1991

Studies on Cigarette Smoke: I. Inactivation of Alpha-1-Pi. II. Oxidation of Methionine by Peroxonitrite. III. Release of Iron From Ferritin by Smoke Solutions.

Juan Jose Moreno

Louisiana State University and Agricultural & Mechanical College

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Studies on cigarette smoke: I. Inactivation of alpha-1-PI. 
II. Oxidation of methionine by peroxynitrite. III. Release of iron 
from ferritin by smoke solutions

Moreno R., Juan José, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991
STUDIES ON CIGARETTE SMOKE:

I. INACTIVATION OF ALPHA-1-PI.

II. OXIDATION OF METHIONINE BY PEROXONITRITE.

III. RELEASE OF IRON FROM FERRITIN BY SMOKE SOLUTIONS.

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 Chemistry

by

Juan José Moreno R.
B.S., Saint Louis University, 1984
August, 1991
ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. William A. Pryor for his guidance and support in the accomplishment of this dissertation, the National Institute of Health and the National Foundation for Cancer Research for financial support, and the Coates Memorial Foundation for financial support in the preparation of this dissertation. I would also like to thank the members of my committee, Profs. J. Selbin, J. M. Jaynes, M. D. Barkley, L. M. Babcock, and former member Prof. R. C. Montelaro, for their generosity and readiness to assist me when I required their help. In addition, I would like to acknowledge Susan F. Hall for simplifying my life as a graduate student, Dr. V.R. Rao for instructing me on protein analysis, Dr. J. Nelson for assistance with NMR interpretations, Dr. D. Vargas for technical support with varied NMR and other not necessarily science-related difficulties, and Dr. R. Cueto for bringing me out of the dark ages by sharing with me his knowledge of computers. Thanks also to Profs. N. Fischer and S. Watkins for their encouragement and moral support.

I would also like to thank my friends and colleagues Mr. M. D. Evans and Dr. J. G. García for invaluable discussions and their priceless company. Finally I would like to also express my gratitude to the Zebouni family for their love and care.
To my mother Manoli,
my father José,
my brother Fernando, and
to Mia
FOREWORD

This dissertation is composed of three chapters that investigate the interaction of cigarette smoke with two proteins: α-1-proteinase inhibitor and ferritin. The main purpose in this investigation is to elucidate possible mechanisms by which cigarette smoking causes a number of pathological conditions, principally pulmonary diseases.

The first chapter, "Inactivation of alpha-1-proteinase Inhibitor by Cigarette Smoke", will be submitted for publication to *Biochemical and Biophysical Research Communications*. This chapter reports on studies done on the mechanism(s) of inactivation of α-1-proteinase inhibitor by cigarette smoke.

The second chapter, "Peroxynitrite Inactivates α-1-proteinase Inhibitor by Oxidation of Methionine", will be submitted for publication to *Chemical Research in Toxicology*. This chapter investigates the mechanism of inactivation of α-1-proteinase inhibitor by peroxynitrite and its connection with the pathogenesis of emphysema.

Finally, chapter three, "Release of Iron from Ferritin by Aqueous Solutions of Cigarette Smoke", was authored by Juan J. Moreno, Mahtab Faroozesh, Daniel F. Church and William A. Pryor. The manuscript has been submitted for publication to *Chemical Research in Toxicology* on May 1991. This chapter reports on the ability of cigarette smoke solutions to release iron.
iron from ferritin and on the identification of possible species in cigarette smoke responsible for the reduction and release of iron from ferritin.
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<td>Alpha-1-PI or α1PI</td>
<td>alpha-1-proteinase inhibitor</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>COSY</td>
<td>two-dimensional correlation spectroscopy</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EIC</td>
<td>elastase inhibitory capacity</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HPLC-ECD</td>
<td>high pressure liquid chromatography with electrochemical detection</td>
</tr>
<tr>
<td>MER10</td>
<td>Pro-Met-Ser-Ile-Pro-Pro-Glu-Val-Lys-Phe</td>
</tr>
<tr>
<td>MER10(SO)</td>
<td>MER10 containing methionine sulfoxide</td>
</tr>
<tr>
<td>MetSO</td>
<td>methionine sulfoxide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAM</td>
<td>pulmonary alveolar macrophages</td>
</tr>
<tr>
<td>PDMS</td>
<td>plasma desorption mass spectrometry</td>
</tr>
<tr>
<td>RELAYED-COSY</td>
<td>relayed coherence transfer spectroscopy</td>
</tr>
<tr>
<td>SANA</td>
<td>N-succinyl-(L-ALA)_3-p-nitroanilide</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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We have studied the interaction of cigarette smoke with two proteins: alpha-1-proteinase inhibitor (α1PI) and ferritin. The inactivation of α1PI, the major plasma inhibitor of serine proteases, by cigarette smoke has been implicated in the etiology of pulmonary emphysema. Release of iron from ferritin, the principal source of physiological iron, by cigarette smoke could explain increased amounts of iron observed in the pulmonary alveolar macrophages of smokers.

These studies show that cigarette smoke reacts with proteins to cause amino acid modifications by two mechanisms: oxidation of methionine and nonoxidative modification of the basic amino acids. Modifications of methionine and lysine result in impairment of the elastase inhibitory activity of α1PI. We have indirectly shown that hydrogen peroxide, formed during the autoxidation of hydroquinones found in smoke, could contribute to oxidation of methionine. Modification of Lys residues could arise from their reaction with formaldehyde and cyanide found in cigarette smoke. Using NMR analysis, we have tentatively identified N-ε-cyanomethyl lysine as the product of the reaction of a synthetic peptide, containing the amino acid sequence of the active site of α1PI, with cigarette smoke.

Peroxonitrite could be formed from the reaction of nitric oxide and superoxide in aqueous solutions of smoke. Kinetic studies show this
peroxide decomposes with a half-life of 4.6 s at physiological pH, and rapidly inactivates a1PI in a dose-dependent manner, suggesting that peroxonitrite is one species responsible for the smoke-induced inactivation of a1PI. Our results also indicate that peroxonitrite is a powerful oxidant, capable of oxidizing methionine residues in proteins without the involvement of hydroxyl radicals or nitrogen dioxide.

Solutions of cigarette smoke induce iron release from ferritin. Under anaerobic conditions, faster rates of iron release are observed and the reducing power of cigarette smoke solutions is prolonged. The concentrations of hydroquinone and catechol in cigarette smoke solutions increase as the smoke is subjected to less filtration. Their increase correlates with higher rates of iron release. These observations indicate that cigarette smoke could release iron from ferritin and alter iron metabolism, increasing the oxidative burden in the lungs of smokers.
INTRODUCTION

Cigarette Smoke Overview. Cigarette smoke is an enormously complex chemical system; about 4000 compounds have been identified (1) in smoke, and this number is constantly increasing as new and more sensitive techniques are developed. Smoke is formed from the incomplete combustion of tobacco and leads to the formation of two types of smoke: mainstream and sidestream (2). Mainstream smoke (MS) is defined as the smoke generated in the burning cone and in other hot zones of the cigarette during puff-drawing which, after travelling through the tobacco column, comes out at the mouthpiece. Sidestream smoke is generated between puffs as the cigarette smolders, and is emitted into the ambient air. The data presented throughout this dissertation was obtained utilizing mainstream smoke generated from reference cigarettes and using standardized smoking parameters (3). The reference cigarettes used (1R1, 1R4F, and 2R1F) are considered international standards for research purposes and have been approved by representatives of commercial manufacturers and by the Tobacco and Health Research Institute (4). For scientists involved in the numerous aspects of smoke research, the use of standard cigarettes provides a common basis for comparison of data.

Of all the effluents of smoke\(^1\), about 30% originates from the

\(^1\)Unless otherwise indicated, "smoke" refers to mainstream cigarette smoke.
tobacco and the remainder comes from air drawn through the porous paper (1). About 500 mg of smoke is obtained from a nonfiltered cigarette (whole smoke) with approximately 83% of the total weight accounted for by nitrogen, oxygen, and carbon dioxide. About 8% is comprised of particulate matter, and 3-4% is carbon monoxide. The remaining 6% of the mass is composed of inorganic and organic vapor phase constituents (1). Table I presents estimates of the number of constituents identified in the major classes of compounds found in cigarette smoke.

By convention the cigarette smoke industry separates whole smoke into gas phase, particulate phase and tar. Gas-phase is defined as the portion of whole smoke that passes through a Cambridge filter, a glass-fiber filter capable of retaining 99.9% of all particles with diameters of 0.2 μm or larger (5). The particulate phase is defined as all the matter retained in the Cambridge filter and tar as the portion of this material minus the weights of nicotine and water.

Cigarette Smoke Chemistry. Although the presence of free radicals in cigarette smoke was first observed at the beginning of 1960 (6,7), we have learned more about their mechanisms of formation, structures, and chemical reactivities in the past fifteen years through the work of Pryor et al. (8-16).

Cigarette smoke contains two classes of free radicals, one found in tar and the other in the gas phase (17). The tar radical is exceptionally stable, allowing direct observation by electron spin resonance (ESR) spectroscopy.
<table>
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<th>Major Classes of Compounds</th>
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<tr>
<td>Amides, imides, lactones</td>
<td>240</td>
</tr>
<tr>
<td>Carboxylic acids, anhydrides</td>
<td>240</td>
</tr>
<tr>
<td>Lactones</td>
<td>150</td>
</tr>
<tr>
<td>Esters</td>
<td>475</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>110</td>
</tr>
<tr>
<td>Ketones</td>
<td>520</td>
</tr>
<tr>
<td>Alcohols</td>
<td>380</td>
</tr>
<tr>
<td>Phenols</td>
<td>285</td>
</tr>
<tr>
<td>Amines</td>
<td>200</td>
</tr>
<tr>
<td>N-Nitrosamines</td>
<td>22</td>
</tr>
<tr>
<td>N-Heterocyclics</td>
<td>920</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>755</td>
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<tr>
<td>Nitriles</td>
<td>105</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>45</td>
</tr>
<tr>
<td>Ethers</td>
<td>310</td>
</tr>
<tr>
<td><strong>TOTAL(^b)</strong></td>
<td>4865</td>
</tr>
</tbody>
</table>

\(^a\) From reference (1).

\(^b\) About 3900 compounds are known; some compounds contain several functional groups (1).
This radical has been identified as a quinone-hydroquinone complex (Q/QH₂) held in a low-molecular weight, tarry matrix (13). The identification of this tar radical as a quinone-hydroquinone complex is corroborated by the high concentrations of quinones and hydroquinones found in cigarette tar (1,18,19). This Q/QH₂ radical can reduce dioxygen to form the superoxide ion which in turn dismutates to hydrogen peroxide, known to form in aqueous solutions of cigarette smoke (20-22). Tar also chelates metals such as iron or copper which can catalyze the decomposition of hydrogen peroxide to form hydroxyl radicals (23). Such tar solutions have been shown to cause single-strand breaks in DNA (24) impeding its synthesis (25).

In contrast, the radicals in gas-phase smoke are much lower in concentration and less stable than the tar radicals (12); consequently, ESR spin trapping techniques have been used for their detection (8). Pryor et al. (8,12,16) identified the radicals in gas-phase smoke as consisting of inorganic radicals such as nitric oxide (NO) and nitrogen dioxide (NO₂), as well as small carbon- and oxygen-centered organic radicals. The apparent half-life of these gas-phase radicals is found to be about 5 minutes (8,12) which is inconsistent with their known stabilities. This inconsistency has been resolved by proposing a steady state mechanism by which the organic radicals in gas-phase smoke are continuously being formed as well as destroyed (16); this mechanism is shown in equations 1 through 5. Also, in
support of this mechanism proposed by Pryor et al., we present in Table II the major constituents identified in gas-phase cigarette smoke.

Nitric oxide (NO) is slowly oxidized to NO₂ (Eq. 1). NO₂ then reacts with an olefin such as isoprene, via allylic hydrogen abstraction (Eq. 2) or addition (Eq. 3), to give carbon centered radicals (R'). These carbon-centered radicals could then be scavenged by oxygen (Eq. 4) to form peroxyl radicals (ROO'). Due to the high concentration of NO in the gas-phase, these peroxy radicals are expected to react with NO (Eq. 5) to give NO₂ and an alkoxy radical (RO').

\[
\begin{align*}
\text{NO} + \text{O}_2 & \rightarrow \text{NO}_2 \quad (1) \\
\text{NO}_2 + RH & \rightarrow \text{HONO} + R' \quad (2) \\
\text{NO}_2 + \text{C}==\text{C} & \rightarrow \text{C} - \text{C}^* \quad (3) \\
R' + \text{O}_2 & \rightarrow \text{ROO}^* \quad (4) \\
\text{ROO}^* + \text{NO} & \rightarrow \text{RO}^* + \text{NO}_2 \quad (5)
\end{align*}
\]
<table>
<thead>
<tr>
<th>Type</th>
<th>Compound</th>
<th>Conc./Cigarette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic Gases</td>
<td>oxygen</td>
<td>67 mg</td>
</tr>
<tr>
<td></td>
<td>carbon monoxide</td>
<td>10-23 mg</td>
</tr>
<tr>
<td></td>
<td>hydrogen cyanide</td>
<td>300 µg</td>
</tr>
<tr>
<td></td>
<td>nitrogen oxides</td>
<td>50-600 µg</td>
</tr>
<tr>
<td></td>
<td>ammonia</td>
<td>50-170 µg</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>acetaldehyde</td>
<td>0.5-1.2 mg</td>
</tr>
<tr>
<td></td>
<td>formaldehyde</td>
<td>100 µg</td>
</tr>
<tr>
<td></td>
<td>acrolein</td>
<td>50-100 µg</td>
</tr>
<tr>
<td>Ketones</td>
<td>acetone</td>
<td>350 µg</td>
</tr>
<tr>
<td>Alkenes</td>
<td>isoprene</td>
<td>400 µg</td>
</tr>
<tr>
<td></td>
<td>ethylene</td>
<td>160 µg</td>
</tr>
<tr>
<td>Aromatic Hydrocarbons</td>
<td>benzene</td>
<td>20-50 µg</td>
</tr>
<tr>
<td></td>
<td>toluene</td>
<td>80 µg</td>
</tr>
<tr>
<td>Organic Halides</td>
<td>methyl chloride</td>
<td>160 µg</td>
</tr>
<tr>
<td></td>
<td>vinyl chloride</td>
<td>1-25 µg</td>
</tr>
<tr>
<td>Others</td>
<td>hydrazine</td>
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</tr>
<tr>
<td></td>
<td>urethane</td>
<td>20-38 ng</td>
</tr>
<tr>
<td></td>
<td>2-nitropropane</td>
<td>0.2-2.2 µg</td>
</tr>
<tr>
<td></td>
<td>methyl nitrate</td>
<td>500 ng</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>6 mg</td>
</tr>
</tbody>
</table>

*This table was composed from data reported mainly in two articles (1,19). Although nitrogen and carbon dioxide are two of the major components of the gas phase of cigarette smoke, they are not included in this table. In addition to the compounds listed, there are about 400-450 minor constituents present in gas-phase smoke.*
Gas-phase cigarette smoke is considered a highly oxidizing mixture and is believed to place an oxidative burden in the lungs of smokers.

