time on the percent PPE inhibited. Doubling the α1PI/PPE incubation time from 5 to 10 min results in an increase the % PPE inhibited. When considering the ACT-damaged α1PI, an increase in the α1PI/PPE incubation time from 5 to 10 min leads to a 5-10% increase in the calculated EIC values. These changes in EIC resulting from an increased α1PI/PPE incubation time are relatively small and the effects are not transmitted to the % protection data (section 3.1.1.1) such that changes in the interpretation of the results are necessary.
Figure 5. The effect of variation of the $\alpha_1$PI/PPE incubation time on the degree of inhibition of PPE. Native $\alpha_1$PI (■); ACT-exposed $\alpha_1$PI (●).
2.2.2.3 Estimation of the $\alpha_I$:PPE ratio in the assay mixture and its effect on EIC determination. In the EIC assay, 20 $\mu$L of a 125 $\mu$g/mL $\alpha_I$ solution is added to 962 $\mu$L assay buffer and 10 $\mu$L PPE stock; 20 $\mu$L 125 $\mu$g/mL $\alpha_I$ in 992 $\mu$L total = 2.52 $\mu$g/mL $\alpha_I$; MW $\alpha_I$ = 52,000. Therefore we have, 2.52 $\mu$g/52,000 = 48.46 pmoles $\alpha_I$/mL.

Thus, $[\alpha_I]$ in assay mixture = 48.46 nM.

For PPE, $\epsilon_{280nm} = 5.74 \times 10^4$ M$^{-1}$ cm$^{-1}$ (194)

$[PPE] = (1/dilution \text{ factor})(A_{280}/\epsilon_{280})$

Dilution factor = 0.5 or 0.75 as appropriate.

For four measurements, 0.5 and 0.75 dilutions of two separate stocks:

$[PPE]$ in stock = 2.19 ± 0.19 $\mu$M

$[PPE]$ in assay = 2.19 x (10/992) = 22.1 nM

Therefore $\alpha_I$:PPE ratio in assay = $(48.46/22.1) = 2.2$

The results of varying the volume of the $\alpha_I$ sample aliquot, and hence the ratio of $\alpha_I$/PPE, are presented in Table 10. The data indicate that lowering the ratio of $\alpha_I$ to PPE from approximately 2 to 1 results in a negligible difference in the EIC value. Finally, the reason for selecting 20 $\mu$L samples for the EIC assay is that this sample size of native $\alpha_I$ gives the greater inhibition of PPE when the pre-incubation time of 5 min was used.
Table 10. The effect of alteration in the $\alpha_1$PI:PPE ratio in the EIC assay mixture on the measurement of EIC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay volume, $\mu$L$^a$</th>
<th>% PPE</th>
<th>% EIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native-$\alpha_1$PI</td>
<td>20</td>
<td>$72 \pm 2$</td>
<td>100</td>
</tr>
<tr>
<td>ACT-$\alpha_1$PI$^c$</td>
<td>20</td>
<td>$31 \pm 5$</td>
<td>$43 \pm 6$</td>
</tr>
<tr>
<td>Native-$\alpha_1$PI</td>
<td>10</td>
<td>$40 \pm 1$</td>
<td>100</td>
</tr>
<tr>
<td>ACT-$\alpha_1$PI</td>
<td>10</td>
<td>$16 \pm 4$</td>
<td>$40 \pm 8$</td>
</tr>
</tbody>
</table>

$^a$ - Volume added to assay cuvette.
$^b$ - Data are means ± av.dev. for two ACT.
$^c$ - $\alpha_1$PI exposed to ACT.
CHAPTER 3

INVESTIGATION OF THE MECHANISM OF DAMAGE TO $\alpha$,PI BY AQUEOUS
CIGARETTE TAR EXTRACT.

3.1 Protection Studies.

Protection studies have been used effectively in numerous
biochemical studies and have been applied to $\alpha$,PI/cigarette smoke
interactions (130,133,135). They are used in this dissertation in an
attempt to elucidate the mechanism of ACT damage to $\alpha$,PI. For the
purposes of this dissertation, modifications of $\alpha$,PI structure that do not
affect EIC are not regarded as damaging, although such modifications could
affect other properties of the protein.

Since there is pre-existing evidence that cigarette smoke causes
oxidative damage to biological targets this was used as a starting point for
the studies. A variety of agents were tested in the ACT system for
protective effects toward $\alpha$,PI. In the majority of cases the experimental
procedure was the same, but there were some variations. Some enzymes
were used to probe the damaging mechanism, and as such it proved
necessary to determine the effects of ACT on the activity of these
enzymes and their assay systems.
3.1.1 Experimental

3.1.1.1 Standard protocol for incubation of α,PI with ACT plus additives.

The incubations contained ACT, α,PI and appropriate amounts of additives to probe the mechanism of damage. The effects of the additives alone on α,PI were also studied using controls that contained 125 μg/mL α,PI and the additives in incubation buffer. Incubations aimed at determining the effect of ACT alone contained 125 μg/mL α,PI in ACT plus incubation buffer to substitute for the additive. Unless otherwise indicated, the incubations were performed in closed vials, under the conditions described in section 2.1.1.3 and the EIC was determined after 24 h.

Data derived from incubations containing additives are reported as percent protection. Percent protection is calculated as shown in equations 13 and 14:

\[
% \text{ protection} = 100 - % \text{ damage} \quad (13)
\]

\[
% \text{ damage} = 100 \cdot \left\{ \frac{100 - %\text{EIC}_A}{100 - %\text{EIC}_B} \right\} \quad (14)
\]

Where, "%\text{EIC}_A" is the % EIC calculated for α,PI incubated in ACT plus an additive and "%\text{EIC}_B" is the % EIC calculated for α,PI incubated in ACT only.

A percent damage of 100 is defined as the effect of ACT alone on α,PI. For α,PI exposed to ACT in the presence of additives: 100% damage implies the additive has no effect; < 100% damage implies the additive protects α,PI; > 100% damage implies the additive enhances ACT damage.
to \( \alpha_1 \text{PI} \). Percent protection, rather than percent damage, is used routinely to make the data compatible with the previous studies of Pryor et al. (130).

3.1.1.2 The effects of free amino acids on the \( \alpha_1 \text{PI} \)-damaging ability of ACT. The amino acids were present in the incubations at 10 mM; methionine and cysteine were also tested at 1 mM. Stock solutions of methionine and cysteine were prepared fresh before each experiment. Due to their poorer solubility, tryptophan and phenylalanine were added as solids to the incubation vials, such that addition of 300 \( \mu \text{L} \) of solution gave 10 mM of each amino acid.

3.1.1.3 The effects of reducing species and hydroxyl radical scavengers on the \( \alpha_1 \text{PI} \)-damaging ability of ACT. All stock solutions were prepared in incubation buffer; the thiol and ascorbate stock solutions were prepared fresh at the start of each experiment. Some incubations of \( \alpha_1 \text{PI} \) with ACT and 10 mM ascorbate, reduced glutathione (GSH), cysteine, dithiothreitol (DTT), methionine or thiourea were performed under air-purged conditions. Air-purged incubations comprised a total volume of 500 \( \mu \text{L} \) in 2 mL glass vials fitted with screw-caps housing teflon septa. Capillaries cut from a used capillary gas-chromatography column were used to introduce compressed air into the vials. Each vial was hooked-up to an air cylinder via a capillary inserted through the septum and each vial was also supplied with a short exhaust capillary. Only the headspace was continually purged, as purging through the solution for 24 h resulted in EIC loss of the control.
The vials were incubated under the standard conditions and EIC was assessed after 24 h.

3.1.1.4 The effects of metal ion chelators and ferric ions on the α,β-
damaging ability of ACT. The standard incubation protocol and conditions were followed as detailed in section 3.1.1.1; after 24 h the EIC was determined. In most cases the stock solutions were prepared in incubation buffer. A few stocks were prepared differently: desferrioxamine mesylate (Desferal®) was prepared in deionized water; diethylenetriamine pentapentaacetic acid (DTPA) was prepared in 0.6 M sodium hydroxide; ferric chloride was prepared in 0.01 M hydrochloric acid. In cases where DTPA and ferric ions were added, the introduction of base or acid, respectively, did not affect the pH of the incubation buffer.

3.1.1.5 Studies using catalase. The activity of catalase (H₂O₂: H₂O₂ oxidoreductase, E.C. 1.11.1.6; from bovine liver, thymol-free, 11,000 U/mg solid, 14,100 U/mg protein; Sigma Chemical Co., St Louis, MO) was determined using the method described by Claiborne (195), in which the disappearance of absorbance at 240 nm is monitored. The concentration of the substrate (hydrogen peroxide) was 20 mM in incubation buffer. The initial hydrogen peroxide concentration was slightly different between experimental runs, but was always ca. 20 mM. Since the results were frequently expressed relative to a control, this slight variation was not a problem. In the assay, an aliquot (2 to 10 µL, depending on the catalase
concentration) of sample was added to hydrogen peroxide substrate to give a 1 mL assay volume. The change in absorbance at 240 nm was followed for a maximum of 30 s, using a hydrogen peroxide substrate reference. Data are expressed as initial rates, $\Delta A_{240} \cdot s^{-1}$ and/or percentage control activity. The catalatic activity of each incubation was assessed in triplicate.

Several preliminary experiments were conducted to determine the effect of single additions of catalase on the activity of $\alpha_1$PI exposed to ACT, and the effect of ACT on these relatively low catalase concentrations. Aliquots (2 to 10 µL) of catalase stock solutions in incubation buffer were added to incubations of $\alpha_1$PI and ACT to achieve the desired concentrations of catalase. The incubations were carried out as outlined in section 3.1.1.1, and the incubates were assessed for EIC or catalase activity. Control incubations were also prepared to determine the effect of catalase per se on $\alpha_1$PI activity.

The activity of 5500 U/mL catalase exposed to ACT was determined at selected intervals over an 8 h incubation period. Incubations consisted of 1 mL (containing 20 µL of a 2.75 x $10^5$ U/mL catalase stock) in Eppendorf vials of 1.5 mL capacity. Control incubations contained catalase in incubation buffer only, while ACT incubations contained 0.5 mL of ACT, 20 µL of catalase and 0.48 mL of incubation buffer. The activity of catalase in each sample was determined in triplicate at each time point.
using a 2 μL aliquot for the assay. A further experiment was performed involving addition of catalase at 0, 4 and 12 h during the 24 h incubation period; a "multiple addition" protocol. The incubations were as described for the previous experiment, except that at 4 and 12 h, 20 μL of 2.75 x 10^5 U/mL catalase stock was added. The catalase activity was determined after 24 h incubation under the standard conditions. After the final addition of catalase, the incubations would contain 15,692 U/mL, if all the catalase remained active.

The same multiple addition protocol was applied to α1PI incubations with ACT. The following incubations (300 μL volume in 500μL Eppendorf vials) were performed: 1. α1PI control; 2. α1PI + catalase; 3. α1PI + ACT; 4. α1PI + ACT + catalase. The initial volumes for incubation 1 were 150 μL of 250 μg/mL α1PI stock plus 150 μL of incubation buffer, for incubation 2, 6 μL of 2.75 x 10^5 U/mL catalase stock was added in place of an equivalent volume of buffer. For the ACT incubations; 3 received 150 μL of α1PI stock, 144 μL of ACT and 6 μL of incubation buffer, 4 received 6 μL of catalase stock instead of buffer. At 4 and 12 h, incubations 1 and 3 received 6 μL of buffer, whereas incubations 2 and 4 received 6 μL of catalase stock. The EIC values were determined after 24 h incubation under the standard conditions.

3.1.1.6 The effect of heat denatured catalase and BSA on the activity of α1PI exposed to ACT. Catalase was prepared as a 25 mg/mL solution in
incubation buffer and inactivated at 100 °C for 3 - 4 min. The mixture was then homogenized by vortexing and brief sonication. The catalase assay (section 3.1.1.5) was used to confirm that the heat-denatured catalase had no activity. Heat-denatured catalase was added to α1PI in incubation buffer only or in ACT by the multiple addition protocol (section 3.1.1.5). The concentration of heat-denatured catalase was thus equivalent to that of active catalase (1.12 mg protein/mL). The EIC values were determined after incubation for 24 h under the standard conditions.

Standard incubation protocols were followed for the α1PI incubations with bovine serum albumin (BSA; essentially fatty acid free, fraction V; Sigma Chemical Co.) in buffer or ACT. The BSA stock solutions were prepared in incubation buffer and the concentrations in the incubations were either 0.58 or 5 mg protein/mL.

3.1.1.7 Studies using superoxide dismutase. The activity of superoxide dismutase (SOD; superoxide: superoxide oxidoreductase, E.C. 1.15.1.1; from bovine erythrocytes, 3360 U/mg solid, 3360 U/mg protein; Sigma Chemical Co.) was determined by the method described by McCord and Fridovich (196). This assay is based on the inhibition, by SOD, of the reduction of ferricytochrome c to ferrocytochrome c by superoxide generated by a xanthine/xanthine oxidase system (X/XO; xanthine: oxygen oxidoreductase, E.C. 1.2.3.22; grade I, from buttermilk, 25 U/1.5 mL, 41 mg protein/ml, 0.42 U/mg protein; Sigma Chemical Co.). The reduction of
ferricytochrome c (Type IV, from horse heart; Sigma Chemical Co.) by superoxide and its inhibition by SOD were monitored by observing ferrocytochrome c formation at 550nm. The true change in the absorbance due to the xanthine dependent reaction was obtained by subtracting a non-xanthine dependent control rate (all components except xanthine present). The reference for all runs was 20 μM cytochrome c in assay buffer.

Since all assay stock solutions were prepared in concentrated form, most of the assay volume was deionized water. Both the assay buffer and water were purged with oxygen before use. The contents of the assay cuvettes were as follows:

1. Control X/XO rate in absence of SOD: a total volume of 1 mL contained 50 mM sodium phosphate, pH 7.6, 1 mM DTPA, 20 μM cytochrome c, 5 mU XO and 250 μM X.

2. The SOD assay contained all the components noted in 1, plus 5 μL of SOD sample in place of an equal volume of water.

Before the addition of X the basal ΔA_{550} was monitored for ~ 120 s, then after addition of X, the ΔA_{550} was followed for a further 120 s, over which time the change was linear.

Studies on the effects of ACT on the SOD assay were done by monitoring ferrocytochrome c formation and XO activity (formation of urate at A_{295}). These studies used ACT which had been incubated under the
standard conditions before use; 0.5% (10μL sample) or 0.25% v/v (5 μL sample) ACT were tested.