**Emphysema and Other Cigarette Smoke Related Diseases.** Significant evidence implicates cigarette smoking in a number of pathological conditions. The three major disorders associated with cigarette smoke are cardiovascular disease, cancer and chronic obstructive pulmonary disease (18).

Four major categories of cardiovascular disease are believed to be accelerated or adversely influenced by cigarette smoke; these are cardiac, disorders of the peripheral circulation, stroke, and venous thrombosis (26). The chronic, high levels of carbon monoxide to which cigarette smokers are exposed cause arterial wall injury and alterations in lipid metabolism leading to hypercholesterolemia (27).

Numerous epidemiological studies have demonstrated higher lung cancer mortality ratios for cigarette smokers than for nonsmokers (18). Oral cancer and cancer of the larynx, esophagus, bladder, kidney and pancreas are also causally related to cigarette smoking (1). The majority of the genotoxic and carcinogenic agents identified in cigarette smoke reside primarily in the particulate phase (1). The most potent carcinogenic fraction of the particulate phase has been shown to be mainly composed of polynuclear aromatic hydrocarbons. This fraction only leads to tumor induction in the presence of cocarcinogens such as catechols, the major
cocarcinogens of the phenolic portion (28). As mentioned above, cigarette smoke has also been shown to form 8-hydroxydeoxyguanosine (29), to induce single-strand breaks in DNA (21,30) and to block DNA synthesis (25). Furthermore, cigarette smoke produces hydrogen peroxide (20-22), superoxide and hydroxyl radicals (23), all believed to be implicated in carcinogenesis.

The suppression of antiprotease activity in the lung by cigarette smoke is probably the most widely accepted explanation for the pathogenesis of pulmonary emphysema (31-34). Alpha-1-proteinase inhibitor (α1PI) is the major serum antiproteinase in humans (35), providing most of the protection against elastolytic activity in the lower respiratory tract (36). Therefore, it is believed that when the antiproteinase/proteinase activity ratio is decreased (more proteinase activity), destruction of the alveolar integrity occurs (32,37). The validity of this hypothesis has been amply corroborated by studies using bronchoalveolar lavage fluids (BAL) (32,37-39).

Alpha-1-proteinase inhibitor loses its elastase inhibitory capacity (EIC) upon oxidation of a methionine residue present in the active site (40-42). Carp et al. (43) determined the presence of methionine sulfoxide in α1PI recovered from BAL of cigarette smokers. Methionine sulfoxide, found in the inhibitor isolated from BAL of smokers, could result from the interaction of α1PI with phagocytic cells, such as neutrophils and macrophages, as well
as from direct reaction with cigarette components (14, 24, 44-56).

In spite of the plethora of evidence that associates cigarette smoking with the pathogenesis of emphysema, the mechanism(s) by which smoke causes disease is(are) still not well established or understood.

Purpose of this Investigation. The research group of Dr. W. A. Pryor has been interested for some time in elucidating the mechanism(s) by which cigarette smoke causes pulmonary diseases. As part of this continuing effort, we have studied the mechanism of inactivation of human alpha-1-proteinase inhibitor, the principal inhibitor against proteolytic degradation of lung tissue, by cigarette smoke. Although it had been extensively shown that exposure of alpha-1-proteinase inhibitor to cigarette smoke resulted in the inactivation of the protein (14, 24, 48-56), little was known about the type of damage sustained by the protein and the identity of the species responsible for the loss of activity.

We have also widened the scope of the existing research by studying the interaction of cigarette smoke with ferritin, this protein being the principal source of physiological iron. This line of research was initiated in an effort to shed light on the causes of accumulation of iron deposits observed in the pulmonary alveolar macrophages of chronic smokers (57). Iron is known to catalyze the formation of reactive oxygen species (58) which have been implicated in a variety of deleterious oxidative processes (59).
It is possible that the elucidation of the mechanisms by which cigarette smoke is believed to cause a variety of diseases could eventually contribute to the development of adequate preventive medicine against cigarette smoke toxicity.
CHAPTER I. Inactivation of Alpha-1-Proteinase Inhibitor by Cigarette Smoke
INTRODUCTION

A significant amount of evidence indicates that cigarette smoking is the major risk factor in the development of pulmonary emphysema (60). The inactivation of alpha-1-proteinase inhibitor (α1PI) by cigarette smoke is believed to be a key factor in the etiology of emphysema (31,61). Inactivation of α1PI causes an excess of free protease activity, mainly that of neutrophil elastase (62), in lung tissue, giving rise to emphysema (61). It is well established that cigarette smoke inactivates α1PI (24,51,53,56). The active site of the inhibitor contains a Met-Ser peptide bond which represents the primary binding site for a variety of proteinases such as elastase and trypsin (40,48). Oxidation of this key methionine residue has been shown to result in the loss of elastase inhibitory capacity (EIC) (40,48). Furthermore, a mutated form of α1PI, containing Val in place of Met in the active site, is more resistant towards oxidants (63) and smoke-induced inactivation (41). In view of these observations, it has often been assumed that oxidation of Met represents the major pathway by which cigarette smoke inactivates α1PI (24,51,53,56).

In spite of much speculation, it has not yet been shown, to our knowledge, that cigarette smoke contributes to the etiology of emphysema by oxidizing methionine. However, it has been found that α1PI recovered from the lungs of cigarette smokers contains 40% lower EIC than α1PI
recovered from the lungs of nonsmokers (64) and that the inhibitor isolated from smokers contains 4 mol methionine sulfoxide per mol inactive protein, while the nonsmokers inhibitor contains no oxidized methionine (43). Although relevant, these findings do not demonstrate that cigarette smoke could directly oxidize methionine residues in proteins. Methionine sulfoxide, found in α1PI isolated from lung fluids of smokers, could result from the action of inflammatory cells present in increased concentrations in the lungs of smokers (45,65,66).

We also studied the possibility of cigarette smoke damaging α1PI by mechanisms other than oxidation of Met. We find that cigarette smoke not only oxidizes Met, but also causes depletions of His and Lys. It had already been shown (67,68) that acylation of Lys results in loss of EIC. Concurrent with our findings, two laboratories published results confirming our observations. Gan and Ansari (69,70) reported that aldehydes found in smoke inactivate α1PI, and Yu et al. (71,72) identified cyanomethyl derivatives of Lys formed from the reaction with formaldehyde and cyanide, both found in cigarette smoke. Although histidine residues in α1PI have been modified by reactions with aldehydes (69) and ozone (73,74), the literature does not indicate a loss of EIC as a consequence of such modifications.

We here present data obtained from studies of the damage sustained by α1PI during its inactivation by cigarette smoke. In an effort to determine
the mechanism of inactivation, we also studied the reaction of a synthetic peptide (MER10), containing the amino acid sequence of the active site of $\alpha_1$PI, with cigarette smoke and three of its major components: hydroquinone, catechol and formaldehyde (1,18,19,75,76).
EXPERIMENTAL

Chemicals. Human α1PI and porcine pancreatic elastase (EC 3.4.21.36) were purchased from Calbiochem Co. (San Diego, CA). N-succinyl-(L-ALA)₃-p-nitroanilide (SANA) was purchased from Sigma Chemical Co. (St. Louis, MO). Chelex-100® resin was purchased from Bio-Rad Laboratories (Richmond, CA). Research cigarettes (1R1 and 1R4F) were purchased from the University of Kentucky, Tobacco and Health Research Institute. The cigarettes were stored at -20°C and humidified in a desiccator over saturated ammonium nitrate at room temperature for at least 24 hr prior to use. All other chemicals were of reagent grade and used without further purification. Unless otherwise indicated 0.1 M sodium phosphate (pH 7.4) pretreated with Chelex-100® was used as buffer.

Synthesis of Decapeptide. The synthesis of MER10 (Pro-Met-Ser-Ile-Pro-Pro-Glu-Val-Lys-Phe) was carried out using a Milligen/Biosearch SAM 2 automatic protein synthesizer apparatus (Milligen/Biosearch, San Rafael, CA). Using tert-butyloxy carbonyl (t-Boc) as the alpha-amino group protecting group, and starting from the C-terminal amino acid which is attached to a copoly(styrene-1%-divinylbenzene) chloromethyl resin, the synthesis cycle consisted of deprotection, base washing, coupling and capping. The crude product of this reaction was passed through a Sephadex G25 column, and further purified by HPLC as described below. This peptide
contains the amino acid sequence of the inhibitory active site of \( \alpha_1 \text{PI} \) from Pro\(^{357} \) to Phe\(^{366} \) (40). The elastase inhibitory capacity (EIC) of this synthetic peptide was tested as described below and found not to be an inhibitor of porcine pancreatic elastase.

**Smoke Preparation and Exposures.** The smoking protocol consisted of drawing 35 mL puffs at one minute intervals into a glass syringe fitted with a three-way stopcock. The smoke of one cigarette (ten 35 mL puffs) was bubbled through the reaction vessel which was kept in an ice-bath. For direct exposures, the reaction vessel contained 125 \( \mu \text{g} \) of \( \alpha_1 \text{PI} \) in 1 mL final volume of phosphate buffer. For indirect exposures, the vessel contained only buffer, and 125 \( \mu \text{g} \) of \( \alpha_1 \text{PI} \) was added after allowing the smoke solution to age at 37 °C for different periods of time as indicated in the pertinent figure legends. Unless otherwise indicated, all solutions were incubated for 24 hours at 37 °C until assayed for elastase inhibitory capacity.

Exposures of MER10 to cigarette smoke were carried out using the protocol for indirect exposure unless otherwise indicated. Usually 20 mg of peptide (final MER10 concentration was 35 mM) was added to 0.5 mL of freshly prepared cigarette solution in buffer. Smoke solutions (gas-phase or filtered) used for reactions with MER10 were prepared with the smoke collected from three cigarettes.

Gas-phase cigarette smoke refers to the smoke obtained from 1R1 cigarettes that passes through a glass fibre Cambridge filter. Cambridge
filters retain 99.9% of all particles with diameters larger than 0.1 μm (5). Filtered-smoke refers to the smoke obtained from 1R4F research cigarettes which has been filtered only by the filter tip that these cigarettes contain. Although gas-phase smoke, due to its relatively simple composition, is convenient for studies on the toxicity of cigarette smoke, it is not a very faithful representation of the smoke delivered by most commercial brand cigarettes. Gas-phase smoke, as stated above, has been subjected to very efficient filtration while commercial cigarettes contain either no filter or a filter which does not retain particulate matter as efficiently as Cambridge filters. A better model for the smoke to which tobacco users are exposed is obtained from 1R4F research cigarettes. This cigarette is equivalent to a low tar commercial cigarette. 1R4F cigarettes deliver approximately 11 mg of total particulate matter, 9.2 mg of tar, and 0.8 mg of nicotine (4).

For the reaction of MER10 with hydroquinone (1,4-benzenediol) and catechol (1,2-benzenediol), 150 μL of a 250 mM solution in each of the benzenediols (75 mM final concentrations) was added to 350 μL of MER10 (35 mM) to give a final volume of 500 μL in buffer. The reaction mixture was incubated for 12 hours at 37 °C in the dark.

**Elastase Inhibition Assay.** Assays for inhibitory activity were performed by measuring the decrease in elastase enzymatic activity resulting from preincubation for 5 minutes at 25 °C with native or smoke-exposed α1PI. The EIC of native α1PI was always taken as 100%. Elastase activity
was determined according to the method of Bieth et al. (77). Briefly, in 952 μL of 0.2 Tris buffer (pH 8.0), 10 μL of elastase (0.14 U/mL) is incubated with 30 μL of α1PI (3.75 μg/mL) for 5 minutes at 25 °C. After the incubation period, 8 μL of a 125 mM solution of SANA in 1-methyl-2-pyrrolidinone is added and absorbance at 410 nm is continuously followed for the first three minutes in a thermostatted cell at 25 °C. SANA in buffer is used as reference.

Amino Acid Analysis. Protein or peptide samples (200 μg) were hydrolyzed with 6 N HCl at 110°C for 24 hr in vacuo. Protein samples exposed to cigarette smoke were pretreated by ultrafiltration before amino acid analysis. Millipore Ultrafree-PF filter units with a molecular weight cutoff of 10,000 were used. Amino acids were separated and quantitated by HPLC in an Amino Quant® column (Hewlett-Packard) using ortho-phthalaldehyde and 9-fluorenylmethylformate (FMOC, used for proline secondary amino group derivatization) precolumn derivatization according to Schuster and Apfel (78). Analysis of cysteine and tryptophan was not performed. Formation of methionine sulfoxide was determined using the method described by Shechter et al. (79) with minor modifications. Briefly, lyophilized samples were dissolved in 80% formic acid and allowed to react with cyanogen bromide (CNBr) (100-fold excess) for 24 hour at room temperature. Cyanogen bromide reacts with the protein, cleaving the peptide bond at the carboxyl end of methionine and forming homoserine
lactone. Methionine sulfoxide (or sulfone) residues do not react with cyanogen bromide. The reaction was stopped by addition of an equal volume of water, then frozen and lyophilized. The cyanogen bromide peptides were hydrolyzed as described above but in the presence of 1% (w/v) 1,4-dithiothreitol in order to reduce methionine sulfoxide back to methionine. In this manner, the value obtained for methionine was taken as methionine sulfoxide, and original methionine concentrations in the protein were obtained from levels of homoserine lactone. Although this analysis for oxidation of methionine gave reproducible and accurate results from control experiments using oxidized α1PI with H₂O₂ and chloramine-T, one complication arose. Homoserine lactone coelutes with glycine in the HPLC separation of the acid hydrolysates, both amino acids eluting as a single peak. We changed the solvent gradient in order to achieve two resolved peaks for these amino acids but noticed that the changes introduced always resulted in losses of resolution for other amino acids. Therefore, without changing the conditions recommended by Shuster and Apfel (78), non-oxidized methionine concentrations were obtained from the difference of glycine plus homoserine levels found in CNBr analyses minus glycine levels obtained from analyses without cyanogen bromide. Response factors used for quantitative analysis of amino acids were calculated from calibration curves derived from four different dilutions of 2.5 mM amino acid standard solutions in 0.1 N hydrochloric acid (Sigma Chemical Co).
HPLC. The products obtained from the different reactions carried out with MER10 were isolated by semipreparative reverse-phase HPLC in an Econosil® C18 10U Alltech column (10 μm, 250x10 mm) (Alltech Associates, Inc., Deerfield, Il) using a detection wavelength of 210 nm. The components of the reaction mixture were separated using a flow rate of 3 mL/min and an initial eluent composition of 80% water, 15% acetonitrile and 5% isopropanol which was linearly increased to 95% acetonitrile and 5% isopropanol over a period of 45 min. All solvents contained 0.1% trifluoroacetic acid. After isolation, organic solvents were evaporated under vacuum, and water was eliminated by lyophilization. Structural studies of these peptide fractions were carried out by amino acid analysis, mass spectrometry and NMR.

NMR Analysis of MER10. The concentrations of the peptide samples, determined from peak areas obtained during the HPLC isolation, were estimated to be between 1.2 and 0.8 mM. All NMR spectra were recorded at 25 °C in D₂O and at 37 °C in DMSO-d₆ on a Bruker AM-400 spectrometer operating at 400.13 MHz equipped with an Aspect 3000 computer. The chemical shifts were relative to internal sodium 3-(trimethylsilyl)[2,2,3,3-d₄] propionate (TSP) in D₂O and, when dimethyl sulfoxide-d₆ was the solvent, the residual signal due to DMSO at 2.49 ppm was taken as reference.

One-dimensional spectra were collected with 8 K complex data points
over a 3600 to 5000-Hz sweep width with a minimum of 64 and a
maximum of 640 scans. The residual HOD and H_2O signals from D_2O and
dimethyl sulfoxide-d_6, respectively, were suppressed by low-power
presaturation prior to data acquisition.

Two-dimensional correlation spectroscopy experiments (COSY) were
obtained in the magnitude mode with 1024-2048 complex data points in _t_2
and 256-300 data points in _t_1, each point being the sum of 32 scan
accumulations. Zero filling in _t_1 and _t_2 dimensions was performed, and both
dimensions were always apodized with an unshifted sine-bell curve before
transformation.

For relayed experiments the standard Bruker microprograms were
used. For example, the following is the pulse sequence used for a one-step
relayed experiment: D1-P1-D0-P1-D2-P2-D2-P1-ACQUISITION, where D1 is
the relaxation time (2.0 s), D0 is the incrementable delay time for two-
dimensional acquisition (3 µs at _t_0), D2 is a delay time (18 ms), P1 is the
90° pulse and P2 the 180° pulse.

**Time-of-Flight MS Analysis.** All mass spectra were obtained on a ^{252}_{\text{Cf}}
Plasma Desorption Time-of-Flight Bio-Ion 20 mass spectrometer (Bio-Ion,
Uppsala, Sweden). Plasma desorption mass spectrometry (PDMS) utilizes a
^{252}_{\text{Cf}} source to ionize molecules, which subsequently are analyzed using the
time-of-flight technique. PDMS provides optimal soft ionization preventing
fragmentation of biological molecules of high molecular weight.
RESULTS

Inactivation of Alpha-1-PI by Direct and Indirect Exposure to Gas-Phase Cigarette Smoke. It has already been reported that direct exposure of \( \alpha_1 \)PI to gas-phase cigarette smoke results in the inactivation of the protein, and that this occurs in a biphasic process (53); this is shown in Figure I.1. An initial rapid loss of elastase inhibitory capacity of about 20% occurs during the first hour of incubation with gas-phase cigarette smoke. This fast inactivation is followed by a slower one. After 24 hours the total loss of EIC is about 60%. To explain this biphasic loss of EIC, it was proposed that the inactivation of \( \alpha_1 \)PI by direct exposure to gas-phase smoke is brought about by two different mechanisms (53). Short-lived species formed by reactions of free radicals in the smoke were proposed to be responsible for the fast \( \alpha_1 \)PI inactivation. These metastable species would be unlikely to be involved in the slow inactivating process observed with solutions of cigarette smoke that require several minutes to be prepared. As to the species responsible for the \( \alpha_1 \)PI inactivation that follows the fast phase, autooxidizable smoke components that would cooxidize with \( \alpha_1 \)PI were proposed. No structural analysis of the smoke-exposed inhibitor has been reported to sustain these hypotheses.

The approach taken to investigate whether distinct mechanisms operate during the fast and slow inactivation of \( \alpha_1 \)PI was to study the amino
FIGURE 1.1. Inactivation of Alpha-1-PI by Direct Exposure to Gas-Phase Cigarette Smoke. Alpha-1-proteinase inhibitor (125 µg/mL) was exposed to gas-phase cigarette smoke (one 1R1 cig/mL) in buffer and incubated at 37 °C. Aliquots were withdrawn at the indicated times and assayed for anti-elastase activity as described in the Experimental section. The insert shows an expansion of the plot over the first 100 minutes. Each data point is the average of duplicate measurements of two independent determinations.
acid composition of α1PI exposed to cigarette smoke by two different types of exposures: direct and indirect (see Experimental section). Indirect exposure of α1PI to gas-phase cigarette smoke does not cause a fast, initial inactivation, and only the continuous, slow loss of EIC is observed (53). The lack of the fast inactivating phase after indirect exposures of α1PI to cigarette smoke suggests that the "early mechanism" does not operate, and inactivation of the inhibitor is brought about by the "slow mechanism".

We allowed the smoke solutions used for the indirect exposures to age for periods of 6 and 24 hours before adding α1PI. The EIC determined for these smoke-exposed α1PI solutions, using both direct and indirect protocols, is shown in Table I.I. After 24 hours of incubation, α1PI exposed directly to gas phase smoke exhibits a decrease in EIC of about 47%. The α1PI exposed to previously prepared solutions of gas phase smoke aged for 6 hours before addition of α1PI shows a decrease in EIC of about 20%, while the inhibitor exposed to smoke that was first aged for 24 hours remains fully active. These results indicate that the inactivating capacity of the smoke decreases with time, and that smoke aged for 24 hr has lost all capacity to impair α1PI as an inhibitor of elastase.

Table I.II shows the amino acid composition of α1PI after having been exposed to cigarette smoke. The direct exposure of α1PI to gas-phase cigarette smoke causes the oxidation of one of the eight methionine residues present in the native protein to methionine sulfoxide. Indirect exposure of
### TABLE I.I. Effect of Aging on the Inactivating Capacity of Gas-Phase Cigarette Smoke.

<table>
<thead>
<tr>
<th>Incubation Time of Smoke Sln* (hr)</th>
<th>% Activity</th>
<th>% Inactivity</th>
<th>%EIC ± S.D. b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control c</td>
<td>6.3</td>
<td>93.7</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>0 d</td>
<td>50.7</td>
<td>49.3</td>
<td>52.6 ± 1.5</td>
</tr>
<tr>
<td>6</td>
<td>24.7</td>
<td>75.3</td>
<td>80.4 ± 0.9</td>
</tr>
<tr>
<td>24</td>
<td>5.1</td>
<td>94.9</td>
<td>101.3 ± 0.6</td>
</tr>
</tbody>
</table>

* Gas-phase smoke solutions (1 cig./mL) were allowed to age in the absence of α1PI for the indicated periods of time at 37 °C. After these aging periods of the smoke solutions, α1PI was added (125 µg) and solutions were incubated for an interval of 24 hrs at 37 °C before determination of EIC.

b Percent elastase inhibitory capacity (%EIC) is indicated as the average of three experiments ± standard deviation.

c The control consisted of a solution of α1PI incubated for 24 hr in the absence of smoke solution at 37 °C.

d Exposures to smoke not aged (0 hour) are equivalent to direct exposure.
TABLE I.II. Amino Acid Analysis of α1PI Exposed to Direct Gas-Phase and to Indirect Aged Cigarette Smoke*.  

<table>
<thead>
<tr>
<th>A.Acid</th>
<th>Literature</th>
<th>Control</th>
<th>0 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>11.1 ± 1.1</td>
<td>12.4 ± 0.3</td>
<td>12.8 ± 0.4</td>
<td>12.6 ± 0.2</td>
<td>12.4 ± 0.3</td>
</tr>
<tr>
<td>Glx</td>
<td>12.9 ± 0.9</td>
<td>12.8 ± 0.3</td>
<td>13.5 ± 0.4</td>
<td>13.5 ± 0.4</td>
<td>13.2 ± 0.4</td>
</tr>
<tr>
<td>Ser</td>
<td>5.3 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>4.6 ± 0.0</td>
<td>4.6 ± 0.0</td>
<td>4.5 ± 0.0</td>
</tr>
<tr>
<td>His</td>
<td>3.3 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>Gly</td>
<td>5.6 ± 0.2</td>
<td>5.7 ± 0.4</td>
<td>5.8 ± 0.4</td>
<td>5.8 ± 0.4</td>
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</tr>
<tr>
<td>Thr</td>
<td>6.8 ± 0.6</td>
<td>8.0 ± 0.3</td>
<td>8.2 ± 0.0</td>
<td>8.2 ± 0.0</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>Ala</td>
<td>6.1 ± 0.3</td>
<td>5.9 ± 0.0</td>
<td>6.2 ± 0.0</td>
<td>6.2 ± 0.1</td>
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</tr>
<tr>
<td>Arg</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.1</td>
<td>2.1 ± 0.0</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Val</td>
<td>6.3 ± 0.7</td>
<td>6.8 ± 0.0</td>
<td>7.0 ± 0.0</td>
<td>6.9 ± 0.1</td>
<td>6.8 ± 0.0</td>
</tr>
<tr>
<td>MetSO</td>
<td>0</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>4.8 ± 0.5</td>
<td>5.5 ± 0.4</td>
<td>5.3 ± 0.2</td>
<td>5.1 ± 0.4</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Phe</td>
<td>6.8 ± 0.6</td>
<td>7.1 ± 0.0</td>
<td>7.4 ± 0.2</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Leu</td>
<td>11.9 ± 1.2</td>
<td>11.4 ± 0.1</td>
<td>11.9 ± 0.2</td>
<td>11.5 ± 0.2</td>
<td>11.8 ± 0.1</td>
</tr>
<tr>
<td>Lys</td>
<td>8.8 ± 1.2</td>
<td>7.0 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Pro</td>
<td>4.8 ± 0.9</td>
<td>4.2 ± 0.1</td>
<td>4.2 ± 0.0</td>
<td>4.2 ± 0.0</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>Met</td>
<td>2.0 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Amino acid amounts are expressed as mol percent ± standard deviation of duplicate determinations.

*b Averaged values of amino acid compositions of human α1PI obtained from previous publications (40,80-82).

c Times indicated correspond to the intervals used to age the cigarette smoke solutions (see Table I.I legend). The compositions of residues which show significant changes are highlighted.
α1PI to gas-phase smoke aged for 6 hr results in the oxidization of two methionine residues, but smoke aged for 24 hr does not oxidize methionine. Table I.II also indicates that direct exposure of α1PI to cigarette smoke causes depletions of about 27% of His residues relative to the control, while protein exposed to smoke aged for 6 or 24 hr shows depletions of this residue of about 14%. Changes in lysine levels are also significant in some of these smoke-exposed protein samples. Direct exposure of α1PI to cigarette smoke causes 27% depletion of Lys; 19% disappearance is detected for α1PI exposed to smoke aged for 6 hours, and the inhibitor exposed to the smoke aged for 24 hours does not show significant changes in lysine levels. Smoke aged for 24 hours modifies His residues but does not affect the elastase inhibitory capacity of α1PI (Table I.I), indicating that the integrity of His residues is not essential for the activity of α1PI. A correlation between amino acid composition and EIC, measured for α1PI exposed to direct smoke and to smoke aged for 6 hours, is not obvious.

It has been extensively documented that alpha-1-proteinase inhibitor loses its elastase inhibitory capacity upon enzymatic (45,66) or chemical (40,42,63,73,74) oxidation of a methionine residue, Met\textsuperscript{348}, present in its active site. Figure I.2 shows the correlation between the number of methionine sulfoxide residues formed in α1PI upon reaction with hydrogen peroxide and the EIC determined for the oxidized inhibitor. Johnson and Travis (40) reported a loss in EIC of 95% with a maximum of two
FIGURE I.2. Correlation Between the Number of Methionine Residues Oxidized to Methionine Sulfoxide and Elastase Inhibitory Capacity for Alpha-1-PI. Alpha-1-PI (2.3 μM) was allowed to react with different concentrations (30 μM to 30 mM) of H₂O₂ (30% solution, w/v) in a final volume of 1 mL phosphate buffer at room temperature. Aliquots of 120 μL were withdrawn, 60 μL of which was used for EIC determination. The remaining volume was immediately frozen and then lyophilized prior to quantitative analysis of methionine sulfoxide (see Experimental section). Each data point is the average of duplicate measurements of one determination.
methionine residues oxidized when α1PI is allowed to react with N-chlorosuccinimide. Figure 1.2 indicates that 8 methionine residues need to be oxidized, using H$_2$O$_2$ as the oxidant, before complete inactivation of α1PI is achieved. This difference in the number of oxidized methionine residues required to accomplish complete inactivation of α1PI could be explained by the different sizes and, therefore, selectivities of the oxidants used in these two experiments. Hydrogen peroxide is a smaller molecule which could probably oxidize Met residues inaccessible to N-chlorosuccinimide. It has not been established that oxidation of Met residues other than Met$^{358}$ renders the protein inactive. Similar differences in the selectivities of several oxidants used during the oxidative inactivation of α1PI have previously been shown (83).

The results obtained with H$_2$O$_2$ (Fig. 1.2) resemble the results we obtain with α1PI exposed to indirect cigarette smoke, where oxidation of two Met residues results in about 25% loss of EIC. By this same rationale, the loss of 47% EIC observed in α1PI after direct exposure to gas-phase smoke cannot be explained solely on the basis of methionine oxidation since the oxidation of one Met should have resulted in only a 20% loss of EIC. This observation suggests that depletion of Lys (Table I.II) by cigarette smoke contributes to the inactivation of α1PI.

**Alpha-1-PI Inactivation by Filtered Smoke.** As stated in the experimental section, filtered smoke represents more faithfully the smoke to
TABLE I.III. Inactivation of α1PI by Direct Exposure to Gas-Phase and Filtered Cigarette Smoke*.