Preliminary studies on the effect of ACT on SOD involved incubation of either 200 or 546 U/mL SOD with ACT under the standard conditions (3.1.1.1). The SOD activity was determined periodically. An incubation of SOD in buffer alone was used as a control. Determination of SOD activity was done at 1, 4 and 8 h from the start of the incubation. The SOD concentrations in the assay cuvettes were 1.0 and 2.73 U/mL for the 200 and 546 U/mL incubations, respectively. Also, 182 U/mL of SOD and 5500 U/mL of catalase were co-incubated in ACT for 1 h, and the SOD activity was determined. These experiments involving co-incubation of SOD and catalase were used with appropriate controls to determine if hydrogen peroxide was involved in damage to SOD caused by ACT.

A multiple addition protocol was used to test the effect of SOD on ACT damage to α1PI. Incubations of SOD in ACT or buffer were used in preliminary experiments to determine survival of SOD in ACT. Aliquots of 5 μL of an SOD stock in incubation buffer (36960 U/mL) were added to the incubations at 0, 4, 8 and 12 h from the start of the incubation. Since the final incubation of SOD (315 μL) contained 20 μL of this stock, the concentration of SOD was 2347 U/mL. SOD activity was measured at 3, 7, 11 and 24 h.
The incubations of α1PI with SOD in 500 μL Eppendorf vials were as follows: 1. α1PI control; 2. α1PI + SOD; 3. α1PI + ACT; 4. α1PI + ACT + SOD. The initial volumes for incubation 1 were 150 μL of 250 μg/mL α1PI stock plus 150 μL of incubation buffer; for incubation 2, 5 μL of 36960 U/mL SOD stock was added in place of an equivalent volume of buffer. For the ACT incubations; 3 received 150 μL of α1PI stock, 145 μL of ACT and 5 μL of incubation buffer, 4 received 5 μL of SOD stock instead of buffer. At 4, 8 and 12 h, incubations 1 and 3 received 5 μL of buffer, whereas incubations 2 and 4 received 5 μL of SOD stock. The EIC values were determined after 24 h incubation under the standard conditions.

3.1.2 Results.

3.1.2.1 The effects of free amino acids on the α1PI-damaging ability of ACT. Only the sulfur containing amino acids show any protection of α1PI. While Met shows protection at 1 mM, Cys protects only at 10 mM (Table 11). The amino acids alone, at the highest concentration used, had no effect on the activity of α1PI.

3.1.2.2 The effects of reducing species and hydroxyl radical scavengers on the α1PI-damaging ability of ACT. Control incubations indicated that 10 mM GSH, DTT, thiourea, 5 mM oxidized glutathione (GSSG), and 50 mM benzoate and mannitol per se, did not affect the EIC of α1PI. Ascorbate alone did affect the EIC of α1PI; at 1 and 10 mM the mean % EIC ± s.d. values were 80 ± 5 and 87 ± 5%, respectively, after a 24 h incubation.
Table 11. The effects of various L-amino acids on the ability of ACT to damage $\alpha_1$PI.

<table>
<thead>
<tr>
<th>Amino Acid*</th>
<th>% Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>4 ± 6</td>
</tr>
<tr>
<td>Arginine</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>Cysteine</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>Cysteine, 1 mM</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>Histidine</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Lysine</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>Methionine</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>Methionine, 1 mM</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Methionine Sulfoxide</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Proline</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Serine</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4 ± 3</td>
</tr>
</tbody>
</table>

*a - All at 10 mM, unless indicated otherwise.
*b - Data are mean ± s.d. of three ACT preparations.
To account for ascorbate exerting this effect in ACT exposures, the % EIC values for α1PI incubated with ACT and ascorbate were calculated using the % I values (see equations 10 to 12, section 2.2.1.1) obtained for incubations of α1PI with ascorbate only. The data on the effects of reducing species and hydroxyl radical scavengers on the α1PI-damaging ability of ACT are shown in Table 12, while Table 13 shows the effect of some of these species under continuously air-purged conditions.

3.1.2.3 The effects of metal ion chelators and ferric ions on the α1PI-damaging ability of ACT. None of the chelators or ferric ions, at the highest concentrations tested, had any effect alone on α1PI activity; the data are shown in Table 14.

3.1.2.4 Studies using catalase. The effect of ~ 5 to 175 U/mL catalase on α1PI exposed to ACT was tested, as was the effect of ACT on ~ 90 to 1450 U/ml catalase. Usually only one run of these experiments was performed, and taken together the experiments show that catalase at these relatively low concentrations is poor at protecting α1PI, probably because the activity is lost during ACT exposure (Tables 15 and 16). In a separate study (using one ACT preparation) catalase concentrations of 1100, 5500 and 11000 U/mL protected α1PI by 33, 33 and 55%, respectively. Catalase alone at these concentrations did not inhibit α1PI activity.
Table 12. The effects of reducing species and hydroxyl radical scavengers on ACT-mediated damage to $\alpha_1$Pl$^a$.

<table>
<thead>
<tr>
<th>Additive</th>
<th>% Protection$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM GSH</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>10 mM GSH</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>5 mM GSSG</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>10 mM DTT</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>1 mM Ascorbate</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>10 mM Ascorbate</td>
<td>86 ± 5</td>
</tr>
<tr>
<td>1 mM Thiourea</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>10 mM Thiourea</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>50 mM Benzoate</td>
<td>0 ± 5</td>
</tr>
<tr>
<td>50 mM Mannitol</td>
<td>6 ± 5</td>
</tr>
</tbody>
</table>

$^a$ - Based on Table 2 in Ref. 190, with permission.

$^b$ - Mean ± s.d. of three ACT preparations.
Table 13. The protective action of GSH, ascorbate, cysteine, DTT, methionine and thiourea toward α,PI in ACT under continuously air-purged conditions.

<table>
<thead>
<tr>
<th>Additive</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>GSH</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>DTT</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Methionine</td>
<td>89 ± 1</td>
</tr>
<tr>
<td>Thiourea</td>
<td>84 ± 2</td>
</tr>
</tbody>
</table>

a - 10 mM.
b - Mean ± s.d. of three ACT preparations.
Table 14. The effects of metal ion chelators and ferric ions on the activity of α,PI exposed to ACT.

<table>
<thead>
<tr>
<th>Additive</th>
<th>% Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM DTPA</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>10 mM DTPA</td>
<td>31 ± 3b</td>
</tr>
<tr>
<td>10 μM EDTA*</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>42 ± 7d</td>
</tr>
<tr>
<td>10 mM EDTA + 50 mM Benzoate</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>10 mM EGTA</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>500 μM Desferal*</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>5 mM Desferal*</td>
<td>-7 ± 4b</td>
</tr>
<tr>
<td>10 mM Bathocuproine Disulfonate</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>10 mM DDC</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>20 mM HIMDA</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>10 μM Fe^{3+}</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>100 μM Fe^{3+}</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>200 μM Fe^{3+}</td>
<td>25 ± 4*</td>
</tr>
<tr>
<td>200 μM Fe^{3+} + 50 mM Benzoate</td>
<td>28 ± 4</td>
</tr>
</tbody>
</table>

a - Mean ± s.d. of three ACT preparations, unless otherwise indicated.
b - Mean ± s.d. of five ACT preparations.
c - Abbreviations: EDTA, Ethylenediamine tetraacetic acid; EGTA, (Ethylenedioxo)diethylenenitrilo tetraacetic acid; HIMDA, N-(2-hydroxyethyl)iminodiacetic acid.
d - Mean ± s.d. of eight ACT preparations.
e - Mean ± s.d. of six ACT preparations.
Table 15. The effect of low concentrations of catalase on the activity of $\alpha_1$PI exposed to ACT.

<table>
<thead>
<tr>
<th>[Catalase], U/mL</th>
<th>% Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>21</td>
</tr>
<tr>
<td>11.0</td>
<td>27</td>
</tr>
<tr>
<td>22.0</td>
<td>23</td>
</tr>
<tr>
<td>44.0</td>
<td>24</td>
</tr>
<tr>
<td>88.0</td>
<td>27</td>
</tr>
<tr>
<td>176.0</td>
<td>29</td>
</tr>
</tbody>
</table>

* - One ACT preparation.
The substrate alone displays a negligible intrinsic decrease in $A_{240}$. Alpha,PI and ACT, either alone or in combination with ACT, have no catalatic activity (Table 16).

Most of the catalase activity (ca. 40%) is lost in the first 4 h and only ca. 5% in the subsequent 4 h of incubation (Table 17). The experiment in which catalase was added to ACT using a multiple addition protocol showed that after 24 h, $101 \pm 8\%$ (mean $\pm$ s.d.; three separate ACT preparations) of the catalase activity remained as compared to the control incubations.

When the multiple addition protocol was used for $\alpha$1PI protection studies the % protection was $82 \pm 1\%$ (mean $\pm$ s.d.; three separate ACT preparations). Additions of catalase alone did not affect the EIC of $\alpha$1PI.

3.1.2.5 The effect of heat-denatured catalase and BSA on the activity of $\alpha$1PI exposed to ACT. Heat-denatured catalase was used to ensure that catalase protection was due to catalytic activity rather than non-specific scavenging of damaging species due to the presence of protein. Also, general protein protective ability was tested using BSA. Heat-denatured catalase and 5 mg/ml BSA protect $\alpha$1PI from ACT damage to a small extent (Table 18). The heat-denatured catalase, and BSA (5mg/mL), per se had no effect on the EIC of $\alpha$1PI.

3.1.2.6 Studies using superoxide dismutase. Similar to the studies with catalase, the effect of ACT on SOD was compared to controls. Therefore,
Table 16. The effect of ACT on the activity of catalase.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Initial Rate* x 10^{-3}</th>
<th>% Control Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>176 U/mL Catalase (rows 1 - 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Catalase + α,PI</td>
<td>6.2 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>2 α,PI + ACT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 α,PI + catalase + ACT</td>
<td>0.5 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td>Catalase, 363 U/mL</td>
<td>35.0 ± 2.0</td>
<td>100</td>
</tr>
<tr>
<td>Catalase, 737 U/mL</td>
<td>70.2 ± 4.7</td>
<td>100</td>
</tr>
<tr>
<td>Catalase, 1100 U/mL</td>
<td>90.1 ± 4.6</td>
<td>100</td>
</tr>
<tr>
<td>Catalase, 1463 U/mL</td>
<td>101.8 ± 4.2</td>
<td>100</td>
</tr>
<tr>
<td>Catalase, 363 U/mL + ACT</td>
<td>1.1 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>Catalase, 737 U/mL + ACT</td>
<td>2.6 ± 1.8</td>
<td>4</td>
</tr>
<tr>
<td>Catalase, 1100 U/mL + ACT</td>
<td>3.4 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>Catalase, 1463 U/mL + ACT</td>
<td>5.4 ± 1.6</td>
<td>5</td>
</tr>
</tbody>
</table>

* Rates are means ± s.d. of triplicate determinations on incubations. In all cases the experiments were run with one ACT preparation.
**Table 17.** The activity of catalase (5500 U/mL) exposed to ACT for up to 8 hours.

<table>
<thead>
<tr>
<th>Time, h</th>
<th>% Control Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>81</td>
</tr>
<tr>
<td>3.5</td>
<td>69</td>
</tr>
<tr>
<td>4.0</td>
<td>62 ± 1*</td>
</tr>
<tr>
<td>6.5</td>
<td>64</td>
</tr>
<tr>
<td>8.0</td>
<td>56 ± 1*</td>
</tr>
</tbody>
</table>

*Mean ± av.dev. of two ACT preparations.*
### Table 18. The effects of heat-denatured catalase and BSA on the activity of α,PI exposed to ACT.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-Denatured Catalase</td>
<td>29 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSA, 0.58 mg/mL</td>
<td>9 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSA, 5 mg/mL</td>
<td>36 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± s.d. for six ACT preparations.
<sup>b</sup> Mean ± s.d. for three ACT preparations.
there was no need to determine absolute activity of SOD. The assay system for SOD was noticeably affected by ACT. The results concerned with these effects are noted in Table 19.

The basal rate of ferricytochrome c reduction is higher in the presence of ACT than in its absence (Table 19). This result is consistent with a previously reported observation (197). Since the basal rate of ferricytochrome c reduction in the presence of ACT is increased, one would expect to see an increased rate of reduction in the presence of X/XO as compared to X/XO alone. However, the rate of reduction by X/XO is approximately 30% lower in the presence of ACT (Table 19). In the presence of ACT, XO activity itself was approximately 10% lower than in the absence of ACT. Thus, a portion of the ACT-mediated decrease in the rate of ferricytochrome c reduction may be due to inhibition of XO. The effect of ACT could also be explained by oxidation of ferrocytochrome c or scavenging of superoxide, although the latter explanation is more likely in view of the aforementioned results (197). Lowering the concentration of ACT leads to a lowered basal rate of ferricytochrome c reduction, but the rate is not restored to control levels, and the X/XO rate is still inhibited. A 5 µL SOD sample was used in the assays (section 3.1.1.7) so as to keep the ACT concentration low.

The results in Table 20 are expressed as "% Reduction". Percent reduction is used as a measure of the rate of cytochrome c reduction in the
Table 19. Studies on the effects of ACT on the SOD assay system.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Basal</th>
<th>Enzymatic</th>
<th>Corrected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>X/XO</td>
<td>4.6 ± 0.1</td>
<td>67.6 ± 1.2</td>
<td>63.0 ± 1.2</td>
</tr>
<tr>
<td>X/XO + 10µL</td>
<td>14.1 ± 0.3</td>
<td>57.5 ± 1.1</td>
<td>43.4 ± 1.1</td>
</tr>
<tr>
<td>ACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µL ACT</td>
<td>13.8 ± 0.3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>X/XO + 5µL</td>
<td>8.4 ± 0.2</td>
<td>51.1 ± 0.4</td>
<td>42.7 ± 0.4</td>
</tr>
<tr>
<td>ACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µL ACT</td>
<td>8.7 ± 0.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>3.8 ± 0.1</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

*Rate x 10⁻³, ΔA₅₅₀.min⁻¹

- Cytochrome c reduction; mean ± av.dev. of two determinations.
- Corrected = enzymatic - basal.
- Urate formation; mean ± av.dev. of two determinations.
<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control SOD, 1 hr</td>
<td>29</td>
</tr>
<tr>
<td>SOD + ACT, 1 hr</td>
<td>79</td>
</tr>
<tr>
<td>Control SOD, 4 h</td>
<td>24</td>
</tr>
<tr>
<td>SOD + ACT, 4 h</td>
<td>73</td>
</tr>
<tr>
<td>Control SOD, 8 h</td>
<td>25</td>
</tr>
<tr>
<td>SOD + ACT, 8 h</td>
<td>87</td>
</tr>
<tr>
<td>Control SOD, 1 h</td>
<td>11</td>
</tr>
<tr>
<td>SOD + ACT, 1 h</td>
<td>54</td>
</tr>
<tr>
<td>Control SOD, 4 h</td>
<td>17</td>
</tr>
<tr>
<td>SOD + ACT, 4 h</td>
<td>58</td>
</tr>
<tr>
<td>Control SOD, 8 h</td>
<td>14</td>
</tr>
<tr>
<td>SOD + ACT, 8 h</td>
<td>49</td>
</tr>
</tbody>
</table>

- One ACT preparation.
presence of SOD as a percentage of the rate in the absence of SOD. The control ferricytochrome c reduction rate for the incubation of SOD in buffer only was that due to X/XO; the control rate for SOD incubated in ACT was that due to X/XO plus 0.25% v/v ACT. Presentation of the data as % reduction allows changes in the control SOD activity to be monitored. The data illustrate that SOD is sensitive to ACT. Over 8 h at 37 °C, native SOD activity remained stable. Instead of using "% Reduction", the % SOD activity is estimated using equation 15:

\[
\%
\text{SOD activity} = 100 \cdot \left( \frac{100 - A}{100 - B} \right)
\]  

(15)

Where "B" is the % reduction in the presence of native SOD and "A" is the % reduction in the presence of SOD treated with ACT. Approximately 50% of the activity of 546 U/mL SOD was lost after 8 h of exposure to ACT. For both 200 and 546 U/mL SOD most of the activity loss occurred within 1 h (~ 60% for 200 U/mL and ~ 50% for 546 U/mL).