<table>
<thead>
<tr>
<th>Addition</th>
<th>%Activity</th>
<th>%Inhibition</th>
<th>% EIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>3.4</td>
<td>96.6</td>
<td>100.0</td>
</tr>
<tr>
<td>Gas-Phase Smoke</td>
<td>52.3</td>
<td>47.7</td>
<td>49.4 ± 6.5</td>
</tr>
<tr>
<td>Filtered Smoke</td>
<td>53.2</td>
<td>46.8</td>
<td>48.4 ± 9.8</td>
</tr>
</tbody>
</table>

* In a final volume of 1 mL of buffer, α1PI (125 μg) was exposed to either gas-phase or filtered smoke from one cigarette. Solutions were incubated for an interval of 24 hr at 37 °C before determination of EIC.  

b The control consisted of a solution of α1PI incubated for 24 hr in the absence of smoke solution at 37 °C.
TABLE I.IV. Amino Acid Analyses of Alpha-1-Proteinase Inhibitor Exposed to Gas-phase and Filtered Cigarette Smoke*.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Control</th>
<th>Gas-Phase</th>
<th>Filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>11.9 ± 0.2</td>
<td>12.1 ± 0.1</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>Glx</td>
<td>12.8 ± 0.4</td>
<td>12.8 ± 0.3</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>Ser</td>
<td>4.7 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>His</td>
<td>3.3 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Gly</td>
<td>5.9 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>Thr</td>
<td>6.8 ± 0.1</td>
<td>6.9 ± 0.3</td>
<td>6.9 ± 0.0</td>
</tr>
<tr>
<td>Ala</td>
<td>6.7 ± 0.2</td>
<td>6.9 ± 0.1</td>
<td>6.8 ± 0.0</td>
</tr>
<tr>
<td>Arg</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.5 ± 0.0</td>
<td>1.5 ± 0.0</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Val</td>
<td>6.2 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>6.5 ± 0.0</td>
</tr>
<tr>
<td>MetSO</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Ile</td>
<td>4.7 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Phe</td>
<td>6.5 ± 0.3</td>
<td>7.0 ± 0.1</td>
<td>6.6 ± 0.0</td>
</tr>
<tr>
<td>Leu</td>
<td>12.1 ± 0.2</td>
<td>12.0 ± 0.1</td>
<td>12.4 ± 0.2</td>
</tr>
<tr>
<td>Lys</td>
<td>8.0 ± 0.1</td>
<td>5.8 ± 0.3</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>Pro</td>
<td>4.3 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Met</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Amino acid amounts are expressed as mol percent ± standard deviation of duplicate determinations of three experiments. The compositions of residues which show significant changes are highlighted.
FIGURE 1.3. Amino Acid Analysis of α1PI Exposed to Direct Gas-Phase Cigarette Smoke. Alpha-1-PI (125 μg) was exposed to direct gas-phase smoke from one cigarette and incubated for 24 hr at 37 °C in a final volume of 1 mL of buffer (chromatogram A). The control (chromatogram B) consisted of a solution of α1PI treated as above but in the absence of cigarette smoke. Amino acid concentrations which exhibit changes with respect to the control are indicated by arrows. Methionine sulfoxide formation is not shown (see Fig. 1.4).
FIGURE 1.4. Amino Acid Analysis of α1PI Exposed to Direct Filtered Smoke After Cyanogen Bromide Treatment. Amino acid analysis of α1PI exposed to filtered smoke (chromatogram A) and of control α1PI (chromatogram B). Experimental conditions are described in Fig. 1.3 legend. As indicated by the arrows, methionine sulfoxide appears as methionine, and unoxidized methionine coelutes with glycine as homoserine lactone (see Experimental section for description of cyanogen bromide treatment).
which cigarette consumers are exposed. This argument leads us to
determine the extent of $\alpha 1$PI inactivation brought about by this type of
smoke. We find that direct exposure of $\alpha 1$PI to filtered smoke produces an
equivalent loss of EIC as that induced by gas-phase smoke; these results are
shown in Table I.III. Amino acid analyses of the protein samples (Table I.IV)
exposed to filtered and gas-phase smoke suggest that, although both types
of smoke cause a similar loss of EIC, the causes for inactivation may differ.
Alpha-1-PI exposed to filtered smoke contains about two methionine
sulfoxide residues as compared to one methionine sulfoxide detected in the
protein exposed to gas phase. Lysine levels in the protein exposed to
filtered smoke are decreased by approximately 10% relative to Lys levels in
the control, while $\alpha 1$PI exposed to gas-phase shows Lys depletion of about
28%. Both smoke-exposed proteins contain decreased amounts of His
(24% decrease) as compared to the control. The HPLC chromatograms
obtained from the amino acid separation of the acid-hydrolysates of the
smoke-exposed $\alpha 1$PI samples and of the control are shown in Figures 1.3 and
1.4. Figure 1.3 clearly shows a decrease in the levels of His and Lys in the
protein exposed to gas-phase smoke, while all other amino acids are not
significantly changed. Samples used for the acquisition of the
chromatograms shown in this figure had not been treated with cyanogen
bromide prior to acid hydrolysis; therefore, methionine oxidation is not
detected. Figure 1.4 shows the extent of methionine oxidation in $\alpha 1$PI
exposed to filtered smoke and illustrates the usefulness of cyanogen bromide treatment in samples containing oxidized methionine residues.

**Reaction of Free Amino Acids with Gas-Phase Cigarette Smoke.** The reaction of cigarette smoke with free amino acids was studied in an effort to identify products which could explain the observed depletions of Lys and His residues in α1PI after exposure to smoke (Tables I.II and I.IV). Identification of these modified residues would give important information regarding the nature of the species, present in cigarette smoke, responsible for the inactivation of α1PI. Using free amino acids in place of the protein allows us to bypass the harsh conditions required for protein hydrolysis (see Experimental) and might reveal the presence of products destroyed during the acid-hydrolysis step. For example, some of the products, such as imines formed from carbonyl compounds found in smoke (1,18,19) and primary amines in the protein, could be labile to acid hydrolysis. Derivatization of the amino acids after the reaction is still necessary due to the low extinction coefficient of the amide group at 210 nm, and also to distinguish amino acids from smoke components that also absorb at this wavelength.

Although we were unable to detect products of His or Lys resulting from exposure to cigarette smoke, the results shown in Table I.V reconfirm our previous observation that these amino acids are depleted by smoke. These results also suggest that the reactivity of the three amino acids tested follows the order: His > Lys > Met. Figure 1.5 shows the
TABLE I.V. Direct Exposure of Free Amino Acids to Gas-Phase Cigarette Smoke*.  

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>% Disappearance(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>His</td>
<td>71 ± 1</td>
</tr>
<tr>
<td>Met</td>
<td>33 ± 6</td>
</tr>
</tbody>
</table>

*a A solution (final volume of 1 mL) containing Lys, His, Met, and Val (all 85 \(\mu\)M) was exposed to gas-phase smoke delivered from one 1R1 cigarette, and incubated at 37 °C for a period of 48 hrs. After the incubation period, direct amino acid analysis was performed as described in the Experimental section.

*b Amino acid concentrations were calculated using Val as internal standard, and percent disappearance values are expressed as the average ± s.d. of two experiments.
FIGURE 1.5. Direct Exposure of Free Amino Acids to Gas-Phase Cigarette Smoke. A solution (final volume of 1 mL) containing approximately 1 mM concentrations of Lys, His, Met and Val was exposed to gas-phase smoke delivered from one 1R1 cigarette and incubated at 37 °C for a period of 48 hrs. After the incubation period, amino acid derivatization with ortho-phthalaldehyde was performed as described in the Experimental section. The initial reaction mixture (chromatogram A) and the amino acid mixture exposed to cigarette smoke (chromatogram B) are shown.
HPLC traces obtained for the separation of the ortho-phthalaldehyde derivatives of the starting mixture and of the amino acids exposed to cigarette smoke. While the concentration of Val remains virtually unchanged after smoke exposure, His, Lys, and Met concentrations sharply decrease. This figure also illustrates the oxidation of Met to MetSO by cigarette smoke.

Assignment of the NMR $^1$H Spin Systems Observed for the Amino Acid Residues in the Decapeptide MER10. The detection of products formed in the reaction of amino acids with cigarette smoke encounters two major difficulties. Firstly, most amino acids do not contain a distinct natural chromophore which would facilitate the detection of products formed in low yields; therefore, they require derivatization of the $\alpha$-amino group before analysis. Since it appears that amine functionalities in $\alpha$1PI undergo reaction with components present in the smoke, detection of these products does not occur, and amino acids must be labeled prior to reaction with cigarette smoke. The use of amino acid derivatives with chromophores that strongly absorb in the visible region, such as N-$\alpha$-$p$-nitrophenyl derivatives, presents new complications since the chromophore itself appears to react with smoke components. The second complication encountered with the use of free amino acids (or derivatives of these) is the separation of the reaction products from cigarette smoke components. Cigarette smoke is an extremely complicated mixture with a large number of compounds absorbing
in the 200 to 440 nm range thereby masking the detection of products formed in the reaction with amino acids.

To circumvent these impediments, we studied the reaction of cigarette smoke with a synthetic peptide (MER10) containing part of the primary sequence found in the active site of alpha-1-PI (40). The use of this decapeptide has several advantages; MER10 is a better model for a1PI than are free amino acids; due to its relatively large size its purification after reaction with smoke is no longer an obstacle, and direct analysis of the products by NMR and MS becomes possible.

Before the NMR spectra of products obtained from reactions involving MER10 could be interpreted, an assignment of all $^1$H NMR resonances of hydrogen atoms in MER10 had to be performed.

When deuterium oxide (D$_2$O) is used as the solvent, all labile protons$^2$ in MER10 are exchanged for deuterium atoms, and only the carbon-bound hydrogens are observable in the $^1$H NMR spectrum. The nonlabile protons of each amino acid residue in MER10 form a unique spin system which allows unambiguous identification. A spin system is defined as the group of spins connected through-bond spin-spin couplings within a residue (84). All nonlabile protons in MER10 (H-Pro-Met-Ser-Ile-Pro-Pro--Val-Lys-Phe-OH) form

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$^2$ Labile protons in MER10 are all the backbone amide protons, $\varepsilon$-NH$_2$ in Lys, and -OH in Ser. These protons undergo rapid exchange with deuterium atoms of the solvent and are not NMR-observable in D$_2$O solution.
a single spin system with the exception of Met and Phe. The $\epsilon \text{CH}_3$ group of Met is not connected with the $\alpha \text{CH}-\beta \text{CH}_2-\gamma \text{CH}_2$ fragment, and the aromatic protons of Phe are not connected with the $\alpha \text{CH}_2-\beta \text{CH}_2$ fragment. The strategy used for the assignment of the NMR signals observed in MER10 is described below.

1. Identification of the spin systems formed by the nonlabile protons for all ten residues in D$_2$O solution was accomplished using COSY, RELAYED-COSY, and DOUBLE-RELAYED-COSY to assign $^1\text{H}-^1\text{H}$ connectivities.

2. Identification of the connectivities for the labile protons in DMSO-d$_6$ solutions was accomplished using RELAYED-COSY. NH hydrogens in MER10 do not exchange with the solvent and are therefore observable by NMR, allowing complete assignment of all residue spin systems.

The COSY spectrum of MER10 in D$_2$O solution is shown in Figure 1.6. To facilitate the reading of this COSY, the more familiar 1D spectrum with the assignment of all resonance lines of MER10 is also shown along the $\omega 2$ axes of the 2D spectrum. Inspection of the COSY connectivities in this spectrum reveals the presence of three easily distinguishable spin systems; these are Val, Ile and Lys. The connectivities for Val are shown below the diagonal and have a pattern containing two intense $\gamma \text{CH}_3-\beta \text{H}$ cross peaks and an intense $\beta \text{H}-\alpha \text{H}$ cross peak. Ile has the most complex spin system but is
FIGURE 1.6. COSY Spectrum of MER10 in D$_2$O Solution. All resonance lines are assigned in the 1D NMR spectrum shown in the upper part of the figure. The connectivities of Phe (dashed lines) and of Glu (solid lines) are shown above the diagonal. The spin system connectivities of Val are shown below the diagonal. The arrow above the diagonal indicates the trans Pro conformer.
easily distinguishable from others, it is composed of two $\delta CH_3-\gamma CH_2$ cross peaks and one $\gamma CH_3-\beta H$ cross peak appearing at high field, two $\gamma CH_2-\beta H$ cross peaks and one $\beta H-\alpha H$ cross peak. The connectivities of the Lys spin system are shown in a latter figure. The connectivities of two other spin systems are shown above the diagonal; these are Glu (dashed lines) and Phe (solid lines). Another prominent feature of this COSY spectrum is the fact that only Phe and all three Pro show both $\alpha H-\beta H$ cross peaks. Methionine, Glu, and Ser spin systems exhibit degeneracy of the $\beta H$ protons and only one of the two $\alpha H-\beta H$ cross peaks is observed. For example, the two $\alpha H-\beta H$ cross peaks of Ser are overlapped since both $\beta H$ protons have nearly identical chemical shifts and the $\beta H-\beta'H$ cross peak is covered by the diagonal.

Figure 1.7 shows the DOUBLE-RELAYED-COSY spectrum of the peptide in D$_2$O. The connectivities of Lys (solid lines) and the amino terminal Pro (dashed lines) are indicated below the diagonal. Above the diagonal, the connectivities for Met are shown. Apart from these three spin systems, RELAYED cross peaks, absent in the COSY spectrum (Fig. 1.6), are labeled in this figure.

Although this NMR analysis of MER10 is not aimed at studying the conformation of the peptide, it is worth noting the different spin systems exhibited by the three Pro residues. Proline residues numbered with Roman numerals superscripts II and III (arbitrarily assigned) are located in the middle
FIGURE I.7. DOUBLE-RELAYED-COSY Spectrum of MER10 in D₂O Solution. The complete spin systems for lysine (solid lines) and amino terminal proline (P', dashed lines) are shown below the diagonal. All connectivities for the spin system of methionine are shown above the diagonal. RELAYED-COSY cross peaks not observed in COSY spectrum (Fig. I.6) are labeled using the one-letter nomenclature system for amino acids.
of the peptide in between Ile and Glu residues. Their spin systems are analogous in that the δCH₂ protons are nonequivalent, each residue having a δ'H-δH cross peak. In the amino terminal Pro residue (P'), the δCH₂ protons are equivalent and the spin system does not contain a δ'H-δH cross peak. Proline residues in proteins usually adopt a trans conformation at the peptide bond (85), but cis peptide bonds have been reported to occur in proline oligomers (86). Scheme 1.1 shows that the α and δ protons of the proline ring change positions with respect to the carbonyl group. By correlating NMR spectral data with optical rotation changes in proline oligomers, it has been shown that in polar solvents the δ protons of the cis conformer appear at higher field than the corresponding protons of the trans conformer (86).

SCHEME 1.1.
Inspection of the structures of these two conformers (Scheme 1.1) suggests that the trans peptide bond should be more favorable since it allows maximum separation between the two chains. Similarly, in MER10 the two internal Pro residues (Pro" and Pro"1) should favor the trans conformation, thus diminishing interaction between the peptide chains. This argument is in agreement with the observation that the resonance signals due to the δH protons in Pro" and Pro"1 appear at lower field than the resonance lines due to the δH protons in Pro1. In the terminal Pro1 residue, there is little energy difference between the two forms and although the cis conformer is usually observable, the trans form is also observed (this shown with an arrow in Fig 1.6, above the diagonal).

The assignment of the spin systems of all amino acid residues in MER10 is completed by identification of the connectivities of the labile protons in DMSO solution. Figure 1.8 shows two expanded regions of the TRIPLE-RELAYED-COSY spectrum of the peptide in DMSO solution. All αH-βH cross peaks, in addition to other signals already discussed above, are shown in the expansion on the upper part of the figure (2.60 to 4.65 ppm along ω1). The expanded region on the bottom of Fig. 1.8 shows the connectivities between the NH-αH cross peaks and the NH-βH cross peaks of all residues except for the two internal Pro residues (Pro" and Pro"1) which do not contain NH protons. The terminal Pro residue shows two NH-αH cross peaks, indicating that the imino group is probably undergoing
FIGURE 1.8. Expanded Regions of TRIPLE-RELAYED-COSY Spectrum of MER10 in DMSO($d_6$) Solution. All spin systems' connectivities are shown. NH-$\alpha$H cross peaks are labeled using the one-letter nomenclature system.
FIGURE 1.9. Expanded Regions of DOUBLE-RELAYED-COSY of MER10 and Methionine Sulfoxide-Containing MER10 in D₂O Solution. The expanded regions shown contain the αH-βH cross peaks for both MER10 (spectrum A) and MER10(SO) (spectrum B).
pyramidal inversion at the nitrogen. The ε-NH protons of Lys are also observable in the RELAYED-COSY in DMSO-\textsubscript{d\textsubscript{6}} solution, allowing the establishment of the complete spin system for this residue.