The reason(s) for the loss of SOD activity in ACT is unknown. However, one experiment demonstrated that the loss of SOD activity was not dependent on hydrogen peroxide. Using two separate ACT preparations, incubation of 182 U/mL SOD in ACT resulted in ~ 60% loss of SOD activity compared to the control. Co-incubation of 182 U/mL of SOD and 5500 U/mL of catalase ACT for 1 h, resulted in the same loss of SOD activity as seen for ACT alone. Controls showed that: catalase did not have any detectable SOD activity; catalase did not interfere with the
activity of SOD; catalase slightly decreased the ACT enhanced rate of ferricytochrome c reduction.

Despite the sensitivity of SOD to ACT, estimated SOD activity varies between ~30 and 70% over 24 h, when SOD is added to ACT using the multiple addition protocol (Table 21). Although these activities were not ideal, they were preferable to a single addition experiment. If superoxide plays some important role in the ACT damage to $\alpha_1$PI, these additions should have an impact on the ACT-mediated damage to $\alpha_1$PI. Addition of SOD, by the multiple addition protocol, to incubations of $\alpha_1$PI with ACT had no effect on the loss of $\alpha_1$PI activity. The additions of SOD alone did not affect the EIC of $\alpha_1$PI.

3.1.3 Discussion

3.1.3.1 The effect of antioxidant enzymes on the ACT mediated damage to $\alpha_1$PI. The activity of catalase is compromised by ACT, hence the use of relatively low concentrations of catalase fail to provide more than ca. 50% protection. The reasons for this loss of activity are unknown, and the mechanism could be complex, given the number of components present in ACT. However, at least one explanation could be the continual exposure of catalase to ACT-derived hydrogen peroxide and reducing species (including superoxide). Such exposure could lead to the generation of catalytically inactive forms of catalase, such as compound II or III (198,199). Also, the low concentrations of catalase could fail to provide
Table 21. The activity of SOD added by multiple addition to ACT.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control SOD, 3 h</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>SOD + ACT, 3 h</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>Control SOD, 7 h</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>SOD + ACT, 7 h</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>Control SOD, 11 h</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>SOD + ACT, 11 h</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Control SOD, 24 h</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>SOD + ACT, 24 h</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

a - Mean ± av.dev. of two ACT preparations.
adequate protection because of the high $K_m$ of catalase for hydrogen peroxide (200). Since hydrogen peroxide is produced in relatively small quantities in ACT (see section 3.2), the low concentrations of catalase could fail to outcompete the $\alpha_1$-PI-damaging reactions dependent on hydrogen peroxide. Maintenance of catalatic activity was achieved by several timed additions of catalase to incubations. As a result of the substantial protective effect of catalase, hydrogen peroxide appears to be an essential component of ACT-mediated damage to $\alpha_1$-PI. Organic hydroperoxides could be responsible for some $\alpha_1$-PI damage, since catalase may use alkyl and acyl hydroperoxides as substrates (200). However, the predominance of hydroxyl radical adducts and apparent absence of alkoxyl radical adducts in spin-trapping studies suggest that hydrogen peroxide is the major hydroperoxide in ACT (22). Hydrogen peroxide is also thought to be involved in damage to $\alpha_1$-PI caused by aqueous extracts of gas phase cigarette smoke (130).

Particulate phase smoke is reducing relative to the gas phase, yet cigarette smoke can oxidize various chemical species. The oxidative ability of smoke is achieved primarily by the products of dioxygen reduction (18, 201-203). The generation of hydrogen peroxide in ACT has been demonstrated, and probably arises mainly from superoxide formed during autoxidation of polyphenols (particularly hydroquinone and catechol) present in the extracts (22-25).
There are a multitude of reactions that can occur involving the autoxidation of polyphenols and the generation of reactive oxygen species. Some probable reactions are illustrated in equations 16 to 24: \( \text{QH}_2 \), \( \text{QH}^- \), quinol; \( \text{Q}^\bullet^- \), semiquinone; \( \text{Q} \), quinone; \( \text{M} \), transition metal complex. Many of these reactions could occur within the context of a "polymeric quinone matrix" as has been suggested by ESR studies on cigarette tar (18, 21).

\[
\begin{align*}
\text{QH}_2 & \rightleftharpoons \text{QH}^- + \text{H}^+ \quad (16) \\
\text{QH}^- + \text{M}^{n+} & \rightarrow \text{Q}^\bullet^- + \text{H}^+ + \text{M}^{(n-1)+} \quad (17) \\
\text{M}^{(n-1)+} + \text{O}_2 & \rightleftharpoons \text{M}^{n+} + \text{O}_2^\bullet^- \quad (18) \\
\text{Q}^\bullet^- + \text{O}_2 & \rightleftharpoons \text{Q} + \text{O}_2^\bullet^- \quad (19) \\
\text{HO}_2^\bullet + \text{H}^+ + \text{O}_2^\bullet^- & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (20) \\
\text{M}^{(n-1)+} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{M}^{n+} + \text{HO}^\bullet + \text{HO}^- \quad (21) \\
2\text{Q}^\bullet^- + 2\text{H}^+ & \rightleftharpoons \text{QH}_2 + \text{Q} \quad (22) \\
\text{QH}_2 + \text{O}_2^\bullet^- & \rightleftharpoons \text{Q}^\bullet^- + \text{H}_2\text{O}_2 \quad (23) \\
\text{Q}^\bullet^- + \text{M}^{n+} & \rightleftharpoons \text{Q} + \text{M}^{(n-1)+} \quad (24)
\end{align*}
\]

The partial protection shown by heat-denatured catalase indicates that a portion of the protective ability of native catalase may be due to non-specific scavenging of damaging species. Mild protection of \( \alpha_{1}\text{PI} \) by 5 mg/ml (75 \( \mu \text{M} \)) BSA is consistent with scavenging of damaging species by proteins at sufficiently high concentration. The reasons for this protective action are likely to include sacrificial oxidation of susceptible amino acid residues, such as Cys and Met if they are accessible to oxidants.
(however, see section 3.2.3.2). The single Cys-SH group of BSA is known to undergo oxidation to the sulfenic acid and higher oxidation states upon exposure to hydrogen peroxide (204,205). By comparison, 1 mM free Cys (Table 8) poorly protects, thus the approximately 75 μM Cys-SH in BSA is expected to be even less protective. However, the sterically isolated BSA Cys-SH is probably less prone to autoxidize and thus may provide more effective scavenging of oxidants compared to a similar amount of free thiol (section 3.1.3.3).

Albumin (and other proteins) in lung lining fluid could provide a protective function in lung ELF by competing for protein damaging species with more critical targets. Albumins have been proposed to serve an antioxidant function in serum (206,207). The antioxidant function of serum proteins may extend to ELF since some serum proteins are likely to have increased concentrations in smokers’ lungs, due to cigarette smoke-induced increases in alveolar permeability (207-209).

Earlier studies showed that SOD could diminish hydrogen peroxide production in ACT (23). Some other studies concerning whole smoke showed that SOD had only minimal effects on the smoke damage to α₁PI (135). The results reported in section 3.1.2.4 show that even if SOD decreases hydrogen peroxide levels in ACT this has no effect on ACT damage to α₁PI as measured in these studies.
The results in section 3.1.2.6 show that SOD is sensitive to water soluble cigarette smoke components and/or species derived from them. This sensitivity of SOD is apparently greater than that shown by either catalase or \( \alpha_1 \)PI. Comparing the damage by ACT to the three proteins studied: 2.4 \( \mu \)M \( \alpha_1 \)PI lost \( \sim \) 80% activity in 24 h; 2.1 \( \mu \)M catalase lost \( \sim \) 40% activity in 4 h; 1.6 \( \mu \)M SOD lost \( \sim \) 60% activity in 1 h. Although hydrogen peroxide has been reported to inactivate copper-zinc-SOD (210,211), the loss of SOD activity in ACT was apparently not related to hydrogen peroxide since catalase did not protect (section 3.1.2.6). One possibility is that since \( \alpha_1 \)PI exposed to cigarette smoke \textit{in vitro} becomes more anionic [this dissertation and refs (134,212)], the same could happen to SOD thus interfering with the electrostatic guidance mechanism crucial to the activity of SOD (213).

\textbf{3.1.3.2 The effect of free amino acids on the ACT mediated damage to \( \alpha_1 \)PI.} The protective ability of free amino acids toward \( \alpha_1 \)PI was tested in earlier studies on gas phase smoke damage to \( \alpha_1 \)PI (130). The amino acid protection studies could provide some information on the amino acid residues in \( \alpha_1 \)PI that are targets of cigarette smoke damage. The effects of eleven L-amino acids were tested which were selected for either demonstrated susceptibility to oxidative attack (Pro, Met, Phe, His, Trp and Cys) (214,215) or involvement in the proteinase inhibitory activity of \( \alpha_1 \)PI (Met, Ser, Arg, Lys) (51,216-218). Alanine was chosen as an unreactive...
aliphatic amino acid, to test the protective effect of the α-carboxyl and α-amino groups.

The results (Table 8) imply that the nature of the damaging species in gas-phase cigarette smoke and ACT show some differences. The protective action of the unprotonated amine group in gas phase smoke exposures of α,PI is not found for ACT (130). Amines probably protect α,PI exposed to gas phase smoke because modification of the ε-amino group of Lys residues in α,PI results in loss of EIC (216,217). Direct exposure of α,PI to gas phase cigarette smoke results in a fast and slow phase of inactivation [Fig 2, and Ref. 188], amines protect equally well in both phases, probably by different mechanisms (130). Protection from fast phase EIC loss was proposed to arise by interception of peroxynitrates by amines (130): authentic peroxynitrates mimic the fast phase of EIC loss by α,PI exposed directly to cigarette smoke (219). Also, carbonyls in cigarette smoke may react with Lys residues to generate Schiff’s bases, and cigarette smoke can modify protein amine groups by generating cyanomethyl derivatives (212,220,221). Amines may protect α,PI from the slow phase of EIC loss due to gas phase smoke exposure by sacrificial oxidation and formation of cyanomethyl derivatives. However, it is unlikely that amines protect in gas phase smoke exposures by scavenging hydrogen peroxide (see section 3.2.3.2). A previously unconsidered mechanism for amine protection in the slow phase is nucleophilic attack on quinones.
generated in smoke solution (polyhydroxybenzenes are abundant in cigarette smoke, see Chapter 1), these quinones could modify susceptible amine groups in α1PI (222, 223).

Methionine and methionine sulfoxide protect α1PI equally from gas phase cigarette smoke damage (130), but only Met protects in ACT (Table 11). The protective action of Met suggests that oxidation of Met in α1PI could contribute to ACT-mediated EIC loss, which is reasonable given the significant loss of EIC when the reactive center Met\textsuperscript{368} of α1PI is oxidized (see Chapter 1). Oxidation of Met to the sulfoxide by ACT is supported by amino acid analyses (sections 3.2.2.1 & 4.1.2) and the fact that 10 mM MetSO does not protect α1PI against ACT damage (Table 11). The effect of Cys on ACT-mediated damage to α1PI is discussed in section 3.1.3.3.

3.1.3.3 The effect of hydroxyl radical scavengers and reducing species on the ACT mediated damage to α1PI. The data so far suggest that hydrogen peroxide is an important component of the mechanism by which ACT damages α1PI. However, it is uncertain whether hydrogen peroxide is the damaging species per se, hydrogen peroxide could be involved in generation of the ultimate damaging agents(s). Since hydrogen peroxide could be a source of more reactive oxidants, such as hydroxyl radicals, the authentic hydroxyl radical scavengers, benzoate and mannitol, were tested for protective effects on ACT mediated α1PI damage. The data (Table 12) show no protection by hydroxyl radical scavengers, suggesting that
hydroxyl radicals generated in ACT do not affect the EIC of \(\alpha_1\text{PI}\). However, in "cleaner chemical systems" \(\alpha_1\text{PI}\) is damaged by hydroxyl radicals (224). Hydroxyl radicals have been detected in ACT by spin-trapping studies (22) and have been proposed to contribute to DNA damage (33) and to the oxidizing capacity of smoke solutions (135). However, the high reactivity of hydroxyl radicals suggests they could be effectively scavenged by more abundant ACT components before reaching critical sites on \(\alpha_1\text{PI}\). The data would be consistent with reactive oxygen species (ROS) generated site-specifically on \(\alpha_1\text{PI}\) (where scavengers have poor access) being responsible for some of the damage to \(\alpha_1\text{PI}\) in ACT (225). Hydroxyl radical scavengers have been demonstrated to react with hydroxyl radicals generated in ACT (22). Thus, damage to \(\alpha_1\text{PI}\) by radicals derived from scavengers could be another explanation for the lack of protection by mannitol and benzoate. However, previous studies have shown that alkyl, alkoxy, and peroxyl radicals do not affect the EIC of \(\alpha_1\text{PI}\) (219). The lack of protection by mannitol and serine (Tables 11 & 12) imply that homogeneously generated hypervalent metal species, such as ferryl, are not involved in damage to \(\alpha_1\text{PI}\) by ACT (226,227).