Since the oxidation of Met residues to MetSO in α1PI has already been shown to result in the inactivation of the inhibitor, we identified the spin system of MetSO in the hydrogen peroxide-oxidized peptide. Figure I.9 shows the regions containing the $\alpha$H-$\beta$H cross peaks for both MER10 and MER10(SO). Examination of this figure reveals the only observable change in this portion of the spectrum upon oxidation of Met to MetSO is a shift to low field of the $\alpha$H-$\beta$H and $\alpha$H-$\gamma$H cross peaks of MetSO when compared to the equivalent cross peaks of Met in MER10. In DMSO-\textsubscript{d\textsubscript{6}} solution (Fig. I.10), the Ser NH-$\alpha$H cross peak in the oxidized peptide shifts towards low field relative to the analogous cross peak in MER10, while all other NH-$\alpha$H cross peaks remained practically unchanged.

**NMR Studies of the Reaction of MER10 with Cigarette Smoke.**

Substantial evidence is provided above indicating that cigarette smoke is capable of oxidizing Met residues in α1PI to the corresponding sulfoxide. Possible species in smoke responsible for the oxidation of Met resulting in the inactivation of alpha-1-PI are benzenediols. Cigarette smoke contains high concentrations of polyhydroxybenzenes (1,18,19,75), the most
FIGURE I.10. Expanded Regions of TRIPLE-RELAYED-COSY Spectrum of Methionine Sulfoxide-Containing MER10 in DMSO Solution. Connectivities are only shown for Met and Ser spin systems.
abundant being catechol and hydroquinone\(^3\), which during autoxidation produce hydrogen peroxide (87,88). Cigarette smoke is known to produce hydrogen peroxide (20,21) at concentrations reaching 130 µg per cigarette after 4 hours (22).

In view of the possible role of benzenediols in the oxidation of Met, we studied the reaction of MER10 with a mixture of cathechol and hydroquinone (see Experimental). Figure 1.11B shows the 1D NMR spectrum of the major product isolated from this reaction (aromatic protons are not shown). The most prominent feature of this spectrum is the presence of a sharp singlet at 2.6 ppm not observed in the spectrum of MER10 (Fig. 1.11A). The ε-CH\(_3\) protons of Met give a singlet at 2.0 ppm (Fig. 1.11A); upon oxidation of the sulfur atom these protons become deshielded and absorb at lower field (2.6 ppm). Figure 1.11C shows the 1D NMR spectrum of a fraction isolated from the reaction of MER10 with filtered cigarette smoke. This spectrum also contains a sharp singlet at 2.6 ppm, indicating oxidation of methionine to the sulfoxide. Oxidation of methionine in both peptides, smoke-exposed and reacted with benzenediols, was also confirmed by mass spectrometry analysis. Figure 1.12A shows the PDMS spectrum of MER10 which indicates a molecular ion peak at 1144 units. The spectra of the products isolated from the reactions of MER10 with benzenediols and

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\(^3\)Refer to Table III.III of this Dissertation for a quantitative determination of catechol and hydroquinone in smoke.
FIGURE I.11. $^1$H 1D NMR Spectra of MER10 Products Isolated from the Reactions with Benzenediols and with Filtered Smoke. Spectra were collected in D$_2$O at 25 °C and chemical shifts are relative to TSP. A: MER10; B: major product isolated from the reaction between MER10 and a mixture of hydroquinone and catechol; C: product isolated from the reaction of MER10 with filtered cigarette smoke.
FIGURE I.12. Mass Spectra of Products Isolated from the Reactions of MER10 with Filtered Cigarette Smoke and Benzenediols. All mass spectra were obtained on a $^{252}$Cf Plasma Desorption Time-of-Flight Bio-Ion 20 mass spectrometer. A: PDMS spectrum of MER10; B: PDMS spectrum of a product isolated from the reaction of MER10 with hydroquinone and catechol; C: PDMS spectrum of a product isolated from the reaction of MER10 with filtered smoke.
with filtered smoke (Fig. 1.12B and 1.12C) indicate the addition of 16 mass units to the molecular weight of MER10. Three peaks, with m/Z values of 1160, 1183 and 1205 are observed in these PDMS spectra, which correspond to the ions (MO + H)⁺, (MO + Na)⁺, and (MO + 2Na)⁺, respectively⁴. These results show that two of the major polyhydroxybenzenes present in cigarette smoke, catechol and hydroquinone, are capable of oxidizing Met residues. Also, we have shown by four different analyses that cigarette smoke oxidizes methionine to methionine sulfoxide.

So far we have only shown disappearances of the basic amino acids His and Lys, and have yet to identify any product formed when α1PI is exposed to cigarette smoke. Since, as shown above, there appears to be no correlation between His depletion and the EIC of α1PI, we studied the reaction of MER10, which does not contain His, with smoke, thereby focusing on modifications of Lys residues. This by no means implies that modifications to His are not relevant when dealing with proteins other than α1PI.

The data in Tables I.II and I.IV suggest that depletions of Lys occur early in the incubation period, and that gas-phase smoke depletes Lys to a greater extent than does filtered smoke. In view of these results, we

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⁴ The notation (MO) denotes the molecular weight of MER10 (M) plus 16 amu.
FIGURE I.13. DOUBLE-RELAYED-COSY Spectrum of MER10 Exposed to Gas-Phase Cigarette Smoke in D₂O. This figure shows the presence of two peptides (see text); the connectivities shown correspond only to amino acids of the modified peptide. The spin systems of Lys (K', solid lines) and Pro (P', dashed lines) are shown below the diagonal. The spin system of Glu (G') is shown above the diagonal.
exposed MER10 to direct gas-phase cigarette smoke by passing the smoke of one 1R1 cigarette every hour for a total interval of four hours, giving a total of four cigarettes smoked. After incubation for 12 hours at 37 °C, we separated by HPLC a fraction that eluted with a different retention time than that of MER10. The DOUBLE-RELAYED-COSY spectrum in D₂O of this fraction is shown in Fig.I.13. Inspection of this figure reveals the presence of two peptides. One peptide is clearly MER10 with the familiar net of amino acid spin systems discussed in the previous section. The presence of a second peptide is evidenced by the appearance of new spin systems attributed to Lys, Glu, and one of the Pro residues; the connectivities of these residues are shown in Fig. I.13. More evidence for the presence of a smoke-modified peptide is the appearance of lower-field cross peaks next to the signals attributed to the side-chain protons for Ile, Val and Phe residues in MER10. The spin system which we have attributed to Lys in the modified peptide contains a δCH₂-εCH₂ cross peak appearing at lower field (ω₁ = 3.05, ω₂ = 1.72) than the analogous δCH₂-εCH₂ cross peak of the MER10 spectrum (ω₁ = 2.9, ω₂ = 1.60). This δCH₂-εCH₂ cross peak is connected to a cross peak (ω₁ = 3.52, ω₂ = 3.00) which is not observed for MER10, suggesting the addition of a -CH₂-R group to the ε-amino group of lysine. The chemical shift of the protons in this methylene group (ω₂ = 3.00, ω₁ = 3.45 ppm) suggests that the R group is an electron withdrawing moiety. The cross peaks arising from the αCH-βCH₂-γCH₂ fragment of this
modified Lys residue are superimposed with the Lys signals of MER10. This is to be expected since the protons of the $\alpha\text{CH-}\beta\text{CH}_2-\gamma\text{CH}_2$ fragment are separated from the protons of the modified NH-$\text{CH}_2$-$R$ fragment by at least five covalent bonds. A feasible explanation for these changes observed in the spin system of Lys would be the addition of a cyanomethyl group (-CH$_2$CN) to the $\varepsilon$-$\text{NH}_2$ moiety. The formation of cyanomethyl derivatives of amines upon reaction with cigarette smoke has been in fact proposed by Yu et al. (71).

As a result of this modification in the lysine side chain, the spin system of Glu in the modified peptide is shifted to low field with respect to the Glu spin system in MER10; the connectivities of the former residue are shown in Fig. 1.13. This effect on the chemical shift of the spin system of glutamic acid suggests that the side chains of Glu and Lys are in close proximity, in spite of the fact that the two amino acids are separated by Val. Lastly, a new Pro spin system is observed (designated as P' in Fig. 1.13) with a strong $\delta'$H-$\delta$H cross peak and a degeneracy of the two $\beta$H protons. This degeneracy of the $\beta$H protons of P' gives rise to a single $\alpha$H-$\beta$H cross peak which appears at lower field ($\omega_1 = 4.90, \omega_2 = 2.33$) than the $\alpha$H-$\beta$H cross peaks of all other spin systems. The data presented here does not allow us to determine unequivocally which of the three Pro residues present in MER10 has been modified by cigarette smoke. However, the chemical shift of the $\alpha$H-$\beta$H cross peak suggests that the nitrogen atom of this residue
FIGURE I.14. DOUBLE-RELAYED-COSY Spectrum of the Product Isolated from the Reaction of MER10 and Formaldehyde in D₂O. The spin system connectivities for the amino terminal Pro residue are shown; all other amino acid cross peaks are labeled using the one-letter nomenclature code.
has undergone reaction with smoke, and the group attached to the nitrogen
deshields the αH proton. Only the terminal proline residue in MER10 could
undergo such reaction since the imino nitrogens of Pro" and Pro"" are part of
the amide backbone.

The DOUBLE-RELAYED-COSY spectrum of the major product isolated
from the reaction between MER10 and formaldehyde, a major component of
gas-phase cigarette smoke (1,18,19), is shown in Fig. 1.14. A comparison of
this figure with Figure 1.13 reveals certain similarities between both spectra.
Figure 1.14 shows a spin system assigned to the terminal Pro residue (P'),
which resembles the Pro spin system of the smoke-modified peptide in
Fig. 1.13. Formaldehyde could react with proline to yield an intermediate
carbinolamine which undergoes elimination to form an iminium ion. Evidence
for this speculation is the presence of a strong cross peak (ω1 = 5.1,
ω2 = 4.90) not coupled with other signals. This cross peak could be
attributed to the vinylic protons of the iminium ion formed from the reaction
of Pro' with formaldehyde. Another similar feature in both spectra is the
presence of the cross peak attributed to the δCH₂-εCH₂ protons of Lys in the
smoke-modified peptide. However, Fig. 1.14 does not exhibit a low-field
cross peak connected to the δCH₂-εCH₂ cross peak, suggesting the
formation of an imine between the εNH₂ group of Lys and formaldehyde.
The data presented here suggest that cigarette smoke inactivates alpha-1-proteinase inhibitor by two different mechanisms: oxidation of methionine and nonoxidative covalent modification of lysine.

Our results show for the first time that components in cigarette smoke oxidize methionine residues to methionine sulfoxide in \( \alpha \)1PI. Only one previous report (43) in the literature has documented the oxidation of methionine in \( \alpha \)1PI as a consequence of cigarette smoking. Carp et al. (43) determined the presence of methionine sulfoxide in \( \alpha \)1PI recovered from bronchoalveolar lavage fluid (BAL) of cigarette smokers. In spite of the significance of these findings, they did not rigorously prove that the oxidation of methionine resulted from the direct interaction of cigarette smoke components with \( \alpha \)1PI. Methionine sulfoxide, found in the inhibitor isolated from BAL of smokers, could result from the interaction of \( \alpha \)1PI with phagocytic cells, present in increased concentrations in the lungs of smokers (65). For example, Matheson et al. (45,66) has shown that neutrophil myeloperoxidase inactivates \( \alpha \)1PI by oxidizing the methionine residue present in the active site.

As shown in Figure 1.1, direct exposure of \( \alpha \)1PI to gas-phase cigarette smoke results in an initial fast inactivation followed by a slow inactivation occurring over 24 hours. Amino acid analysis of \( \alpha \)1PI exposed to cigarette
smoke, using protocols which would mimic these two inactivation processes, shows that oxidation of Met occurs during both the short-term and long-term inactivations. These results indicate that these two mechanisms of inactivation cannot be distinguished solely by amino acid analysis of the exposed protein.

Nevertheless, several lines of evidence suggest that hydrogen peroxide, known to be produced by cigarette smoke solutions (20-22), is the species responsible for the inactivation of α1PI in the slow phase. Cigarette smoke contains high concentrations of polyhydroxybenzenes (1,18,19,75), the most abundant being catechol and hydroquinone, which produce hydrogen peroxide during autoxidation (87,88). We observe that a mixture of hydroquinone and catechol oxidizes the methionine residue of the synthetic peptide MER10. Also, the pattern of inactivation of α1PI by hydrogen peroxide (Fig. 1.2) resembles the results obtained from indirect exposure of the protein to cigarette smoke. These findings confirm previous speculations (53) that the long-term inactivation of α1PI by cigarette smoke is a result of the cooxidation of the inhibitor with autooxidizable species present in cigarette smoke.

On the other hand, the data presented here does not allow us to identify the species responsible for the oxidation of Met in the short-term inactivation. The gas-phase of cigarette smoke is known to contain inorganic radicals such as nitric oxide (NO), nitrogen dioxide (NO₂), as well
as small carbon- and oxygen-centered organic radicals (8,12). Pryor et al. (53) proposed short-lived species such as peroxonitrates, formed in smoke by the reaction of nitrogen dioxide and organic peroxyl radicals, as the oxidants responsible for the fast inactivation of α1PI. Although this theory remains largely unchallenged, the results presented in Chapter II suggest that peroxonitrite, formed by the reaction of superoxide and nitric oxide, could play a role in the short-term inactivation of α1PI. In chapter II we show that peroxonitrite is a short-lived, strong oxidant capable of inactivating α1PI by oxidizing methionine.

Concurrent with oxidation of Met, we observe depletions of His and Lys residues in α1PI exposed to cigarette smoke. Since it had already been reported that modification of His has no effect on the inhibitory activity of α1PI (67), in agreement with our observations, we did not investigate the mechanism by which components of cigarette smoke react with this residue. Although modification of His residues in α1PI appears not to be essential for the activity of the inhibitor, modification of His residues of other proteins by cigarette smoke may have significant consequences. For example, it has been reported that oxidative modification of one His residue in glutamine synthetase causes its inactivation and subsequent proteolytic degradation (89-91).