Ascorbate and the thiols could exert their protective action in ACT by several mechanisms. The direct reaction of thiols with hydrogen peroxide has been determined to be slow (205,228), but over the extended incubation times used in the studies with ACT direct reaction could be of
some importance. Since ascorbate and the thiols are effective reducing agents they could reduce adventitious transition metal ions in ACT which could then participate in the Fenton reaction, to decompose hydrogen peroxide to more reactive species, such as hydroxyl radicals. Even though the incubation buffer is treated with Chelex®-100, adventitious transition metal ions are likely to be present (229); the cigarettes will also supply transition metals (6). The incubations were performed under air, therefore oxygen is likely to compete very effectively with hydrogen peroxide for reduced transition metals. As discussed below, ascorbate and the thiols could autoxidize in ACT and deplete oxygen in the closed vial incubations; the decomposition of hydrogen peroxide by Fenton reactions would therefore be more pronounced during the later stages of the incubations.

The oxygen content of the closed vials is sufficient to support autoxidation of ascorbate and thiols. Autoxidation will not only deplete oxygen in the vials, but will decrease the concentration of reducing groups and generate ROS (230). Clearly (Table 12) under closed vial incubations the protective abilities of ascorbate and thiols at 10 mM predominate over any contribution their autoxidation may make to the loss of α, β activity by generation of ROS, or their potential to regenerate autoxidizable ACT components such as hydroquinones (223). The protective action of ascorbate and the thiols at 1 mM is negligible (Table 12), probably due to a combination of exhaustion of reducing ability through autoxidation and
poorer competition with $\alpha_1$PI for oxidants. The minimal protective ability of GSSG (Table 12) demonstrates that the sulfhydryl group of GSH is responsible for the protective action of this compound.

In air-purged incubations the protective abilities of the reducing agents are noticeably different (Table 13), the order of $\alpha_1$PI protection is:

Met ~ thiourea > GSH > ascorbate ~ Cys > DTT. This order of protection can be explained by the differing autoxidation properties of the various compounds. Thiourea and Met likely autoxidize the slowest, Cys autoxidizes faster than GSH, and DTT autoxidizes faster than both of the monothiols (231). Under continual air-purging, the differing degrees of autoxidation of the protective species will generate differing quantities of ROS and at the same time deplete differing amounts of protective groups.

Thiourea, like Met, protects at both 1 and 10 mM, which is probably due to slower autoxidation and the demonstrated ability of thiourea to react directly with hydrogen peroxide (232-234). The hydroxyl radical scavenging and metal complexing properties of all of the reducing species probably contributes minimally to their protective ability in ACT, since benzoate, mannitol and high concentrations of potent metal complexing agents fail to provide more than 50% protection (Tables 12 and 14). Even though thiols, ascorbate and thiourea have been reported to react with superoxide (235-237), such reactions are probably unimportant for protection of $\alpha_1$PI against damage by ACT.
Data to be presented later indicate that MetSO formation in $\alpha_1\text{Pl}$ exposed to ACT is the probable explanation for the EIC loss. Thiols, at high concentrations, long (> 15 h) incubation times and often high temperature (> 50 °C), are able to reduce MetSO to Met (238). Since long incubation times are used in the ACT studies and the reducing agent:$\alpha_1\text{Pl}$ molar ratio is about 4000:1 (10 mM reducing species), the protective effect of thiols could partly be due to "repair" of oxidized $\alpha_1\text{Pl}$. Such a repair action may also be feasible for ascorbate and free methionine (239).

3.1.3.4 The effect of chelators on the ACT mediated damage to $\alpha_1\text{Pl}$.

Metals in ACT could participate in damage to $\alpha_1\text{Pl}$ in several ways. More reactive oxidants (such as hydroxyl radicals or hypervalent transition metal species) could be generated from reactions of transition metals with hydrogen peroxide (226). The $\alpha_1\text{Pl}$ could be damaged site-specifically by the interaction of hydrogen peroxide with transition metal-$\alpha_1\text{Pl}$ complexes (225). Also, transition metals are likely to be catalysts of polyphenol autoxidation and subsequent generation of hydrogen peroxide (230). In ACT all of these factors may be involved to differing extents in the actions of the various chelators tested. As noted earlier, adventitious transition metals are likely to be present in the incubation buffer, and the cigarettes may provide a maximum of 0.5 $\mu$M iron and copper in ACT (6,229).

Chelators only exert effects in ACT at high concentrations, this could be due partly to competition between added chelators and chelators in ACT.
(e.g. polyphenols). However, other factors such as limiting concentrations of transition metal ions and reducing species could also be important.

Previous studies demonstrate that 30 mM EDTA added to ACT results in an approximately 16-fold increase in the hydroxyl radical/spin-trap adduct signal (22). This increase in hydroxyl radical flux was proposed to occur via a metal catalyzed Haber-Weiss reaction (equations 18 and 21 below), although quinol/semiquinone driven Fenton reactions could also be involved (equations 17, 18, 21 and 24, below) (22,33,34).

\[
\begin{align*}
M^{n+} + O_2{^*} & \rightarrow M^{(n-1)+} + O_2 \quad (18) \\
M^{(n-1)+} + H_2O_2 & \rightarrow M^{n+} + HO^* + HO^- \quad (21) \\
QH^- + M^{n+} & \rightarrow Q^{*} + H^* + M^{(n-1)+} \quad (17) \\
Q^{*} + M^{n+} & \rightleftharpoons Q + M^{(n-1)+} \quad (24)
\end{align*}
\]

In the absence of EDTA, hydroxyl radical spin adduct signals in ACT are low (22). Thus, transition metal-ACT component complexes cause minimal hydrogen peroxide decomposition, or EDTA serves to transfer hydroxyl radical generation to locations accessible to the spin-trap. Unlike EDTA, 30 mM DTPA abolishes hydroxyl radical spin-adduct signals in ACT (22). Thus it has been concluded that metal catalyzed Haber-Weiss or quinol/semiquinone driven Fenton reactions are not supported by DTPA-bound metals in ACT (22). Protection by DTPA (Table 14) could therefore
occur by inhibition of transition metal-catalyzed polyphenol autoxidation and/or the removal of protein-bound metals from sensitive sites on \( \alpha_1 \text{PI} \). In addition to the mechanisms noted for DTPA, EDTA probably protects \( \alpha_1 \text{PI} \) by degrading hydrogen peroxide to more reactive and less selective ROS via an EDTA-metal ion complex. The failure of benzoate to improve the protective effect of EDTA suggests that hydroxyl radical generation by EDTA-metal complexes does not compromise the protective effects of EDTA.

It is unlikely that DTPA or EDTA protect by the direct scavenging of \( \alpha_1 \text{PI} \)-damaging species. Firstly, additives that would be considered more effective scavengers of \( \alpha_1 \text{PI} \)-damaging agents (Lys, His, Trp and Pro for example) do not protect \( \alpha_1 \text{PI} \) from ACT damage (Table 11). Secondly, EGTA and HIMDA, which are structurally similar to DTPA and EDTA but less effective chelators of transition metals, show poorer protective ability (Table 14).

Ferric ions added to ACT show some protection of \( \alpha_1 \text{PI} \) (Table 14), probably via the degradation of hydrogen peroxide by an iron-ACT component complex. The addition of ferric chloride to cigarette smoke solutions has been shown to increase the decomposition of hydrogen peroxide to more reactive species (25). The fact that benzoate does not enhance protection by ferric ions (Table 14) suggests that the protective effects of ferric ions are not compromised by an increased flux of free
hydroxyl radicals. Other factors, such as binding of ferric ions to \( \sigma_1 \)PI, may negate some of the protective potential of the ferric ions.

In contrast to EDTA and DTPA, Desferal* did not protect \( \sigma_1 \)PI (Table 14), which could indicate that ferric ions are not involved in ACT damage to \( \sigma_1 \)PI. However, the effects of Desferal* in ACT would be expected to be similar to the effects of DTPA, i.e. the inhibition of both the decomposition and generation of hydrogen peroxide. Some studies have shown that 0.5 mM Desferal* inhibits the generation of ROS in cigarette smoke solutions (25). Any protective actions of Desferal* could be compromised by reaction with superoxide in ACT to produce a protein damaging nitroxide radical (240,241).

Copper has received particular attention with regard to site-specific damage to proteins including \( \sigma_1 \)PI (242-245), and could feasibly be involved in \( \sigma_1 \)PI damage by ACT. Both DTPA and EDTA are reasonable cupric ion chelators [log stability constants for Cu (II) of 21.4 and 18.8 respectively (246)], thus their effects could also be explained on the basis of copper complexation. The most protective of all the chelators studied is DDC (Table 14), which because of its structural similarity to thiourea is likely to protect mostly by sacrificial oxidation rather than copper complexation. Bathocuproine disulfonate, a chelator of cuprous ions, does not protect \( \sigma_1 \)PI (Table 14).
Since EDTA, DTPA, Desferal* and histidine were all unable to protect \( \alpha_1 \)PI from damage by ACT, then site-specific damage to critical residues in \( \alpha_1 \)PI by metal catalyzed ROS generation seems unlikely or plays a minor role (225,247-249). Although, if multiple mechanisms operate in ACT to damage \( \alpha_1 \)PI, all of which are dependent on hydrogen peroxide, then elimination of any one mechanism will not prevent the loss of EIC.

3.2 Modelling Aqueous Cigarette Tar Extract-Mediated Damage to \( \alpha_1 \)PI.

The overall aim of the studies in this section was to model ACT-mediated EIC loss of \( \alpha_1 \)PI using simpler systems. Much of the data reported so far implies that the damage to \( \alpha_1 \)PI by ACT contains a substantial oxidative component, dependent on hydrogen peroxide. The studies in this section are designed to determine whether or not hydrogen peroxide \textit{per se} could be responsible for most of the EIC loss of \( \alpha_1 \)PI exposed to ACT.

Studies on ACT/hydrogen peroxide combinations were performed to detect any synergism between ACT components and hydrogen peroxide. Incubations of \( \alpha_1 \)PI with ACT under low oxygen tensions assessed the importance of oxygen in the ACT damaging mechanism.

The above experiments depended on reasonable estimates of the amount of hydrogen peroxide generated by ACT. Some previous studies were helpful in estimating hydrogen peroxide formation in ACT (23).
However, there are some difficulties in using the data from the earlier study (23) and experiments reported in this section of the dissertation were designed to address these difficulties. Since a preliminary study indicated that MetSO was the product of ACT-methionine interaction, experiments were geared toward detection and quantitation of MetSO. The quantitative data could be used to estimate the oxidative capacity of ACT toward Met.

As will be noted in section 3.2.2 the hydrogen peroxide exposures show responses to protective species similar to those observed for the ACT exposures. Hydrogen peroxide exposures adequately account for MetSO formation in \( \alpha_1 \)PI exposed to ACT (section 4.1), but do not reproduce the ACT-damage to \( \alpha_1 \)PI in terms of changes in electrophoretic mobility (section 4.2). Studies using catechol/hydroquinone may provide a better model for ACT damage to \( \alpha_1 \)PI, since the quinols provide oxidants and are reducing species, a situation more akin to ACT.

3.2.1 Experimental.

3.2.1.1 Estimation of the oxidizing capacity of ACT: Analyses of methionine, methionine sulfoxide and valine exposed to ACT. Incubations of 500 \( \mu \)L total volume were performed under the standard conditions in 1 mL Eppendorf vials. Control incubations of 15.6 mM Val + 8.4 mM MetSO or 16.4 mM Val + 11.8 mM Met were done in incubation buffer only. After 24 h incubation the samples were frozen and lyophilized. Following lyophilization the residues were dissolved in 1 mL of 0.4 N boric
acid, pH 10.2 and diluted 1/100 with the same buffer. The samples were then filtered through individual filter syringes (LID/X filter syringes, 0.45 μm, nylon-66 filter; Xydex Corp., Bedford, MA) and the filtrate transferred to HPLC vials.

The HPLC analyses were performed on a Hewlett-Packard, HP 1090 liquid chromatograph equipped with a diode array detector, ternary solvent delivery system, variable-volume auto-injector, auto-sampler, thermostatically controlled column compartment and a 200 x 2.1 mm, 5 μ, Hypersil ODS microbore amino acid analysis column (Hewlett-Packard, Palo Alto, CA). Samples were subjected to an on-line pre-column derivatization procedure, using an injection program, with 25 mM mercaptopropionic acid and 25 mM o-phthaldialdehyde prior to injection (250). Injections of 26 μL were made, containing 5 μL of sample solution mixed with 0.4 N borate, pH 10.2 and the derivatizing agents. Analyses were performed at 35 °C and the chromophore was detected at 338 nm. The mobile phase was composed of a binary solvent system: solvent A, 20 mM sodium acetate, pH 7.2, containing 0.1 mM EDTA, 0.5% v/v tetrahydrofuran and 0.015% v/v triethylamine; solvent B, 80% v/v acetonitrile/20% v/v 0.1 M sodium acetate. Solvent A was decreased non-linearly from 98% to 0% over 15 min, at a flow rate of 0.44 mL/min (250). Calibration curves were constructed using 5, 50 and 100 μM standards containing a mixture of valine, methionine and MetSO. The chromatograms were processed using
software supplied with the HPLC system.

3.2.1.2 The effect of hydrogen peroxide at concentrations relevant to those produced in ACT on the activity of \( \alpha,\Pi \).

The effect of hydrogen peroxide alone on the EIC of \( \alpha,\Pi \) was determined by incubating 1.3 mM hydrogen peroxide with 125 \( \mu \)g/mL \( \alpha,\Pi \) under the standard conditions. Hydrogen peroxide was added as three aliquots, 5, 5 and 10 \( \mu \)L of an ~20 mM stock at 0, 4 and 6 h, to give concentrations of 0.33, 0.66 and 1.3 mM hydrogen peroxide respectively. The final incubation volume was 315 \( \mu \)L. This repetitive addition protocol was adopted to avoid exposing \( \alpha,\Pi \) to hydrogen peroxide in one step.

A time course of the loss of EIC of \( \alpha,\Pi \) exposed to 1.3 mM hydrogen peroxide by the multiple addition protocol was constructed by simply determining the EIC at selected time points. The effects of some of the additives that protected in ACT were also tested in this cleaner hydrogen peroxide system. In all cases incubations were under the standard conditions, using hydrogen peroxide at 1.3 mM added according to the protocol described in the above paragraph.

3.2.1.3 The effect of ACT/hydrogen peroxide combinations, and ACT under low oxygen tension, on EIC loss of \( \alpha,\Pi \).