With Lys, however, we observe a correlation between the extent of depletion of this amino acid and the inhibitory capacity of α1PI. Pryor et al.
(56) found that amines protect α1PI against loss of elastase inhibitory capacity by cigarette smoke, and Fretz and Gan (67) reported loss of activity of α1PI brought about by chemical modification of Lys residues. Our data, shown in Tables I.II and I.IV, indicate that depletions of Lys occur early in the reaction of α1PI with cigarette smoke, and that direct gas-phase smoke depletes Lys to a greater extent than does filtered smoke. We also observe that depletion of Lys in α1PI is significantly reduced when aged smoke is used (Table I.II). NMR analysis of a product, isolated from the reaction of gas-phase smoke with MER10, suggests the ε-amino group of Lys and the secondary amino group of Pro react with components of smoke. We have tentatively identified these modified amino acids as being the products of the reaction of the amino group with formaldehyde, in the case of Pro, and with formaldehyde and cyanide in the case of Lys. These results agree with reports by Yu et al. documenting the formation of cyanomethyl derivatives of biogenic amines (71) and of basic amino acids in proteins (72) by reaction with formaldehyde and cyanide. However, these authors argued that no products were formed when formaldehyde, in the absence of cyanide, was allowed to react with amines. Our results indicate that this reaction takes place, as shown in Fig. I.14, contradicting this observation by Yu et al. It has also been shown that reaction of the ε-amino group of Lys with acrolein and other carbonyl compounds found in cigarette smoke results in the inactivation of alpha-1-proteinase inhibitor (68,69).
CONCLUSION

We have shown that cigarette smoke reacts with α1PI to cause modifications in its amino acid composition by two different mechanisms: oxidation of methionine to methionine sulfoxide and nonoxidative covalent modification of amines. These amino acid modifications result in impairment of the elastase inhibitory activity of alpha-1-proteinase inhibitor, the major plasma inhibitor of serine proteases (62). We have indirectly shown that hydrogen peroxide, formed during the autoxidation of hydroquinones found in smoke (1,18,19,75), is responsible for the oxidation of methionine, and that modification of amines results from their reaction with formaldehyde and cyanide. It is possible that the identification of these compounds in cigarette smoke, as the species responsible for the inactivation of α1PI, could aid in the development of adequate preventive medicine against emphysema.
CHAPTER II. Peroxynitrite Inactivates Alpha-1-Proteinase Inhibitor by Oxidizing Methionine.
The reaction of peroxonitrite\(^5\) with alpha-1-proteinase inhibitor (\(\alpha1\)PI) and with a synthetic decapeptide (MER10) containing the sequence of amino acids found in the active site of \(\alpha1\)PI (Pro-Met-Ser-Ile-Pro-Pro-Glu-Val-Lys-Phe) has been studied. Peroxonitrite inactivates \(\alpha1\)PI under both physiological and basic pH conditions. Thiourea and methionine protect \(\alpha1\)PI against inactivation by peroxonitrite, while mannitol, benzoate and urea failed to afford effective protection. The major product isolated from the reaction between peroxonitrite and MER10 was analyzed by NMR, mass spectrometry, and amino acid analysis. These analyses indicate that peroxonitrite primarily oxidizes the methionine residue in the peptide. We do not detect smaller molecular weight peptides that would indicate cleavage of MER10 by peroxonitrite, nor hydroxylation or nitration of the phenylalanine residue. These observations imply that the hydroxyl radical is not the species responsible for the inactivation of \(\alpha1\)PI in this system. Our results suggest that peroxonitrite is a powerful oxidant capable of oxidizing methionine residues in proteins without the requisite of forming hydroxyl radicals and nitrogen dioxide through the decomposition of peroxonitrous acid. The implications of these observations on lung diseases attributed to

\(^5\) The nomenclature often used for ONOO\(^-\) is peroxynitrite. Here the recommended name of peroxonitrite (94) will be used.
cigarette smoke are discussed.

INTRODUCTION

The suppression of antiprotease activity in the lung by cigarette smoke is one of the most widely accepted explanations for the pathogenesis of pulmonary emphysema in smokers (31), but the mechanism(s) by which cigarette smoke causes this suppression is (are) still not well understood. Among the major components present in cigarette smoke, nitric oxide (NO) has attracted very little attention.

Nitric oxide (NO) is one of the major components in the gas phase of cigarette smoke with concentrations of up to 1000 parts per million (ppm) depending on the type of tobacco from which cigarettes are made (19). Nitric oxide is slowly oxidized to nitrogen dioxide (NO₂) in cigarette smoke, and this can only be detected upon aging of the smoke (92) since fresh smoke does not contain NO₂ (93). The reason for this is that at 600 °C (the temperature of the cigarette burning tip) the decomposition of NO₂ is complete (94). The half-life of 1000 ppm NO in air is about three minutes (95), and it has been suggested that the oxidation of NO in the respiratory airways does not proceed faster (96). In spite of the fact that during smoking the smoke is held in the mouth and the lungs for short periods of time, very little NO₂ probably is formed. These facts have caused
investigators to conclude that NO plays a minor role in the pathogenesis of emphysema and only through its oxidation to NO₂ (96).

Pulmonary alveolar macrophages (PAM) have been implicated in the pathogenesis of emphysema, the main evidence being that clusters of these inflammatory cells collect in the bronchioles of smokers before and during tissue destruction and fibrosis (97,98). The presence of cigarette smoke in the lung causes increased concentrations of neutrophils, another type of inflammatory cells, suggesting that emphysematous lesions may also result from the destruction of lung tissue by these cells (65). Furthermore, superoxide (O₂⁻) production by PAM is significantly increased in the lungs of chronic smokers (99,100). Thus nitric oxide from cigarette smoke could react with cellulary-produced superoxide ion to produce peroxonitrite (ONOO⁻) which has been shown to be a powerful oxidant capable of oxidizing thiols (101) and deoxyribose (102). The reaction of O₂⁻ and NO is diffusion-controlled in the gas phase (103) and a rate constant of about 3.7 x 10⁷ M⁻¹s⁻¹ has been measured under physiological conditions (104). Alternatively, the formation of peroxonitrite in the lungs of smokers could arise from the reaction of NO produced by macrophages and neutrophils (105-107), and O₂⁻ derived from PAM, which as stated above have an increased production of O₂⁻ in smokers.

To investigate if peroxonitrite could play a role in the pathogenesis of emphysema, we studied the reaction of peroxonitrite with alpha-1-proteinase
inhibitor (α1PI). This protein is the most abundant extracellular antiprotease in the lung and provides most of the protection against neutrophil elastase in the lower respiratory tract (36,108). We also report on the reaction of a synthetic decapeptide (MER10), a model for the active site of α1PI, with peroxonitrite. The use of this smaller molecule in place of α1PI in the reaction with peroxonitrite enabled us to conduct product analysis by NMR, mass spectrometry and amino acid analysis.
EXPERIMENTAL

Chemicals. Human α1PI, and porcine pancreatic elastase (EC 3.4.21.36) were purchased from Calbiochem Co. (San Diego, CA). Bovine liver catalase (EC 1.11.1.6), bovine serum albumin, mannitol, thiourea, urea, methionine, sodium benzoate and N-succinyl-(L-ALA)₃-p-nitroanilide (SANA) were all purchased from Sigma Chemical Co. (St. Louis, MO). Chelex-100® resin was purchased from Bio-Rad Laboratories (Richmond, CA). MER10 (H-Pro-Met-Ser-Ile-Pro-Pro-Glu-Val-Lys-Phe-OH) was synthesized in a Milligen/Biosearch SAM 2 automatic protein synthesizer apparatus (Milligen/Biosearch, San Rafael, CA) as described in the Experimental section in Chapter I. All other chemicals were of reagent grade, and used without further purification. Unless otherwise indicated sodium phosphate pretreated with Chelex-100® was used as buffer.

Synthesis of Peroxonitrite. Peroxonitrite was synthesized using a quenched flow reactor (Fig.II.1) as described by Reed et al. (109). The purpose of the flow reactor is to quench the acid catalyzed reaction between NaN₃O₂ and H₂O₂ with an excess of NaOH. Base quenching of the acid catalyzed reaction gives the more stable peroxonitrite ion by deprotonation of peroxynitrous acid. Solutions consisting of 0.6 M NaN₃O₂, 0.7 M H₂O₂ in 0.6 M HCl, and 1.5 M NaOH were pressurized with nitrogen and passed through the reactor at equal flow rates (25 ± 5 mL/min). The product was
FIGURE 11.1. Quenched Flow Reactor Used for the Synthesis of Peroxonitrite. Flask A contained 0.6 M NaN0₂, flask B 0.7 M H₂O₂ in 0.6 M HCl, and flask C 1.5 M NaOH. All three containers were pressurized with nitrogen and the solutions passed through the reactor at flow rates of 25 ± 5 mL/min. The product was collected in a flask kept at 0 °C in an ice water bath.
collected in a flask immersed in ice water. Excess H₂O₂ was removed by passing the reaction mixture through a column (10 x 1.5 cm) packed with manganese oxide (MnO₂); the resulting solution containing a large excess of alkali was stored at -20 °C for periods no longer than two weeks. The use of MnO₂ in the removal of excess H₂O₂ resulted in loss of peroxonitrite; this decomposition of peroxonitrite has previously been attributed to MnO₂ catalyzing a reaction with H₂O₂ to give nitrite as a product (110). Therefore, our peroxonitrite solutions were contaminated with nitrite and nitrate ions, but no attempts for further purification were made. Other investigators had unsuccessfully attempted such purification in the past using several different techniques and observed that decomposition of peroxonitrite was unavoidable (111). Peroxonitrite solutions form a deep yellow top layer by freeze fractionation, aliquots obtained from this top layer were used in the experiments. The concentration of peroxonitrite in the top layer, determined spectrophotometrically at 302 nm using an extinction coefficient of 1670 M⁻¹ cm⁻¹ (111), was typically about 0.10 ± .02 M. A cell containing previously decomposed peroxonitrite in phosphate buffer was used as reference to correct for interference by other compounds (e.g. nitrite) absorbing at 302 nm.

**Decomposition of Peroxonitrite.** Peroxonitrite decomposition was followed at 25 °C in 0.1-0.5 M sodium phosphate buffer for at least three half-lives by measuring absorbance changes at 302 nm every 0.1 s after
addition of peroxonitrite (0.25 to 3.00 mM) to a thermostatted cell. The pH of the solutions was measured at the end of each experiment. First order kinetics were observed over at least three half-lives.

**Exposures.** Solutions of α1PI (125 μg/mL, 2.4 μM using a MW of 52 kDa) were incubated with peroxonitrite (1 to 2 mM) in 0.1 M or 0.5 M sodium phosphate buffer under constant stirring for 2 to 12 hr at 25 °C in dark glass vials. Other additives were added as indicated in table legends. The pH of solutions was measured at the end of each incubation period and found to be 10.4-11.5 ± 0.5 using 0.1 M buffer, and 7.40 ± 0.05 using 0.5 M buffer. Exposures of the synthetic peptide, MER-10, were carried out in a 2:1 (mM) molar ratio of peptide:peroxonitrite in 0.1 M phosphate buffer with incubation periods of 24 hr at 37 °C. Protein or peptide samples incubated and treated under the same conditions as peroxonitrite-treated samples but in the absence of peroxonitrite were used as controls.

**Elastase Inhibition Assay.** Assays for inhibitory activity were performed by measuring the decrease in elastase enzymatic activity resulting from preincubation for 5 min at 25 °C with native or peroxonitrite-treated α1PI. Elastase activity was measured according to the method described by Bieth et al. (77) and has already been described in the Experimental section of Chapter I.

**Amino Acid Analysis.** Refer to Experimental section of Chapter I.

**HPLC.** The products of the reaction between peroxonitrite and
MER10 were isolated by semipreparative reverse-phase HPLC in a Econosil® C18 10U Alltech column (10 μm, 250x10 mm) (Alltech Associates, Inc., Deerfield, IL). The mobile phase consisted of a ternary system of water, acetonitrile, and iso-propanol; all solvents contained 0.1% trifluoroacetic acid. The components of the reaction mixture were separated using a flow rate of 3 mL/min and an initial eluent composition of 80% water, 15% acetonitrile and 5% isopropanol which was linearly increased to 95% acetonitrile and 5% iso-propanol over a period of 45 min. Isolated fractions were evaporated under vacuum and their molecular weights and NMR spectra obtained as described below. Two major fractions were collected from the reaction of MER10 and peroxonitrite comprising 65% and 35% of the starting material.

NMR Analysis of MER10. The concentration of the peptide samples, determined from peak areas obtained during the HPLC isolation, was estimated to be 1.2 and 0.8 mM. Refer to the Experimental section of Chapter I for further details.

Time-of-Flight MS Analysis. Refer to the Experimental section of Chapter I.

RESULTS

Decomposition of Peroxonitrite. The kinetics of decomposition of
peroxonitrite to nitrate is perhaps the most thoroughly studied chemical aspect of this compound (101,102,111-116). In our case, the purpose of measuring the rates of decomposition of peroxonitrite was to estimate the duration of incubation times of peroxonitrite with either α1PI or MER10 necessary to allow complete decomposition (or disappearance) of peroxonitrite. Due to the simplicity of the method utilized in these measurements, we were limited to following the decay of peroxonitrite within the pH range of 7.00 to 10.75. The disappearance of the 302 nm band due to peroxonitrite is too fast at pH values below this range.

The kinetics for the decomposition of peroxonitrite were fitted to a first order reaction equation by nonlinear regression. A typical plot generated by following the decay of absorbance at 302 nm is shown in Figure II.2. We observe first-order kinetics over at least three-half lives as previously reported (111,113). The dependence of the apparent first-order rate constant on pH is shown in Figure II.3. It has been proposed (111,113) that this pH dependence is consistent with the reaction sequence shown in eqs 1 and 2, and that this process can be described by eq 3, where $k_{HA}$ is the first-order constant for the decomposition of peroxonitrous acid (HOONO), and $K_a$ is the ionization constant for peroxonitrous acid.
FIGURE II.2. Decomposition of Peroxonitrite. Typical curve obtained for the decomposition of peroxonitrite by following the change in absorbance at 302 nm at 25 °C in 0.1 M phosphate buffer (pH 7.80). The curve drawn through the data points is the nonlinear regression fit obtained using the equation: $y = a \times \exp(-bt)$. 
FIGURE II.3. The Effect of pH on the Apparent Rate Constant of Peroxonitrite Decomposition. Apparent rate constants for the decomposition of peroxonitrite were fitted to a first order reaction equation by nonlinear regression. The pH of the solutions was determined at the end of each run. Each data point represents the average of two to four determinations. Error bars are shown only where they are larger than symbols.
\[
\begin{align*}
\text{ONOO}^- + H^+ & \rightleftharpoons \text{ONO}OH \quad (1) \\
\text{HOONO} & \rightarrow \text{NO}_3^- + H^+ \quad (2)
\end{align*}
\]

\[
k_{\text{obs}} = \frac{(k_{HA} \times [H^+])}{([H^+] + K_a)} \quad (3)
\]

A plot of \(1/k_{\text{obs}}\) against \(1/[H^+]\) is linear (Figure II.4), and gives values of \(0.44 \pm 0.10 \text{ s}^{-1}\) and \(7.1 \pm 0.3\) for \(k_{HA}\) and \(pK_a\), respectively. At pH 7.4 this rate constant value corresponds to a half-life of 4.6 s, and at pH 10.4 to a half-life of 50 min. These results are consistent with recent studies by Beckman \textit{et al.} \(101,102\) who determined the rate constant \(k_{HA}\) for the decay of peroxonitrite and the \(pK_a\) of peroxonitrous acid at pH 7.4 and 37 °C to be \(0.65 \pm 0.05 \text{ s}^{-1}\) and \(6.8\), respectively.