Studies on the interaction of hydrogen peroxide with ACT components involved exposures of 125 \( \mu \)g/mL \( \alpha,\Pi \) to ACT for 24 h under normal or low oxygen tensions in the presence of 0.9 mM hydrogen peroxide. Hydrogen peroxide was added as
three aliquots, 5, 5 and 10 µL of an 18.5 mM stock at 0, 4 and 6 h, to give concentrations of 0.23, 0.46 and 0.89 mM hydrogen peroxide respectively. The final incubation volume was 415 µL. Also, 4 hr incubations of α,PI with hydrogen peroxide and ACT were performed; hydrogen peroxide was added at the start of the experiment to give a concentration of 0.4 mM in a 400 µL incubation volume. In all cases where hydrogen peroxide solutions were added over time, control incubations received equal volumes of deionized water. Incubations under low oxygen tensions were continuously purged with argon, whereas incubations under normal oxygen tensions were continuously purged with air (see section 3.1.2.2).

3.2.1.4 Catechol and hydroquinone as a model for the effects of ACT on α,PI. Alpha,PI at 125 µg/ml was exposed to 230 µM catechol and 230 µM hydroquinone, the exposures also contained 0.1 µM Fe(III) and 0.3 µM Cu(II). The catechol and hydroquinone stocks were prepared fresh at the start of every experiment. The incubations were done under the standard conditions and the EIC determined after 24 h. The time course of the loss of EIC of α,PI exposed to catechol/hydroquinone was simply determined by assessing EIC at selected time points. In some cases additives were used to assess protective effects toward α,PI exposed to the catechol/hydroquinone mixture.
3.2.2 Results.

3.2.2.1 Estimation of the oxidizing capacity of ACT: Analyses of methionine, methionine sulfoxide and valine exposed to ACT. The data from this experiment are presented in Table 22. When Met is incubated with ACT under the standard conditions, MetSO, and not methionine sulfoxide, is produced. The ACT contains no species that are detectable by the amino acid analysis procedure, as determined when only ACT is injected into the HPLC. The production of MetSO can be used to estimate the oxidizing capacity of ACT toward Met. After 24 h, 1.28 μmoles of Met are oxidized to MetSO/ml ACT (Table 22).

The negligible change in Val and MetSO concentrations after exposure to ACT (Table 22) suggests that ACT has little affect on the integrity of the α-amino or carboxyl groups. Since only about 60% of the decrease in Met content after exposure to ACT is accounted for by the increase in MetSO (Table 22), a portion of Met is depleted in ACT by uncharacterized pathways. If ACT were able to reduce MetSO to methionine, this could lead to an underestimation of the oxidizing capacity of ACT; however, incubation of MetSO with ACT for 24 h does not result in the measurable formation of Met (Table 22).

3.2.2.2 The effect of hydrogen peroxide alone, and in combination with ACT, on the activity of α1-Pl. Under low oxygen tensions, α1-Pl activity is unaffected by ACT (Table 23). Co-incubation of ACT and 0.9 mM hydrogen peroxide
Table 22. Analysis of Met, MetSO and Val exposed to ACT.

<table>
<thead>
<tr>
<th>Incubation*</th>
<th>% change in concentrationb</th>
<th>µmol MetSO formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Val</td>
<td>Met</td>
</tr>
<tr>
<td>Val + Met +</td>
<td>- 1.5</td>
<td>- 18.9 ±</td>
</tr>
<tr>
<td>ACT</td>
<td>± 0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Val + MetSO +</td>
<td>+ 3.1</td>
<td>N.D.*</td>
</tr>
<tr>
<td>ACT</td>
<td>± 0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a - See Experimental.
b - After 24 h, relative to amino acids incubated in buffer only:
   +, indicates increase; -, indicates decrease. Mean ± s.d. of three ACT preparations.
c - As a percentage of initial Met concentration.
d - Mean ± s.d. for 3 ACT preparations.
e - Not detected.


Table 23. The effect of ACT and hydrogen peroxide alone and in combination under normal and low oxygen tensions on the EIC of α,PI.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>[O₂]</th>
<th>Time, h²</th>
<th>% EICᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT Normal</td>
<td>24</td>
<td>15 ± 4ᵉ</td>
<td></td>
</tr>
<tr>
<td>ACT Lowᵈ</td>
<td>24</td>
<td>96 ± 4ᵉ</td>
<td></td>
</tr>
<tr>
<td>ACT Normal</td>
<td>4</td>
<td>70 ± 3</td>
<td></td>
</tr>
<tr>
<td>ACT Low</td>
<td>4</td>
<td>102 ± 2</td>
<td></td>
</tr>
<tr>
<td>ACT + 0.4 mM H₂O₂ Normal</td>
<td>4</td>
<td>55 ± 4</td>
<td></td>
</tr>
<tr>
<td>ACT + 0.4 mM H₂O₂ Low</td>
<td>4</td>
<td>87 ± 3</td>
<td></td>
</tr>
<tr>
<td>ACT + 0.9 mM H₂O₂ Normal</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ACT + 0.9 mM H₂O₂ Low</td>
<td>24</td>
<td>35 ± 2</td>
<td></td>
</tr>
<tr>
<td>0.4 mM H₂O₂ Normal</td>
<td>4</td>
<td>94 ± 3ᵉ</td>
<td></td>
</tr>
<tr>
<td>0.9 mM H₂O₂ Normal</td>
<td>24</td>
<td>33 ± 6ᵉ</td>
<td></td>
</tr>
<tr>
<td>1.2 mM H₂O₂ Normal</td>
<td>24</td>
<td>42 ± 4ᶠ</td>
<td></td>
</tr>
<tr>
<td>1.3 mM H₂O₂ Normal</td>
<td>24</td>
<td>33 ± 14 степени</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ - Incubation time.
b - Unless indicated, data are means ± s.d. of three separate ACT preparations.
c - Five separate ACT preparations.
d - Flushed continually with argon; see Experimental.
e - Six ACT preparations.
f - Five ACT preparations.
g - Seven ACT preparations.
peroxide leads to abolition of EIC after 24 h under normal oxygen tensions, but not under low oxygen tensions (Table 23). Some data were obtained at 4 h incubation to study further the synergistic interactions between ACT and hydrogen peroxide. Under normal oxygen tensions, ACT enhances the damaging effects of 0.4 mM hydrogen peroxide (Table 23). However, under low oxygen tensions ACT only minimally increases the damage to $\alpha$-PI by 0.4 mM hydrogen peroxide (Table 23).

Data for the effects of additives on the activity of $\alpha$-PI exposed to 1.3 mM hydrogen peroxide are presented in Table 24. The percent protection values were calculated according to equations 4 and 5 in section 3.1, with "hydrogen peroxide" substituted for "ACT" in these equations. Figure 6 shows a time course of the loss of EIC of $\alpha$-PI exposed to 1.3 mM hydrogen peroxide added by the multiple addition protocol.

3.2.2.3 Catechol and hydroquinone as a model for the effects of ACT on $\alpha$-PI. The concentrations of catechol, hydroquinone, Fe(III) and Cu(II) used for the exposures of $\alpha$-PI were calculated according to the levels of these species found in 1R1 cigarettes:

- Fe: 0.042 $\mu$g/cig.
- Cu: 0.19 $\mu$g/cig.
- Catechol: 110-300 $\mu$g/cig.
- Hydroquinone: 100-300 $\mu$g/cig. (12).
Table 24. The effect of various compounds on the activity of α,Π exposed to 1.3 mM hydrogen peroxide.

<table>
<thead>
<tr>
<th>Additive*</th>
<th>% Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>Thiourea</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>Methionine</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>DDC</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>DTPA</td>
<td>-2 ± 3</td>
</tr>
<tr>
<td>50 mM Benzoate</td>
<td>-2 ± 3</td>
</tr>
<tr>
<td>EDTA</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>EDTA + 50 mM Benzoate</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>5 mg/mL BSA</td>
<td>-10 ± 10</td>
</tr>
<tr>
<td>GSH</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>Ala</td>
<td>-10 ± 2</td>
</tr>
</tbody>
</table>

*a - All at 10 mM unless indicated otherwise.
*b - Mean ± av.dev. for two independent runs.
Figure 6. Time course of the EIC loss of 125 μg/ml α1PI exposed to 1.3 mM hydrogen peroxide. The hydrogen peroxide was added at three times (see Experimental).
The concentrations used in the exposures assume complete extraction of all four species (250 µg/cig. for each of the dihydroxybenzenes) into ACT, thus the exposures represent upper levels. In hindsight, the addition of the transition metals was perhaps pointless, as phosphate is the most abundant complexing agent in the incubations (100 mM).

Alpha,PI exposed to catechol/hydroquinone [plus Fe(III)/Cu(III)] retained 47 ± 6% EIC (mean ± s.d.; 9 separate incubations). Omission of the Fe(III) and Cu(II) resulted in the retention of a further 10 ± 1% EIC (mean ± av.dev.; 2 separate incubations). The data concerning the effects of various species tested for protective ability are presented in Table 25.

Figure 7 shows a time course of the loss of EIC of α,PI exposed to the catechol/hydroquinone system. The rate of loss of EIC is comparable to that of the hydrogen peroxide and ACT exposures.

3.2.3 Discussion.

3.2.3.1 Estimation of the oxidizing capacity of ACT. If hydrogen peroxide is the primary oxidant of Met in ACT, then MetSO formation can be used to estimate the hydrogen peroxide content of ACT. Peroxides oxidize sulfides with a 1:1 stoichiometry (251), thus data on Met oxidation shown in Table 22 would indicate the formation of 0.64 ± 0.02 µmoles of hydrogen peroxide in the ACT incubations (i.e. 1.28 ± 0.04 mM hydrogen peroxide). A previous study has quantitated hydrogen peroxide production in ACT (23),
Table 25. The effect of various compounds on the activity of α1Pi exposed to 230 μM catechol, 230 μM hydroquinone.a.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>% Protection</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Thiourea</td>
<td>54 ± 1</td>
<td></td>
</tr>
<tr>
<td>10 mM Methionine</td>
<td>96 ± 4</td>
<td></td>
</tr>
<tr>
<td>10 mM DTPA</td>
<td>- 5 ± 5</td>
<td></td>
</tr>
<tr>
<td>10 mM GSH</td>
<td>94 ± 2</td>
<td></td>
</tr>
<tr>
<td>5 mg/ml BSA</td>
<td>92 ± 4</td>
<td></td>
</tr>
<tr>
<td>50 mM Benzoate</td>
<td>- 16 ± 1</td>
<td></td>
</tr>
<tr>
<td>10 mM Methionine sulfoxide</td>
<td>59 ± 1</td>
<td></td>
</tr>
<tr>
<td>10 mM Methionine sulfone</td>
<td>65 ± 1</td>
<td></td>
</tr>
<tr>
<td>10 mM Ala</td>
<td>69 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

a - Plus 0.1 μM Fe(III) and 0.3 μM Cu(II).
b - Mean ± av.dev. for 2 runs.
Figure 7. Time course of the loss of EIC of 125 μg/ml α₁PI exposed to 230 μM catechol/230 μM hydroquinone.
but depending on how the data are interpreted, different quantities of hydrogen peroxide can be calculated to have formed in 24 h. If we assume there is no turnover of hydrogen peroxide in ACT then after 24 h, $0.84 \pm 0.21$ mM hydrogen peroxide would be calculated to be present. On the other hand, if the hydrogen peroxide is continually generated and destroyed, then a minimum of $2.86 \pm 0.24$ mM hydrogen peroxide would be estimated to form in ACT.

It was noted in section 3.2.2.1 that only about 60% of the Met was detected as MetSO. The remaining 40% is unaccounted for and at least two possibilities for the fate of this 40% can be ruled out: Met was not oxidized to methionine sulfone as this would have been detected by HPLC; modification of the $\alpha$-amino or carboxyl groups is unlikely, since Val underwent no overall change. Underestimation of the amount of hydrogen peroxide in ACT would have little impact on the studies of damage to $\alpha$,PI, since higher levels of hydrogen peroxide would incur even greater damage than that observed in the studies presented in section 3.2.2.2. Reduction of MetSO to Met by ACT is undetectable, thus ruling out one possible reason for underestimation.

Gross overestimation of the hydrogen peroxide content of ACT by the Met oxidation method is more troublesome. A major contributor to overestimation would be a lack of specificity of Met oxidation. Most of the oxidizing capability of cigarette smoke solutions is ultimately attributable to
hydrogen peroxide (25). Hydroxyl radical is an obvious candidate as a Met oxidant since this has been suggested to contribute to the oxidizing capacity of cigarette smoke extracts, and hydroxyl radical can oxidize Met to the sulfoxide (135,252). Since the data of Nakayama et al. (23) indicate 0.84 ± 0.21 mM as a lower level for hydrogen peroxide concentration in ACT, the value of 1.28 mM suggested by the Met oxidation data does not seem unreasonable.

3.2.3.2 Hydrogen peroxide and α,PI. If hydrogen peroxide can act alone in ACT to damage α,PI, then a concentration of hydrogen peroxide equal to that formed in ACT over 24 h should depress α,PI activity to a similar extent to that seen for ACT. The data (section 3.2.2.2) suggest hydrogen peroxide could account for most of the damage to α,PI caused by ACT. The time course (Fig. 6) shows that the rate of EIC loss is comparable with ACT-mediated EIC loss. Earlier (section 2.1.3) it was suggested that damaging species could accumulate in aged ACT and the damaging reaction could be slow. The oxidant quantitation data and the slowness of EIC loss upon exposure to hydrogen peroxide supports the idea of the buildup of a slowly damaging species. Although it seems reasonable that hydrogen peroxide could directly damage α,PI in ACT, the response of α,PI to hydrogen peroxide is variable, causing about 70-80% loss in EIC. Hydrogen peroxide could account for most of the damage to α,PI and its damaging effects may be enhanced by interaction with other ACT components.
Some synergism is observed between the damaging actions of hydrogen peroxide and ACT. The apparent synergism between 0.9 mM hydrogen peroxide and ACT is likely due to the additive damaging effect of the exogenous (i.e. added) and endogenous (i.e. from autoxidation of ACT components) hydrogen peroxide. Under argon, the loss of EIC due to the ACT/0.9 mM hydrogen peroxide mixture is attributable to the exogenous hydrogen peroxide alone. The damaging effect of the ACT/0.4 mM hydrogen peroxide mixture cannot be explained by the combined action of exogenous and endogenous hydrogen peroxide, since 0.4 mM hydrogen peroxide alone has no effect on the EIC. The incubation of $\alpha_1$PI with ACT/0.4 mM hydrogen peroxide under argon results in a 13% loss of EIC. Note that the loss of EIC is 15% greater when $\alpha_1$PI is incubated with ACT/0.4 mM hydrogen peroxide under air as compared to $\alpha_1$PI incubated under air with ACT alone (Table 23). Thus hydrogen peroxide can synergize with an unidentified component(s) in ACT to cause enhanced damage to $\alpha_1$PI. These data support an earlier suggestion that cigarette smoke could enhance the damaging ability of hydrogen peroxide produced from inflammatory cells in smokers' lungs (135).

The complete preservation of the EIC of $\alpha_1$PI incubated with ACT under argon confirms the previously assumed dependence on oxygen of ACT damage to $\alpha_1$PI. It is reasonable to conclude that an oxidative mechanism(s) alone accounts for the loss of EIC of ACT-exposed $\alpha_1$PI.
The data in Table 23 allow one other point to be addressed, i.e. is there any difference between the rate of EIC loss when \(\alpha_1\text{PI}\) is exposed to ACT in open as opposed to closed incubation vessels? The answer is no; compare data for 4 and 24 h air-purged ACT exposures (Table 23) and the time course (Fig 1).

Several of the species tested for protective ability in ACT were also tested in the hydrogen peroxide exposures. All of the reducing species protect completely, probably for the same reasons noted in section 3.1.3.3 for ACT. The protection by DDC supports the earlier comment that it can act by scavenging oxidants. If the action of DDC was to remove metals bound to \(\alpha_1\text{PI}\) then EDTA and DTPA would be expected to exert similar protection against hydrogen peroxide damage. Since BSA does not protect \(\alpha_1\text{PI}\) exposed to hydrogen peroxide, this suggests the protective role of BSA in ACT is more than simply by scavenging oxidants. Both EDTA and DTPA do not protect against hydrogen peroxide damage to \(\alpha_1\text{PI}\). Transition metals play a negligible role in the hydrogen peroxide-mediated damage to \(\alpha_1\text{PI}\). Also, the absence of reducing species implies that Fe(III)-EDTA, for example, cannot participate in a radical driven Fenton reaction. Since Ala does not protect \(\alpha_1\text{PI}\) against hydrogen peroxide damage, the protective action of the \(\alpha\)-amino group toward \(\alpha_1\text{PI}\) in gas phase extract exposures (130) is not due to sacrificial oxidation by hydrogen peroxide.
Despite this information it is still not possible to state categorically that hydrogen peroxide alone causes the EIC loss of $\alpha_1$PI exposed to ACT, since hydrogen peroxide could still react with one or more ACT components to generate the ultimate damaging species. Such an idea must account for the slowness of the EIC loss in ACT, e.g. components in ACT that interact with hydrogen peroxide could be limiting to the production of damaging species, or the ultimate damaging species could damage $\alpha_1$PI as slowly as hydrogen peroxide.

The majority of the ACT incubations were performed in closed vials. Since the damaging mechanism is dependent on oxygen, it seems prudent to assess the oxygen content of the incubation vials. The 500 $\mu$L incubation vials contained 300 $\mu$L of incubate and a 200 $\mu$L headspace. Air is 21% $O_2$, and the density of $O_2 = 1.31$ mg/ml at 25 °C.

Vol. $O_2$ in headspace = $(21/100) 	imes 200 \mu$L = 42 $\mu$L.

Weight 42 $\mu$L $O_2 = 1.31 \mu$g/$\mu$L $\times 42 \mu$L = $5.5 \times 10^6$ g.

MW $O_2 = 32$, therefore $5.5 \times 10^6$ g = $1.72 \times 10^6$ mole $O_2$.

Molarity of $O_2$ in headspace = $[(1.72 \times 10^6 \times 1000)/0.2]$

= 8.6 mM

$[O_2]$ in water at 25 °C = 0.26 mM

Therefore incubation vials contain (8.6 + 0.26) ~ 9 mM $O_2$.

The calculation indicates that there is sufficient oxygen present to account for the estimated hydrogen peroxide to be produced in ACT.
As was noted earlier (section 3.1.3.1), much evidence suggests that polyphenols are the major source of hydrogen peroxide in cigarette smoke solutions. The amount of autoxidizable species estimated to be present in ACT will place a limit on the quantity of hydrogen peroxide that could possibly be generated. Phenols are found at about 0.6 mg/cig (6) in the particulate phase and if we assume complete extraction and an average molecular weight of 110 (dihydroxybenzenes) then we would estimate ca. 1.1 mM of these species in a 500 µl incubation of ACT (i.e., as used for the Met oxidation studies). Since it is highly unlikely that the extraction is 100% efficient, 1.1 mM is an upper estimate. The MetSO formation studies estimated at least 1.3 mM "oxidant" in ACT. Therefore there are probably additional autoxidizable species in ACT and oxidized quinones may be reduced after autoxidation; e.g., addition of water to p-benzoquinone generates further autoxidizable species (223).

3.2.3.3 Catechol/hydroquinone and α,PI. Initially it was thought that exposures of α,PI to catechol plus hydroquinone would model more closely the effects of ACT on α,PI than hydrogen peroxide alone. The degree and rate of EIC loss of α,PI exposed to catechol/hydroquinone is comparable with that for the hydrogen peroxide exposures (sections 3.2.2.2 & 3). However, like the hydrogen peroxide exposures the changes in electrophoretic mobility of catechol/hydroquinone-exposed α,PI did not mimic those of ACT-exposed α,PI (section 4.2.2). Also, the effects of agents tested for protective ability
were noticeably different from their effects in ACT. Overall, the catechol/hydroquinone system was a poorer model than the hydrogen peroxide exposures.

It is likely that catechol/hydroquinone damages $\alpha_1$PI by a combination of lysyl group modification and oxidation (of Met). Since MetSO$_2$, methionine sulfone and Ala protect $\alpha_1$PI by ca. 60%, they all share a common protective feature. The $\alpha$-amino group is the probable protective functional group, most likely by nucleophilic attack on quinones. There is precedent in the literature for the addition of amines to quinones, and for loss of EIC of $\alpha_1$PI after modification of lysyl residues (216,217,222). The higher protective ability of Met and GSH would be due to both sacrificial oxidation and reaction with quinones. Also, BSA is very protective and this is likely to be due to effective competition with $\alpha_1$PI for oxidants and quinones. The lack of protection by DTPA implies that metal-catalyzed Haber-Weiss reactions are not involved in the damage to $\alpha_1$PI. This conclusion is supported to some extent by the failure of benzoate to protect. As with the ACT studies other explanations for the failure of benzoate to protect $\alpha_1$PI can be invoked, such as site-specific generation of ROS or homogeneous production of ferryl. Ferric-DTPA complexes, for example, can be reduced by catechols, and presumably $p$-hydroxyphenols, and their semiquinones [the slower reaction of $o$-semiquinones with oxygen implies these would be more effective.
reducing species for Fe(III) (253) ) thus DTPA is not expected to abolish polyphenol autoxidation although it may inhibit it (229,253-255).

Although the catechol/hydroquinone system is a poor model with regard to ACT, the apparent protective effect of amine groups is reminiscent of gas phase cigarette smoke (GPE) studies (130). Damage to α,PI by gas phase cigarette smoke extract is inhibited by unprotonated amine groups and catalase; hydrogen peroxide was proposed as a damaging species (130). Loss of EIC by α,PI exposed to GPE could also involve derivatization of lysyl residues by formaldehyde/hydrogen cyanide (212,220), carbonyls (221) and, on the basis of the studies presented above, quinones (222). One notable difference between the damage due to GPE and catechol/hydroquinone is that the former is not inhibited by the thioether functionality of Met, only the α-amino group (130).
CHAPTER 4

STRUCTURAL DAMAGE TO α,PI BY AQUEOUS CIGARETTE TAR EXTRACT.

4.1 Amino Acid Analysis of α,PI Exposed to Aqueous Cigarette Tar Extract and to Hydrogen Peroxide.

The cyanogen bromide cleavage technique (256) was used to study Met oxidation in α,PI. This analytical procedure does not interfere with the detection of other amino acids, apart from glycine, which under the HPLC conditions employed co-elutes with homoserine generated from the cyanogen bromide-methionine reaction. This amino acid analysis procedure was adopted on the basis of the data already presented, indicating Met oxidation by ACT, and the established fact of EIC loss associated with Met\textsuperscript{358} oxidation in α,PI (see Chapter 1). Tryptophan, proline and cysteine were not determined in these analyses.

4.1.1 Experimental.

Alpha,PI at 125 \( \mu g/ml \) was exposed to ACT (3.1.1.1) or 1.2 mM hydrogen peroxide (3.2.1.2). The EIC loss was determined prior to amino acid analysis.

The samples were filtered through a 10,000 nominal molecular weight limit, low-protein binding, cellulosic membrane filter. The residue was then washed with 2 x 500 \( \mu l \) of water, and the washed residue

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suspended in 210 μl 70% v/v formic acid. The samples in formic acid were transferred to glass screw-cap vials and 100 μl of a 123 mM cyanogen bromide solution in 70% v/v formic acid was added. Since cyanogen bromide is volatile and highly toxic it was always handled in a fume hood with extreme caution. The samples were left to incubate with cyanogen bromide for 24 h at room temperature. After the 24 h incubation, the samples were transferred to hydrolysis tubes and the formic acid removed under vacuum. To the dried samples 100 μl of 6N hydrochloric acid (containing 1.8 mg/ml DTT) was added to each hydrolysis tube, the tubes were placed in a hydrolysis vial. Hydrolysis was done at 110 °C, in vacuo for 24 h. After hydrolysis, the hydrochloric acid was removed under vacuum and the hydrolysate was suspended with agitation in 300 μl of 0.4N borate buffer, pH 10.2. The samples were then filtered through individual filter syringes (0.45 μm, nylon) and the filtrate transferred to HPLC vials.

The amino acid analyses by HPLC were performed as described in 3.2.1.1. Calibration curves were constructed using standards made by dilutions of a commercially prepared 2.5 mM stock (Pierce Chemical Co., Rockford, IL). The chromatograms were processed using software supplied with the HP 1090 liquid chromatograph system.
4.1.2 Results.

The amino acid analysis data for the individual residues are presented as percentages of the total amino acid content (Table 26). The "Literature" values were derived by considering data from references 121 and 257-259: the total number of residues in $\alpha_1$PI was calculated (excluding Cys, Trp and Pro) to be 375; the percentage for Gly + homoserine was derived using the sum of the number of Met (which will produce homoserine after reaction with cyanogen bromide) and Gly residues.

The residue percentages for the native $\alpha_1$PI samples agree well with the literature values. The supplier of the $\alpha_1$PI indicated that the product contains <5% impurities, but this has negligible impact on the amino acid analyses. The only explicable difference in the amino acid content of $\alpha_1$PI exposed to the two treatments is the formation of MetSO. The Leu content of hydrogen peroxide-exposed $\alpha_1$PI was decreased compared to native $\alpha_1$PI. The decrease in Leu is an inexplicable result since hydrogen peroxide would not be expected to modify this or other aliphatic residues. Statistical analysis of the data seems redundant, since the differences between treatments were obvious, however a t-test conducted on all of the data revealed no new insights on the data, apart from a significant difference ($P > 0.05$) in the MetSO contents of the hydrogen peroxide- vs ACT-exposed $\alpha_1$PI.
Table 26. Comparison of amino acid residue contents of native $\alpha_1$-PI, $\alpha_1$-PI exposed to ACT and $\alpha_1$-PI exposed to hydrogen peroxide.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Percentage of total residues*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lit.</td>
</tr>
<tr>
<td>Asx</td>
<td>11.70 (0.77)</td>
</tr>
<tr>
<td>Glx</td>
<td>13.60 (0.8)</td>
</tr>
<tr>
<td>Ser</td>
<td>5.60 (0.53)</td>
</tr>
<tr>
<td>His</td>
<td>3.20 (-)</td>
</tr>
<tr>
<td>Gly + hSer$^a$</td>
<td>8.00 (0.8)</td>
</tr>
<tr>
<td>Thr</td>
<td>7.47 (0.54)</td>
</tr>
<tr>
<td>Ala</td>
<td>6.40 (0.27)</td>
</tr>
<tr>
<td>Arg</td>
<td>1.87 (0.27)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.60 (-)</td>
</tr>
<tr>
<td>Val</td>
<td>6.40 (0.53)</td>
</tr>
<tr>
<td>MetSO</td>
<td>-</td>
</tr>
<tr>
<td>Ile</td>
<td>5.07 (0.54)</td>
</tr>
<tr>
<td>Phe</td>
<td>7.20 (0.53)</td>
</tr>
<tr>
<td>Leu</td>
<td>12.27 (1.07)</td>
</tr>
<tr>
<td>Lys</td>
<td>9.60 (1.07)</td>
</tr>
</tbody>
</table>

a - Mean (s.d.).  b - Sample size = 4.
c - 1.2 mM hydrogen peroxide; sample size = 5.
d - Sample size = 7.  e - hSer; homoserine.
4.1.3 Discussion.

The detection of MetSO in both the hydrogen peroxide- and ACT-exposed \( \alpha_1 \)PI is significant given the demonstrated loss of EIC of \( \alpha_1 \)PI when Met\(^{368} \) is oxidized (55,72,91). Although a number of earlier reports support the idea that Met in \( \alpha_1 \)PI is oxidized after exposure to aqueous cigarette smoke solutions \textit{in vitro}, Met oxidation has not been directly demonstrated (124,130,138,142). The detection of MetSO in hydrogen peroxide-exposed \( \alpha_1 \)PI is not unexpected; MetSO formation is the primary reason for the loss of EIC of \( \alpha_1 \)PI after hydrogen peroxide exposure (260). The population of damaged \( \alpha_1 \)PI resulting from treatment with hydrogen peroxide or ACT is possibly a mixture of \( \alpha_1 \)PI molecules containing none, one or more than one MetSO. Since the hydrogen peroxide and ACT exposures result in EIC loss, then Met\(^{368} \) is the residue that is oxidized predominantly. Treatment of \( \alpha_1 \)PI with oxidants such as Chloramine-T, N-chlorosuccinimide and myeloperoxidase/hydrogen peroxide/chloride results in the oxidation one to two Met, namely Met\(^{368} \) and Met\(^{351} \), both of which are exposed to the solvent on the reactive site loop in \( \alpha_1 \)PI (120,121,261). Thus, the demonstrated susceptibility of Met\(^{351} \) to oxidation makes it reasonable that this residue could also be oxidized in \( \alpha_1 \)PI exposed to ACT, and to hydrogen peroxide. However, it is believed that oxidation of Met\(^{351} \) does not affect EIC (138,262). Other Met residues in \( \alpha_1 \)PI, not accessible to the solvent, could be oxidized after ACT or hydrogen peroxide exposure, since some
studies have indicated that solvent accessibility is not necessarily a determinant of the susceptibility of Met residues to oxidation (263).