Decomposition of peroxonitrite in the Presence of Methionine. We observe that when peroxonitrite is incubated in the presence of methionine the initial rate of disappearance of the 302 nm band due to peroxonitrite increases linearly with the concentration of Met added (Figure II.5 and Table II.1). Methionine sulfoxide or methionine sulfone, the two possible oxidation products formed in this reaction, do not absorb significantly at 302 nm, and therefore do not interfere with these measurements.

Methionine promotes the rate of disappearance of peroxonitrite at pH values larger than the measured \(pK_a\) for peroxonitrous acid which suggests that the reacting species is predominantly peroxonitrite ion rather than its
FIGURE II.4. Determination of the Equilibrium Constant and Pseudo-First Order Rate Constant for the Decomposition of Peroxonitrous Acid. Plot of $1/k_{\text{obs}}$ against $1/[H^+]$ using the expression $k_{\text{obs}} = (k_{\text{HA}} \times [H^+])/([H^+] + K_a)$. 
FIGURE II.5. Peroxonitrite Decomposition in the Presence of Methionine. Initial rates of decomposition of peroxonitrite as a function of methionine concentration at pH 8.8. Error bars are only shown where larger than symbols. Each data point represents the average of two determinations.
TABLE II.I. The Effect of Methionine on the Initial Rates of Decomposition of Peroxonitrite*.

<table>
<thead>
<tr>
<th>Met (mM)</th>
<th>Peroxonitrite (mM)</th>
<th>pH</th>
<th>$k_{obs} \times 10^{-3}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.84</td>
<td>8.8</td>
<td>9 ± 0</td>
</tr>
<tr>
<td>0.25</td>
<td>0.84</td>
<td>8.8</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>0.50</td>
<td>0.84</td>
<td>8.8</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>0.75</td>
<td>0.84</td>
<td>8.8</td>
<td>64 ± 1</td>
</tr>
<tr>
<td>1.00</td>
<td>0.84</td>
<td>8.7</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>0.00</td>
<td>1.68</td>
<td>10.7</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>1.00</td>
<td>1.68</td>
<td>10.7</td>
<td>0.6 ± 0.0</td>
</tr>
</tbody>
</table>

* Approximate initial rates of peroxonitrite decomposition were determined at 25 °C in 0.1 M sodium phosphate buffer by measuring absorbance changes at 302 nm every 0.1 s after addition of peroxonitrite (0.25 to 3.00 mM) to a thermostatted cell containing different concentrations of Met. The pH of the solutions was measured at the end of each experiment.
FIGURE 11.6. Inactivation of Alpha-1-PI as a Function of Peroxonitrite Concentration. Alpha-1-proteinase inhibitor (125 µg/mL) was exposed to different concentrations of peroxonitrite at 37 °C in phosphate buffer (pH 10-11.5 ± 0.5), and assayed for anti-elastase activity as described in the Experimental Section. Each data point is the average of duplicate measurements of two independent determinations.
conjugated acid; these results are shown in Table II.1.

**Inactivation of Alpha-1-PI by Peroxonitrite.** Peroxonitrite inactivates \( \alpha1\text{PI} \) in a dose-dependent manner (Figure II.6). At the lowest peroxonitrite concentration used, 170 \( \mu \text{M} \) (about 70:1 excess), a 24% decrease in EIC is observed. As stated in the experimental section, solutions of peroxonitrite are contaminated with nitrite and nitrate ions, and possibly \( \text{H}_2\text{O}_2 \). Although most of the \( \text{H}_2\text{O}_2 \) is probably removed by treating the crude peroxonitrite with \( \text{MnO}_2 \), traces of hydrogen peroxide left after this treatment could oxidize the methionine residue in the active site of \( \alpha1\text{PI} \) (Met\(^{358}\)) causing its inactivation (40). In order to assure ourselves that \( \alpha1\text{PI} \) is inactivated by peroxonitrite and not by either \( \text{H}_2\text{O}_2 \) or a mixture of nitrite and nitrate ions, \( \alpha1\text{PI} \) was incubated with peroxonitrite, an equivalent solution of peroxonitrite decomposed prior to addition, and a mixture of nitrite and nitrate ions. Figure II.7 shows that addition of 2 mM peroxonitrite causes complete inactivation, while an equivalent concentration of a decomposed peroxonitrite does not inactivate \( \alpha1\text{PI} \). Similarly, the anti-elastase activity of \( \alpha1\text{PI} \) is not affected upon incubation with nitrite and nitrate ions. These results illustrate that inactivation of the protein is caused by peroxonitrite and not by either a mixture of nitrite and nitrate ions nor by \( \text{H}_2\text{O}_2 \) not removed by \( \text{MnO}_2 \). Moreover, Table II.II shows that addition of catalase provides minimum protection (ca. 9%) against inactivation of \( \alpha1\text{PI} \). About the same degree of protection observed with catalase is obtained with BSA.
FIGURE II.7. Effect of Decomposed Peroxonitrite, Nitrate, and Nitrite Ions on the Anti-Elastase Activity of Alpha-1-PI. All solutions (final volume of 250 μL) were incubated at 25 °C for 3 hr and contained 125 μg/mL α1PI, 2 mM peroxonitrite (■), or 0.3 M nitrate + 0.3 M nitrite (□), or 2 mM decomposed peroxonitrite (◇) in 0.5 M phosphate buffer (pH 7.4). The EIC of α1PI was determined as described in the Experimental Section. Decomposed peroxonitrite was prepared by incubating it in buffer at 40 °C until the absorbance band at 302 nm had disappeared. Uninhibited elastase (○) and untreated α1PI (●) are shown for comparison purposes. Error bars appear only where they are larger than symbols, and they represent the standard deviation of duplicate runs of two determinations.
(ca. 4.8%), suggesting that catalase protects by a sacrificial role rather than by its catalytic activity, and that $\text{H}_2\text{O}_2$ removal, after the synthesis of peroxonitrite, is effective.

Peroxonitrite is stable in alkaline solutions \( (111) \) but has a $pK_a$ of 6.8 and decomposes rapidly after it is protonated. It has been proposed \( (102,115) \) that peroxonitrite decomposition generates a strong oxidant with reactivity similar to that of the hydroxyl radical \( (\text{HO}^\cdot \text{ in eq 1}) \), and $\text{NO}_2^\cdot$, also a radical and a strong oxidant.

To investigate if $\text{HO}^\cdot$ is responsible for the observed inactivation of $\alpha_1\text{PI}$ by peroxonitrite, we added hydroxyl radical scavengers to the incubation mixtures; these results are shown in Table II.II. Out of the $\text{HO}^\cdot$ scavengers used, thiourea is the most effective, affording complete protection against peroxonitrite-induced inactivation of $\alpha_1\text{PI}$. Benzoate and mannitol, also strong $\text{HO}^\cdot$ scavengers, do not protect $\alpha_1\text{PI}$ significantly even in a 50-fold excess with respect to peroxonitrite. Urea, a relatively weak scavenger of $\text{HO}^\cdot$, also fails to protect $\alpha_1\text{PI}$. On the other hand, methionine which is not considered a specific scavenger of $\text{HO}^\cdot$ completely prevents $\alpha_1\text{PI}$ inactivation. Similar results are obtained under basic and physiological pH suggesting that the same mechanism of inactivation operates under both conditions.

Table II.III shows estimated rates of reaction between the hydroxyl
### TABLE II.II. Protection by Hydroxyl Radical Scavengers and Catalase Against Inactivation of Alpha-1-PI by Peroxynitrous Acid*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in final system</th>
<th>[ONOO⁻] (mM)</th>
<th>% Protection&lt;sup&gt;b&lt;/sup&gt; (pH 10.4-11.5)</th>
<th>% Protection&lt;sup&gt;b&lt;/sup&gt; (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100 U/mL</td>
<td>2</td>
<td>0.0</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>300 U/mL</td>
<td>1</td>
<td>ND</td>
<td>9.2 ± 1.5</td>
</tr>
<tr>
<td>BSA</td>
<td>5.33 µg/mL</td>
<td>1</td>
<td>ND</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>20 mM</td>
<td>2</td>
<td>99.6 ± 1.4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>25 mM</td>
<td>1</td>
<td>100.0 ± 2.8</td>
<td>ND</td>
</tr>
<tr>
<td>Thiourea</td>
<td>20 mM</td>
<td>2</td>
<td>99.0 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>1</td>
<td>ND</td>
<td>99.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>1</td>
<td>97.7 ± 0.6</td>
<td>104.0 ± 0.0</td>
</tr>
<tr>
<td>Urea</td>
<td>50 mM</td>
<td>1</td>
<td>7.0 ± 5.0</td>
<td>4.8 ± 2.4</td>
</tr>
<tr>
<td>Benzoate</td>
<td>20 mM</td>
<td>1</td>
<td>ND</td>
<td>19.3 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>1</td>
<td>10.0 ± 5.2</td>
<td>15.6 ± 2.4</td>
</tr>
<tr>
<td>Mannitol</td>
<td>50 mM</td>
<td>1</td>
<td>11.8 ± 0.5</td>
<td>10.9 ± 4.8</td>
</tr>
</tbody>
</table>

* Reaction mixtures containing α1PI (125 µg/mL), added compounds in the indicated concentrations, and peroxonitrite (1 to 2 mM) were incubated for periods of 3 hr (25 °C) in 500 mM phosphate buffer (final pH 7.40 ± .05) or 12 hr (25 °C) in 100 mM phosphate buffer (final pH 10.4 to 11.5).

<sup>b</sup> Percent protection was calculated as 100x[(100 - %EIC<sub>A</sub>)/(100-%EIC<sub>B</sub>)], where "%EIC<sub>A</sub>" is the EIC measured for α1PI plus peroxonitrite plus additive and "%EIC<sub>B</sub>" is the EIC measured for α1PI plus peroxonitrite only. Values are presented as average % protection ± standard deviation calculated from duplicate runs of at least two independent experiments.

<sup>c</sup> 100 U/mL catalase corresponds to 7.1 µg/mL, and 300 U/mL to 21.3 µg/mL.

<sup>d</sup> ND stands for not determined.
radical and different components present in the incubation mixtures. These calculations indicate that all scavengers used, except for urea, should effectively protect α1PI against peroxonitrite-induced inactivation if HO− were the inactivating species.

**Oxidation of Methionine.** Product analysis of the reaction between MER10 and peroxonitrite was conducted to gain some insight into the mechanism of inactivation of α1PI by peroxonitrite.

Figure II.8 shows the PDMS spectra obtained for untreated MER10 (Fig. II.8A), and for the two HPLC fractions isolated (see Experimental) from the reaction of MER10 with peroxonitrite (Fig. II.8B and 8C). The spectrum of MER10 shows two intense peaks with masses of 1145 and 1167 corresponding to the (M+H)+ and (M+Na)+ ions respectively, and a less intense peak of 1189 corresponding to the (M+2Na)+ ion. Inspection of spectrum 8B suggests that this HPLC fraction is unreacted peptide with masses corresponding to the (M+H)+, (M+Na)+, (M+2Na)+, and (M+3Na)+ ions. Spectrum 8C indicates the addition of 16 mass units to the molecular weight of MER10 and consists of four peaks with mass values of 1160, 1182, 1205, and 1225 corresponding to the ions (MO+H)+, (MO+Na)+, (MO+2Na)+ and (MO+3Na)+, respectively⁶. We do not observe ions of lower molecular weight than that of MER10 in the PDMS.

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⁶ The notation (MO) indicates the molecular weight of MER10 (M) + 16 amu.
**TABLE II.III.** Rate Constants and Reaction Rates Between the Hydroxyl Radical and Various Components Present in Incubation Mixtures of Alpha-1-PI and Peroxonitrite.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Rate Constants* (L mol⁻¹ s⁻¹)</th>
<th>Concentration (mol L⁻¹)</th>
<th>Reaction Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO⁻⁺ + Protein</td>
<td>10¹¹</td>
<td>2.3 x 10⁻³</td>
<td>2.3 x 10⁸</td>
</tr>
<tr>
<td>HO⁻⁺ + Hydrogen phosphate</td>
<td>1.5 x 10⁶</td>
<td>0.4</td>
<td>6.1 x 10⁴</td>
</tr>
<tr>
<td>HO⁻⁺ + Dihydrogen phosphate</td>
<td>~ 2 x 10⁴</td>
<td>0.1</td>
<td>1.9 x 10³</td>
</tr>
<tr>
<td>HO⁻⁺ + Thiourea</td>
<td>3.9 x 10⁸</td>
<td>2.0 x 10⁻²</td>
<td>7.8 x 10⁷</td>
</tr>
<tr>
<td>HO⁻⁺ + Benzoate</td>
<td>5.9 x 10⁶</td>
<td>5.0 x 10⁻²</td>
<td>3.0 x 10⁸</td>
</tr>
<tr>
<td>HO⁻⁺ + Mannitol</td>
<td>1.7 x 10⁸</td>
<td>5.0 x 10⁻²</td>
<td>8.5 x 10⁷</td>
</tr>
<tr>
<td>HO⁻⁺ + Urea</td>
<td>7.9 x 10⁶</td>
<td>5.0 x 10⁻²</td>
<td>4.0 x 10⁴</td>
</tr>
<tr>
<td>HO⁻⁺ + Methionine</td>
<td>8.3 x 10⁶</td>
<td>2.0 x 10⁻²</td>
<td>1.7 x 10⁸</td>
</tr>
</tbody>
</table>

* All rates constants (pH 7, 25 °C) were obtained from reference (117).
FIGURE II.8. Mass Spectra of the Products Isolated from the Reaction of Peroxonitrite with MER10. HPLC fractions obtained from the reaction between peroxonitrite and MER10 are designated using the same nomenclature of Table II.IV. A: MER10; B: HPLC fraction 1B; C: HPLC fraction 1C. All spectra were obtained on a $^{232}$Cf Plasma Desorption Time-of-Flight Bio-Ion 20 mass spectrometer.
spectra of either fraction (1B and 1C) indicating that fragmentation of the peptide does not occur upon reaction with peroxonitrite. Similarly, no evidence of nitration which would give a molecular weight of 1190 amu (1144 + 46) is observed in Fig. II.8. The most probable site for nitration in MER10 is the phenyl ring of phanylalanine. Some low intensity peaks in spectra 8B and 8C are not labeled since they are also present in the spectrum of MER10 (Fig. II.8A), and therefore are considered of no relevance. The presence of Na in these samples is probably due to the fact that peroxonitrite solutions are highly contaminated with the alkali (see preparation of peroxonitrite in Experimental Section).