Although the above discussion has focused on the oxidation of Met, modification of Lys and Tyr residues in \(\alpha_1\)PI are known to diminish EIC, whereas Cys or Arg modification affect other proteinase inhibitory functions of \(\alpha_1\)Pl (217,218,257,264,265). There is no evidence from the amino acid analyses that either Lys or Tyr are affected by hydrogen peroxide or ACT exposure. Despite the apparent absence of changes to amino acids other than Met, this does not imply that other residues are unaltered by exposure of \(\alpha_1\)PI to hydrogen peroxide or ACT. For example, the sample treatment and harsh protein hydrolysis conditions could reverse some amino acid residue modifications, this is probably more applicable to the ACT-exposed \(\alpha_1\)PI. Some amino acid residue modifications may be irreversible, but could be beyond the limit of detection of the analytical procedure.

Hydrogen peroxide at neutral or slightly alkaline pH is capable of oxidizing thioether, sulfhydryl, imidazole, phenol and indole groups (266). Studies with hydrogen peroxide (Table 26) show no apparent modification of groups other than thioethers, and if other groups are modified, previously published studies imply that they are of little importance with regard to loss of EIC (260).

Addressing the more complex situation with ACT, we would perhaps expect more than just modification of Met to occur. However, the amino
acid analysis reflect no other substantial, irreversible amino acid residue modifications (Table 26). The amino acid protection studies (section 3.1.2.1) would imply that modification, irreversible or otherwise, of amine (ε-amino of Lys), aromatic (Phe), indole (Trp), guanidino (Arg) and imidazole (His) groups, if they occur in ACT-exposed α1PI, are of no consequence with respect to EIC loss. This conclusion necessarily assumes that the free amino acids have similar reactivities to the residues in their various chemical environments in the protein. Interaction of a protein bound metal with hydrogen peroxide can produce highly localized damage affecting very few susceptible residues, however such damage can be devastating to protein function (248); such relatively small changes in amino acid composition may go undetected in the analyses presented in Table 26. Several types of residue may undergo oxidation by this so-called "metal catalyzed oxidation" pathway; a contribution by this pathway to ACT damage to α1PI cannot be discounted.

In summary, the amino acid analysis results, in conjunction with the protection data from the free amino acid studies, suggest that oxidation of Met is the primary cause of EIC loss of α1PI exposed to ACT. Direct oxidation of Met by hydrogen peroxide is a probable mechanism for this modification, although metal catalyzed oxidation of Met could also be involved. Other amino acid residue modifications cannot be disregarded, but it is likely that such modifications are not related to the loss of EIC.
4.2 Analysis of Native and Damaged \( \alpha_1 \) Pi by Denaturing and Non-Denaturing Polyacrylamide Gel Electrophoresis.

4.2.1 Experimental.

Samples of native \( \alpha_1 \) Pi, \( \alpha_1 \) Pi exposed to ACT (2.1.1.3), hydrogen peroxide (3.2.1.2), or catechol/hydroquinone (3.2.1.4), with and without additives were subjected to electrophoresis under both the denaturing and non-denaturing conditions. The EIC values were determined prior to the electrophoresis. The gels were cast in an HSI (Hoefer Scientific Instruments, San Francisco, CA) gel casting unit and run using an HSI SE 250 vertical slab gel electrophoresis unit. The various components of the gel systems were as follows, electrophoresis grade reagents were used throughout. The denaturing gel had the following composition; 0.1% w/v SDS, 11% w/v acrylamide, 0.29% w/v bis-acrylamide, 11% w/v sucrose and 0.37 M Tris*-HCl, pH 8.8. The stacking gel had the following composition; 0.1% w/v SDS, 3.8% w/v acrylamide, 0.1% w/v bis-acrylamide, 0.13 M Tris*-HCl, pH 6.8. The reservoir buffer contained 0.1% w/v SDS, 0.19 M glycine and 0.025 M Tris*-HCl. For denaturing electrophoresis, 100 \( \mu l \) of incubate was mixed with 50 \( \mu l \) of incubation buffer and 150 \( \mu l \) of sample buffer. The samples were heated at 95 °C for 5 min. Blanks consisting of sample buffer and incubation buffer only were also prepared in the same way, as were molecular weight markers appropriately diluted from a commercial concentrate (SDS-PAGE molecular weight standards, low range; Bio-Rad
The final composition of the sample for electrophoresis consisted of: 42 μg/ml α1PI, 2% w/v SDS, 0.1% v/v glycerol, 0.05% v/v mercaptoethanol, 0.001% w/v bromophenol blue and 0.06 M Tris-HCl, pH 6.8. The samples were cooled and 5 μL aliquots were applied to wells in the stacking gel. The gels were run in the anodal direction, at a stable temperature (ca. 23 °C), using 7.5 mA/gel for the stacking gel and 15 mA/gel for the separating gel.

The non-denaturing gels had the following composition; 8% w/v acrylamide, 0.21% w/v bis-acrylamide, 5.5% w/v sucrose and 0.37 M Tris-HCl, pH 8.8. The other components of the electrophoresis system were the same as noted for the denaturing gels, except that SDS was excluded. The sample buffer contained neither SDS nor mercaptoethanol.

After electrophoresis, the gels were immediately fixed and stained for 1 h with a solution of 50% v/v methanol, 10% v/v acetic acid and 0.1% w/v Coomassie Brilliant Blue R-250. After 1 h, gels were destained overnight or until the background was sufficiently clear, with 5% v/v methanol, 7% v/v acetic acid. In most cases the gels were stained further using a silver staining kit (Sigma Chemical Co.) to intensify the bands. After conditioning in 2% v/v glycerol the gels were dried using an HSI gel drier, then photographed.
4.2.2 Results.

The gels are displayed in Figures 8 to 13, the anode is at the bottom of the gel in all cases, where two lanes on the same gel apparently have the same material in them, these were from separate incubations; only one incubation of native α₁PI was used per experiment. Unless indicated otherwise, the catechol/hydroquinone incubations contained Cu(II) and Fe(III).

On the SDS-PAGE gels, the native α₁PI band migrated to a position consistent with its molecular weight (52 kDa) (Fig 8-10). There was no detectable change in band position for the ACT or hydrogen peroxide treatments, but the catechol/hydroquinone exposed α₁PI showed band broadening with some movement to slightly lower molecular weights (Fig 9). Methionine and DTPA had no impact on the ACT exposed α₁PI bands (Fig 10), whereas methionine but not DTPA caused the catechol/hydroquinone band to "tighten" into a native α₁PI band (Fig 10). In the absence of Cu(II) and Fe(III) the catechol/hydroquinone exposure showed no difference in band structure compared to the exposures in the presence of Cu(II) and Fe(III) (Fig 10).

The three types of exposure showed different responses on the non-denaturing gels. The native α₁PI consistently gave two bands, one more intense and anionic than the other (Fig 11 - 13). The identity of the less anionic band is unknown; α₁PI exists in two major isoforms in normal human
Figure 8. SDS-PAGE of native \( \alpha_1 \)PI, \( \alpha_1 \)PI exposed to ACT and \( \alpha_1 \)PI exposed to 1.3 mM hydrogen peroxide. Gel lanes are as follows: lane 1, MW standards; lane 2, native \( \alpha_1 \)PI; lane 3, \( \alpha_1 \)PI exposed to ACT; lane 4, \( \alpha_1 \)PI exposed to 1.3 mM hydrogen peroxide; lane 5, \( \alpha_1 \)PI exposed to ACT; lane 6, native \( \alpha_1 \)PI; lane 7, \( \alpha_1 \)PI exposed to 1.3 mM hydrogen peroxide; lane 8, MW standards.
Figure 9. SDS-PAGE of $\alpha_1$PI exposed to ACT and $\alpha_1$PI exposed to catechol/hydroquinone. Gel lanes are as follows: lane 1, MW standards; lane 2, $\alpha_1$PI exposed to ACT; lane 3, $\alpha_1$PI exposed to catechol/hydroquinone; lane 4, native $\alpha_1$PI; lane 5, $\alpha_1$PI exposed to ACT; lane 6, $\alpha_1$PI exposed to catechol/hydroquinone; lane 7, native $\alpha_1$PI; lane 8, MW standard.
Figure 10. SDS-PAGE of $\alpha_1$PI exposed to ACT and $\alpha_1$PI exposed to catechol/hydroquinone in the presence of DTPA and methionine. Gel lanes are as follows: lane 1, MW standard; lane 2, $\alpha_1$PI exposed to catechol/hydroquinone [no Cu(II) or Fe(II)]; lane 3, $\alpha_1$PI exposed to catechol/hydroquinone plus 10 mM DTPA; lane 4, $\alpha_1$PI exposed to catechol/hydroquinone plus 10 mM methionine; lane 5, native $\alpha_1$PI; lane 6, $\alpha_1$PI exposed to ACT plus 10 mM DTPA; lane 7, $\alpha_1$PI exposed to ACT plus 10 mM methionine; lane 8, MW standard.
Figure 11. Non-denaturing PAGE of native $\alpha_1$PI, $\alpha_1$PI exposed to ACT and $\alpha_1$PI exposed to 1.3 mM hydrogen peroxide. Gel lanes are as follows: lane 1, native $\alpha_1$PI; lane 2, $\alpha_1$PI exposed to ACT; lane 3, native $\alpha_1$PI; lane 4, $\alpha_1$PI exposed to 1.3 mM hydrogen peroxide; lane 5, native $\alpha_1$PI; lane 6, $\alpha_1$PI exposed to ACT; lane 7, native $\alpha_1$PI; lane 8, $\alpha_1$PI exposed to 1.3 mM hydrogen peroxide.
Figure 12. Non-denaturing PAGE of α₁-PI exposed to ACT plus catalase and α₁-PI exposed to ACT under argon. Gel lanes are as follows: lane 1, α₁-PI exposed to ACT plus catalase; lane 2, α₁-PI exposed to ACT; lane 3, α₁-PI exposed to ACT under argon; lane 4, native α₁-PI; lane 5, catalase; lane 6, α₁-PI exposed to ACT plus catalase; lane 7, α₁-PI exposed to ACT under argon; lane 8, α₁-PI exposed to ACT.
Figure 13. Non-denaturing PAGE of $\alpha_1$PI exposed to catechol/hydroquinone plus 10 mM alanine, and $\alpha_1$PI exposed to ACT plus 10 mM amino acids. Gel lanes are as follows: lane 1, $\alpha_1$PI exposed to catechol/hydroquinone; lane 2, $\alpha_1$PI exposed to catechol/hydroquinone plus 10 mM Ala; lane 3, native $\alpha_1$PI; lane 4, $\alpha_1$PI exposed to ACT; lane 5, $\alpha_1$PI exposed to ACT plus 10 mM Lys; lane 6, $\alpha_1$PI exposed to ACT plus 10 mM Arg; lane 7, $\alpha_1$PI exposed to ACT plus 10 mM Met; lane 8, $\alpha_1$PI exposed to ACT plus 10 mM Ala.
serum with pi values differing by 0.2 units. It seems unlikely that the two bands represent two isoforms since the difference in pi is probably too small to have an effect on mobility in these gels. The $\alpha_1$PI was used as received from the supplier, thus this minor band could represent the < 5% contamination. Since the SDS-PAGE gels gave only one band for $\alpha_1$PI it is evident that the contaminant has a similar molecular weight to $\alpha_1$PI. The gels were all stained with silver stain, so the less anionic band is likely present at very low amounts relative to the more anionic band. The results and discussion presented in the following sections pertain to the more intense anionic band.

Exposure of $\alpha_1$PI to ACT results in smearing of the native band in the anodal direction, indicating an increase of overall negative charge on the protein (Fig 11-13). The hydrogen peroxide exposures have no effect on the $\alpha_1$PI band position and intensity. The catechol/hydroquinone exposures also cause smearing of the $\alpha_1$PI band toward the anode (Fig 13). Under argon, or in the presence of catalase, the native $\alpha_1$PI band still smears toward the anode; catalase is more cationic relative to $\alpha_1$PI and does not contain any contaminants that co-migrate with the $\alpha_1$PI (Fig 12). The effect of catechol/hydroquinone on the electrophoretic behavior of the $\alpha_1$PI is mildly reversed by 10 mM Ala (Fig 13). The effect of ACT on the electrophoretic behavior of the $\alpha_1$PI is mildly reversed by 10 mM Lys, Ala, Arg or Met (Fig 13).
4.2.3 Discussion.

The evidence so far indicates that ACT damages $\alpha_1$PI by an oxidative mechanism, primarily oxidation of Met. In an attempt to reproduce the effects of ACT on $\alpha_1$PI the hydrogen peroxide and catechol/hydroquinone models were used. The additional data from electrophoresis has substantiated the inadequacy of the two models. The hydrogen peroxide exposures account reasonably well for the MetSO formation in ACT-exposed $\alpha_1$PI, but do not reproduce the non-denaturing gel changes of ACT-exposed $\alpha_1$PI. On the other hand, the catechol/hydroquinone system does not show the same changes as ACT-exposed $\alpha_1$PI on the SDS gels, but does more closely resemble the changes on the non-denaturing gels.

The increase in the anionic nature of the $\alpha_1$PI exposed to ACT indicates that ACT components mask or eliminate positive charge on the $\alpha_1$PI. An increase in the anionic mobility on non-denaturing gels has been noted for $\alpha_1$PI exposed in vitro to whole smoke extracts (134,136). The $\alpha_1$PI exposed to hydrogen peroxide does not show increased movement toward the anode. Thus, oxidation of Met is not a suitable explanation for the increase in anionic mobility. Since the incubation of $\alpha_1$PI with ACT plus catalase or ACT under argon does not affect the band smearing, then oxidative processes are probably not a involved in this increased anionic movement on non-denaturing gels. Also, the increase in anionic behavior of $\alpha_1$PI after exposure to ACT is not related to EIC loss because under argon
ACT causes the same electrophoretic changes even though the EIC is completely preserved (section 3.2.2.2). One cannot discount, however, that the mechanisms for the band smearing under argon are different for those under air. The ability of 10 mM Lys, Ala or Arg to partly restore native $\alpha_1$PI band character to the ACT-exposed $\alpha_1$PI, yet the failure of these three amino acids at 10 mM to protect against loss of EIC (section 3.1.2.1), supports the idea that the band smearing of $\alpha_1$PI caused by ACT is unrelated to loss of $\alpha_1$PI activity. The band smearing in non-denaturing gels is possibly due to either non-covalent interaction of ACT components with positive charges on $\alpha_1$PI (Lys and Arg side chains) or reversible covalent modification of Lys and/or Arg residues. Covalent modification of 20% of the Lys residues in $\alpha_1$PI (ca. 7 residues) results in a 20% loss of EIC, whereas modification of Arg residues does not affect EIC (217). Evidently ACT components do not affect Lys residues critical to EIC, if such residues are modified by ACT components.

Alpha-1-PI exposed to catechol/hydroquinone shows increased movement toward the anode on non-denaturing gels. In the presence of 10 mM Ala the catechol/hydroquinone-exposed $\alpha_1$PI slightly more like native $\alpha_1$PI. It was suggested in section 3.2.3.3 that catechol/hydroquinone may damage $\alpha_1$PI by a combination of oxidation of Met and covalent modification of Lys. The increase in anionic behavior of the catechol/hydroquinone-exposed $\alpha_1$PI is consistent with modification of positively charged residues.
The protective effect of Ala is probably due to sacrificial modification of the α-amino group which would minimize the electrophoretic changes. Purely oxidative changes would not cause the altered mobility of catechol/hydroquinone-exposed α,PI, since the hydrogen peroxide alone had no effect on α,PI mobility.

On the SDS gels there is no indication of significant aggregation or fragmentation of α,PI exposed to ACT or hydrogen peroxide. The exposure of α,PI to catechol/hydroquinone does shift the native α,PI band to a slightly lower molecular weight range which is indicative of fragmentation of the α,PI. If the SDS gel changes for the catechol/hydroquinone-exposed α,PI are indicate fragmentation then redox active metals could play a role in the generation of species responsible for polypeptide chain scission. Hydroxyl radicals or hypervalent transition metal species generated on, or close to, the protein could cause such scission and the damage would also be dependent on hydrogen peroxide. The addition of 10 mM DTPA to the catechol/hydroquinone incubations does not affect the apparent fragmentation (neither does the exclusion of the Fe(III) and Cu(II) additions), suggesting the following: metals are not involved in the damage; DTPA cannot compete for metals with α,PI (at a DTPA:α,PI molar ratio of 4000:1, this is unlikely); even with DTPA present metals can still participate in reactions such as the radical driven Fenton reaction. Adventitious metal ions can be sufficient to cause protein fragmentation in the presence of hydrogen.
peroxide (249), so, even if the Cu(II) and Fe(III) are not added to the incubations this may have little effect on the damage to \( \alpha_1 \)PI. The dihydroxybenzenes are a source of reducing ability for metal ions to produce more reactive species from hydrogen peroxide that could fragment \( \alpha_1 \)PI. Hydroxyl radicals are produced in solutions containing catechols, DTPA and ferric ions (254). Thus, inclusion of DTPA in the \( \alpha_1 \)PI incubations with catechol/hydroquinone does not eliminate the possibility of ROS generation.

Protein fragmentation mediated by ROS, especially those produced site-specifically, usually results in specific fragmentation patterns, and seldom produces complete dissolution of the protein (215, 243, 248, 249, 267). The shift in the mean band position for the catechol/hydroquinone-exposed \( \alpha_1 \)PI is consistent with loss of fragments of a few kDa. The changes seen for catechol/hydroquinone-exposed \( \alpha_1 \)PI on SDS gels are not related to increased anionic mobility on non-denaturing gels since the ACT-exposed \( \alpha_1 \)PI is also more anionic and yet shows no change in mobility on the SDS gels.

Unlike DTPA, 10 mM Met protects \( \alpha_1 \)PI from catechol/hydroquinone damage and restores the mobility of \( \alpha_1 \)PI on SDS gels to that of the native protein. The protection by methionine is most likely due to scavenging of oxidants, thus preventing the generation of species that could fragment \( \alpha_1 \)PI. Covalent attachment of quinones to \( \alpha_1 \)PI via amino groups, and the
subsequent reduction of the quinones, allows for localized autoxidation of the protein-attached quinols with the generation of ROS.

4.2.4 Summary and future studies.

Aqueous cigarette tar extract, causes a slow steady loss of the EIC of $\alpha_1$PI at 37 °C, pH 7.4, to give at least a 60% loss of EIC in 24 h. The ACT retains its ability to damage $\alpha_1$PI for many hours. Changes in pH and adsorption of $\alpha_1$PI onto particulates are discounted as reasons for the loss of EIC.

The damage to $\alpha_1$PI by ACT is dependent on hydrogen peroxide, but superoxide plays no direct role in the damaging mechanism. Hydroxyl radicals or hypervalent metal species generated in the homogeneous phase are not $\alpha_1$PI-damaging species in ACT, compatible with their high reactivity and unselectivity. The production of reactive oxygen species site-specifically on $\alpha_1$PI cannot be discounted.

The loss of EIC of $\alpha_1$PI is due to oxidative damage. Non-oxidative modification does occur when $\alpha_1$PI is exposed to ACT, resulting in a more anionic protein, but such modification does not cause loss of EIC. The non-oxidative changes are not reflected in an altered content of amino acid residues in $\alpha_1$PI, suggesting such changes are reversible or unrelated to amino acid modification. Additionally, no major fragmentation of $\alpha_1$PI exposed to ACT is detected. Hydrogen peroxide could account for the major portion of damage to ACT-exposed $\alpha_1$PI, by direct oxidation Met$^{358}$. 
Oxidation of Met\(^{368}\) is sufficient to cause a substantial loss of EIC. In support of this, free Met but not MetSO protects against damage to \(\alpha_1\)PI by ACT, and ACT-exposed \(\alpha_1\)PI contains noticeable quantities of MetSO. At concentrations relevant to those produced in ACT, hydrogen peroxide causes appreciable formation of MetSO in \(\alpha_1\)PI and the rate of EIC loss is comparable to that seen for ACT exposure.

Hydrogen peroxide appears to be a reasonable model for the oxidative changes in \(\alpha_1\)PI, but does not reproduce the non-oxidative modifications. Exposure of \(\alpha_1\)PI to catechol/hydroquinone was used to try to mimic the EIC loss, non-oxidative changes and the effects of protective agents in the ACT exposures. Catechol/hydroquinone causes a similar rate of EIC loss to ACT and also increases the anionic mobility \(\alpha_1\)PI, but otherwise this system bears minimal resemblance to ACT. Damage to \(\alpha_1\)PI by catechol/hydroquinone probably occurs by a combination of oxidation of Met and modification of Lys.

The protective action of sulfhydryls and ascorbate in ACT varies with the incubation conditions, and could be due to various factors, \textit{e.g.} direct reaction with hydrogen peroxide. There are many potential roles for transition metals in the mechanism of ACT damage to \(\alpha_1\)PI, but none of the chelators tested protect completely. Protection by the chelators is probably related to the inhibition of production, or the degradation of, hydrogen peroxide. Removal of protein bound metals from \(\alpha_1\)PI, to inhibit metal
catalyzed protein oxidation of Met for example, probably would not affect the loss of EIC since hydrogen peroxide could still directly oxidize critical methionyl residues.

Several factors need to be taken into account concerning the potential for cigarette smoke particulate components to damage \( \alpha_1 \)PI in vivo as addressed in the following discussion. Damage to \( \alpha_1 \)PI is thought to be a highly localized event. One would not expect homogeneous distribution of \( \alpha_1 \)PI, antioxidants or cigarette smoke components in the lung. Thus, the chance for direct damage to \( \alpha_1 \)PI by cigarette smoke components is greater at sites in the lung where the \( \alpha_1 \)PI and antioxidant concentrations are low and the concentration of cigarette smoke components is high. The detection of very localized damage difficult because the bronchoalveolar lavage techniques currently used do not enable the sampling of lung microenvironments.

Lung ELF is endowed with several low molecular weight antioxidants, such as glutathione, ascorbate and vitamin E and the variation in the amount of some of these antioxidants in smokers’ ELF has been measured (268-270). Also, the ELF contains macromolecular antioxidants; ceruloplasmin, transferrin, catalase and SOD have been detected (269,271,272). The various antioxidants in ELF are likely to hinder not only any damage to \( \alpha_1 \)PI caused by the direct action of cigarette smoke, but also damage due to inflammatory cell oxidants (273). Hydrogen peroxide can
diffuse across membranes, and therefore in addition to extracellular scavenging, hydrogen peroxide generated extracellularly can be degraded intracellularly (137, 274-276).

Cigarette smoking imposes a chronic oxidative burden on the lung. In areas where there is ongoing oxidative insult to the lung lining fluid, and thus perhaps poorer antioxidant protection, cigarette smoke components would be expected to have a more direct impact on \( \alpha_1 \)PI. Smokers almost continually introduce cigarette smoke material into their lungs to replace that which is slowly removed by the impaired clearance mechanisms. Under normal circumstances, \( \alpha_1 \)PI, HNE and elastin are present simultaneously, and the presence of the elastin ensures that even relatively mild decreases in \( \alpha_1 \)PI activity can markedly increase the degradation of elastin by HNE (72, 131).

The studies in this dissertation arose from previous studies \textit{in vitro} by the Pryor research group on gas phase smoke interaction with \( \alpha_1 \)PI and the interaction of ACT with DNA. Further examination of ACT damage to \( \alpha_1 \)PI would probably serve only to fine tune the study. Additional studies of cigarette smoke component/\( \alpha_1 \)PI interaction \textit{in vitro} are probably of more use in conjunction with \textit{in vivo} studies. Human studies are beyond the immediate scope of the Pryor group, but some more useful human studies await the development of substantially improved lung fluid sampling techniques. Two further studies related to cigarette smoke/\( \alpha_1 \)PI interaction that could be done with experimental animals are presented below.
One of the most outstanding problems to date concerning the direct interaction \emph{in vivo} of $\alpha_1$PI with cigarette smoke components is whether it actually occurs! Many studies have implied that oxidation of $\alpha_1$PI occurs \emph{in vivo} after acute and chronic cigarette smoke inhalation (see Chapter 1). However, the relative contribution to such supposed oxidation from cigarette smoke vs inflammatory cells is unknown. Those studies that show no apparent oxidation of $\alpha_1$PI in smokers' lungs frequently do not discount oxidation for a variety of reasons including poor sampling of lung microenvironments or reluctance to discount oxidation. Unless cigarette smoke oxidants cause a unique and reproducible pattern of Met oxidation then searching for oxidized $\alpha_1$PI is a poor way to assess its direct interaction of cigarette smoke components. One could search for evidence of some unique protein modifications caused by cigarette smoke such as cyanomethylation quinone addition to Lys side chains. \emph{In vitro} studies are required to detect if authentic quinones or those in smoke can react with amino groups in $\alpha_1$PI.

Another avenue of \emph{in vivo} research is related to determining the macromolecular antioxidant status of lung ELF after cigarette smoke inhalation. Ideally, this would be done after both acute and chronic smoke exposures using a suitable animal model. Whereas studies of low molecular weight antioxidants in ELF are ongoing in other laboratories, the macromolecular antioxidant status is less well studied (268,269). Part of
the reason for this poorer study of macromolecular antioxidants in ELF is that their presence has only recently been appreciated (271,277,278). The ELF provides the initial antioxidant defense for the lung and so the ability of this fluid to diminish oxidant levels obviously places less of a burden on the underlying cell layer as well as protecting important components of the ELF. Since the macromolecular antioxidants are proteins their function could be impaired by the action of cigarette smoke and inflammatory cell-derived oxidants. This study unfortunately suffers from the same problem of microenvironment sampling, but this has not hindered the acquisition of useful data on the low molecular weight antioxidants.

Obviously the best preventative measure for cigarette smoke-induced disease is not to smoke, but this approach has evidently failed in numerous cases. Since cigarette smoking is unlikely to disappear in the near future, continued research on the chemistry of cigarette smoke and the pathological mechanisms of cigarette smoking-induced disease is justified. Smoking is a major public health problem that proves costly both in terms of life and healthcare. Research into the basic mechanisms of smoking-induced disease should ultimately enable the development of suitable measures to reduce the risk to smokers' and non-smokers' health, until such a time as the smoking habit is abandoned.


APPENDICES

APPENDIX A

Alpha,PI, or $\alpha_{PI}$ - Alpha-1-proteinase inhibitor.

ACT - Aqueous cigarette tar extract.

AM - Alveolar macrophage(s).

Av.dev. - Average deviation.

BPTI - Bovine pancreatic trypsin inhibitor.

BSA - Bovine serum albumin.

DDC - Diethyldithiocarbamate.

DGP - Direct gas phase.

DTPA - Diethylenetriamine pentapentaacetic acid.

DTT - Dithiothreitol.

EDTA - Ethylenediamine tetraacetic acid.

EGTA - (Ethylenedioxy)diethylenenitrilo tetraacetic acid.

EIC - Elastase inhibitory capacity.

ELF - Epithelial lining fluid.

ESR - Electron spin resonance spectroscopy.

GPE - Gas phase extract.

GSH - Glutathione.

GSSG - Oxidized glutathione.
HIMDA - N-(2-hydroxyethyl)iminodiacetic acid.

HNE - Human neutrophil elastase.

HPLC - High pressure liquid chromatography.

MetSO - Methionine sulfoxide.

NE - Neutrophil elastase.

PMN - Polymorphonuclear leukocyte(s); neutrophil(s).

PPE - Porcine pancreatic elastase.

ROS - Reactive oxygen species.

SANA - N-succinyl-(L-Ala)₃-p-nitroanilide.

s.d. - Standard deviation.

SOD - Superoxide dismutase.

TPM - Total particulate matter.

X/XO - Xanthine/xanthine oxidase.
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Dr. Mark D. Evans
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*JCI 52:1343-1352, 1990, Fig. 1A*
September 24 1991

Ronald G. Crystal, M.D.
Chief, Pulmonary Branch
National Heart Lung and Blood Institute
National Institutes of Health
Bethesda, MD 20892.

Dear Dr. Crystal,

I am writing to you in reference to two of your articles: R.G. Crystal, "Alpha-1-antitrypsin deficiency, emphysema, and liver disease: genetic basis and strategies for therapy", published in The Journal of Clinical Investigation (1990) volume 52, number 5, pages 1343-1352, and R.C. Hubbard and R.G. Crystal "Antiproteases" in "The Lung: Scientific Foundations. Vol.2", edited by R.G. Crystal, J.B. West, P.J. Barnes, N.S. Cherniack and E.R. Weibel, 1991, pages 1775-1787. I am writing a PhD dissertation and would like to modify and use Figure 1A from article in The Journal of Clinical Investigation and Table 1 from the article in "The Lung: Scientific Foundations. Vol.2". I have enclosed a copies of both the modified Table and Figure as they would appear, should you grant me permission to use them.

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Mark Dennis Evans was born in Wolverhampton, England on December 16th, 1962. He attended Highfields Comprehensive School, Wolverhampton, graduating in June 1981. Afterwards he attended Brunel University, Uxbridge, England, where in 1985 he obtained a Bachelor of Science (Hons.), Second Class (Upper Division) in Applied Biochemistry. He came to Louisiana State University in 1986 to pursue a graduate education. Mark Dennis Evans is currently a candidate for the degree of Doctor of Philosophy in the Department of Biochemistry.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Biochemistry

Title of Dissertation: In vitro Studies on Cigarette Smoke-mediated Damage to Human Alpha-1-proteinase Inhibitor

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