The amino acid analysis of the HPLC fractions isolated from the reaction of MER10 with peroxonitrite is presented in Table II.IV. The amino acid composition of fraction C again shows no evidence of peptide cleavage or modification of any other residue except for complete oxidation of methionine to methionine sulfoxide. These results are in agreement with the M + 16 peak detected by PDMS for fraction 1C (Fig. II.8C). Fraction 1B shows about 10% oxidation of total methionine, suggesting that this fraction is mostly composed of the unreacted peptide with a small contamination of the oxidized peptide. In agreement with this explanation the PDMS spectrum of this fraction (Fig. II.8B) does not show the presence of the oxidized peptide or of any smaller molecular weight peptides, indicating that this fraction is mainly composed of unreacted MER10.
TABLE II.IV. Amino Acid Composition of MER10 Exposed to Peroxonitrite*.

<table>
<thead>
<tr>
<th>Residue</th>
<th>MER10</th>
<th>Product 1B</th>
<th>Product 1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU</td>
<td>10.5±0.4</td>
<td>10.2±1.2</td>
<td>10.4±0.7</td>
</tr>
<tr>
<td>SER</td>
<td>9.9±0.6</td>
<td>8.8±0.8</td>
<td>8.6±0.3</td>
</tr>
<tr>
<td>MET</td>
<td>10.4±0.7</td>
<td>7.6±0.0</td>
<td>0.4±0.3</td>
</tr>
<tr>
<td>VAL</td>
<td>9.8±0.4</td>
<td>10.5±0.3</td>
<td>10.3±0.2</td>
</tr>
<tr>
<td>METSO(^b)</td>
<td>0.1±0.1</td>
<td>1.6±0.5</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td>ILE</td>
<td>10.0±0.1</td>
<td>10.5±0.2</td>
<td>10.3±0.2</td>
</tr>
<tr>
<td>PHE</td>
<td>10.0±0.1</td>
<td>10.2±0.4</td>
<td>9.8±0.6</td>
</tr>
<tr>
<td>LYS</td>
<td>10.5±0.5</td>
<td>10.4±0.5</td>
<td>9.2±0.9</td>
</tr>
<tr>
<td>PRO</td>
<td>29.3±3.0</td>
<td>30.2±0.5</td>
<td>32.5±2.1</td>
</tr>
</tbody>
</table>

* Residue amounts expressed as % mol ± s.d. of duplicate runs of two independent experiments.

\(^b\) Methionine sulfoxide was determined by the cyanogen bromide method (see experimental).
Further confirmation for the oxidation of methionine to methionine sulfoxide (MetSO) as the major product of the reaction between MER10 and peroxonitrite is revealed by $^1$H NMR$^7$. Figure II.9 shows the one-dimensional NMR spectra (1D NMR) of fraction 1C and MER10. The most noticeable change in the spectrum of fraction C (panel A in Fig. II.9) is the disappearance of the sharp singlet at 2.00 ppm due to the $\varepsilon$-CH$_3$ moiety of Met observed in MER10. Instead, a lower field resonance line at 2.65 ppm is observed; this signal corresponds to the protons in the $\varepsilon$-CH$_3$ group of MetSO confirming sulfur oxidation. The rest of the protons in MetSO ($\beta$-CH$_2$ and $\gamma$-CH$_2$) are also shifted to lower field positions compared to the Met signals in MER10. The aromatic protons of the phenylalanine residue (inserts in Fig. II.9) in fraction 1C show the same chemical shift and pattern of those corresponding to Phe in MER10, indicating absence of nitration of this residue in the product. These results also suggest that hydroxylation of the benzene ring by peroxonitrite, as previously reported (116), does not occur in the major product isolated from this reaction.

The resonance lines corresponding to the $\alpha$-protons in these peptides (except for Val $\alpha$H) are not shown in the 1D NMR spectra displayed in Fig. II.9. The signals due to both $\beta$H and $\alpha$H protons of most residues are poorly resolved in 1D spectra. The assignment of these resonances is enabled

---

$^7$ Assignments for all the resonances observed in MER10 have been accomplished by the use of COSY and RELAYED COSY experiments shown in Chapter I.
FIGURE 11.9. $^1$H 1D NMR Spectra of MER10 and the Major Product Isolated from the Reaction with Peroxonitrite. Amino acids are represented using the single-letter code. Panel A shows the spectrum of the major product resulting from the reaction between MER10 and peroxonitrite; panel B shows the spectrum of MER10. Inserts in both panels show the resonance lines of the aromatic protons of Phe. Spectra were collected in D$_2$O at 25 °C and chemical shifts are relative to TSP.
through the use of a two-dimensional COSY experiment. Figure II.10 shows an expansion of the COSY spectra obtained from fractions B and C. The cross peaks in this region result from interactions between the $\alpha$H and $\beta$H protons of all ten residues, and the $\beta$H and $\gamma$H, and $\delta$H and $\epsilon$H of the three prolyl residues. Most spin systems in this peptide show a degeneracy in the two $\alpha$H-$\beta$H cross peaks. For example; the two $\alpha$H-$\beta$H cross peaks of Ser are overlapped and appear as a single cross peak since both $\beta$H protons have nearly identical chemical shifts and the $\beta$H-$\epsilon$H cross peak is covered by the diagonal. However, this degeneracy does not hinder the interpretation of the spectra.

The COSY spectra in Fig. II.10 confirm the observations made from all product analyses discussed above. The COSY spectrum of fraction 1C only differs from that of MER10 (not shown) in that the $\alpha$H-$\beta$H cross peak of Met is not present and instead a cross peak due to the $\alpha$H-$\beta$H protons in MetSO is observed. The spectrum of fraction 1B shows an $\alpha$H-$\beta$H cross peak characteristic of Met indicating, as stated above, this is predominantly unreacted MER10. Again no indication of nitration or hydroxylation of the Phe residue is observed in these spectra.

We observe that when MER10 is exposed to peroxonitrite in the presence of a large excess of dimethyl sulfoxide (DMSO) no oxidation of Met to MetSO occurs. DMSO is a powerful hydroxyl radical scavenger, it interacts with HO$^-$ to produce methyl radicals (\textsuperscript{•}CH\textsubscript{3}) which ultimately form
FIGURE II.10. Expansion of COSY Spectra Obtained from Fractions 1B and 1C Isolated from the Reaction of MER10 and Peroxynitrite. NMR spectra of fractions C (panel A) and B (panel B) were collected in D$_2$O at 25 °C. The cross peaks shown in this region result from interactions between $\alpha$H and $\beta$H protons in all ten residues of the peptide, $\beta$H and $\gamma$H, and $\delta$H and $\delta'$H of the prolyl residues. Amino acids are represented using the single-letter code.
methane by hydrogen abstraction or formaldehyde (118) after reaction with molecular oxygen and subsequent decomposition. On the other hand, DMSO can also be oxidized by strong oxidants such as HOCl and H₂O₂ (119).

We conducted a simple experiment in order to determine whether DMSO prevents oxidation of the methionine residue in MER10 by scavenging HO⁻ or by being oxidized to the sulfone reacting directly with peroxonitrite. DMSO (0.2 M) was allowed to react with peroxonitrite (2.5 mM) in deuterium oxide (D₂O) at pH 10.5 in an NMR tube, and the reaction mixture was analyzed by NMR after 30 min, 1 hr, and 24 hr. The NMR spectra obtained after these intervals show a singlet at 2.9 ppm indicating that peroxonitrite oxidizes dimethyl sulfoxide to dimethyl sulfone. At this pH, we do not detect any resonance lines suggesting formation of formaldehyde.

DISCUSSION

There is ample evidence in the literature indicating that peroxonitrous acid (ONO₂H), the protonated form of peroxonitrite, rapidly and quantitatively isomerizes to nitric acid (104,111-114,120). However, there is confusion as to whether the mechanism of isomerization proceeds by homolysis of the O-O bond with the formation of the hydroxyl radical and nitrogen dioxide as intermediates (eqs 4 and 5), or via an intramolecular
rearrangement without the production of radical intermediates (eq 6).

\[
\begin{align*}
\text{ONO} & \quad \leftrightarrow \quad [\text{NO}_2^\bullet + \bullet \text{OH}] \quad (4) \\
[\text{NO}_2^\bullet + \bullet \text{OH}] & \quad \rightarrow \quad \text{HONO}_2 \quad (5) \\
\text{ONO} & \quad \rightarrow \quad \text{HONO}_2 \quad (6)
\end{align*}
\]

Halpheny and Robinson (116) first proposed a radical mechanism based on the observations that peroxonitrite at pH 0.5-1.2 hydroxylated aromatics in very low yields and also initiated the polymerization of methyl acrylate. This mechanism of isomerization through radical intermediates has been opposed by Hughes and Nicklin (111) who calculated the activation energy for the isomerization of peroxonitrite to be 12.5 kcal mol\(^{-1}\), a value very different from the usual one calculated for O-O homolyses (e.g. 30-40 kcal mol\(^{-1}\)).

Our results suggest that peroxonitrite inactivates α1PI by oxidizing the Met residue in the active site (40), and that the oxidizing agent most probably is peroxonitrite and not HO\(^-\).

Methionine promotes the decomposition of peroxonitrite at pH values above the calculated pK\(_a\) for peroxonitrouric acid indicating that peroxonitrute ion reacts with Met, and not HO\(^-\) or NO\(_2\) which would only form through the
decomposition of peroxonitrous acid (eq 4). Under basic conditions (e.g. pH 10 to 11.5) peroxonitrite ion is stable (111), and production of HO· and NO₂ is not pertinent since peroxonitrous acid is mostly dissociated (pKₐ 6.8 ± 0.3). We observe that peroxonitrite inactivates α₁PI in a pH range of 10.0-11.5 again suggesting that the anion reacts with α₁PI causing its inactivation. Under both physiological and basic pH conditions, benzoate and mannitol do not prevent α₁PI inactivation by peroxonitrite confirming that the oxidizing agent is not HO·.

It has been observed that HO· reacts with proteins causing gross structural modifications and spontaneous fragmentation (121-124). However, product analysis of the reaction between peroxonitrite and MER10 by PDMS indicates that fragmentation of the peptide does not occur at significant levels. Furthermore, we do not observe either nitration or hydroxylation of phenylalanine in the major product isolated from the reaction between MER10 and peroxonitrite. Both nitrated and hydroxylated peptides would be expected to form as a result of the reaction of NO₂ or HO· with the Phe residue in MER10.

We observe that thiourea, a powerful HO· scavenger, affords α₁PI complete protection against peroxonitrite-induced inactivation. However, this observation does not unequivocally confirm the presence of HO· since it has been shown that thiourea is not specific as a scavenger of HO· (119) and can be oxidized, for example, by H₂O₂ (125). The protection observed
with thiourea and Met, both present in large excess with respect to α1PI, probably arises because these compounds are being oxidized and therefore depleting peroxonitrite before oxidation of the protein takes place. This assumption was verified by the observation that peroxonitrite reacts with DMSO at basic pH to form primarily the sulfone. We do not detect other oxidation products, such as formaldehyde, which would indicate that DMSO has reacted with HO⁻. This line of reasoning leads us to speculate that urea, mannitol and benzoate do not protect α1PI since they cannot be easily oxidized.

CONCLUSION

Our results indicate that at physiological pH peroxonitrite is a powerful oxidant⁸ on its own without the requisite of forming HO⁻ (or NO₂) through the decomposition of peroxonitrous acid as previously suggested (102). We show that peroxonitrite is capable of oxidizing methionine residues in proteins such as α1PI. The oxidation of Met to MetSO has been reported to cause loss of biological activity in a wide variety of proteins (126,127). The methionine residue, which upon oxidation renders α1PI inactive, is situated in the active inhibitory site of the protein (Met³⁵⁸-Ser³⁵⁹) (40). This region of

⁸ An oxidation potential of E°' = 1.5 v has been estimated for the reduction of ONO₂⁻ to NO₂ (W.H. Koppenol, unpublished results).
\( \alpha 1\)PI is a highly stressed loop protruding from the protein and is, therefore, very accessible (128). We suggest that, due to its relatively small size, peroxonitrite could also oxidize less accessible methionine residues in other proteins. In fact, peroxonitrite ion was recently shown to mediate the oxidation of sterically hindered sulfhydryl groups in albumin (101).

In addition, we suggest that NO, present in cigarette smoke, could participate in the development of emphysema and other lung diseases related to cigarette smoking (60,129) by a previously unconsidered pathway. Cigarette smoke could increase transient concentrations of cellularly-produced NO (105,106) which upon reaction with PAM-derived superoxide (99,100), already increased in smokers, forms the strong oxidant peroxonitrite. As we have shown, peroxonitrite could oxidize \( \alpha 1\)PI, leaving the lung susceptible to an unopposed proteolytic attack on its extracellular matrix.
CHAPTER III. Release of Iron from Ferritin by Aqueous Solutions of Cigarette Smoke
This study demonstrates the ability of aqueous solutions of cigarette smoke to reduce iron and cause its release from ferritin. SOD increases the rates of iron release with the less filtered smoke solutions, but has no effect on the rate of iron release caused by aqueous solutions of gas-phase cigarette smoke. Faster rates of iron release are observed under anaerobic conditions and the reducing power of the cigarette smoke solutions is prolonged when incubated in argon. Hydroquinone and catechol, two of the major polyhydroxybenzenes in cigarette smoke, increase in concentration in the smoke as this is subjected to less filtration. Higher concentrations of these benzenediols correlate with higher rates of iron released from ferritin. Concomitant with iron release, depletions of amino acids in ferritin are observed. Depletion of histidine is partially prevented by bathophenanthroline sulfonate and mannitol, while lysine and arginine depletions remain unaffected. These observations suggest that cigarette smoke components react directly with these amino acid residues in ferritin. Cigarette smoke-induced release of iron could alter iron metabolism in the lungs of chronic smokers and contribute to the increase in the total oxidative burden on the lungs of smokers.
INTRODUCTION

Cigarette smoking has been implicated in a variety of lung diseases, such as emphysema and lung cancer (18,129), but the basic mechanisms by which tobacco smoke causes disease are still not well understood. Prolonged exposure to cigarette smoke also causes inflammation in the lower respiratory tract, a condition associated with larger numbers of pigment-laden macrophages found in the bronchiole, alveolar ducts, and alveoli of smokers (60). It has been shown that the pulmonary alveolar macrophages (PAM) of smokers contain increased amounts of iron and ferritin (57). The reason for these increased levels of iron and ferritin in the PAM of chronic smokers is not known. McGowan and Henley (130) have suggested that the majority of this iron is insoluble and seems not to be associated with the iron storage protein ferritin. Also, Qian and Eaton (131) recently showed that cigarette smoke solutions were able to reduce salts containing ferric ions and to bind the resulting ferrous ions. Preliminary electron spin resonance studies of lung tissue from smokers suggest iron accumulation that is proportional to the dark pigmentation of the lung (132).

These considerations prompted us to study the ability of aqueous solutions of cigarette smoke to release iron from ferritin, the principal source of physiological iron. Cigarette smoke contains high concentrations of polyhydroxybenzenes (1,18,19) with reduction potentials (133) lower than
ferritin (134); these compounds could theoretically reduce and release iron from ferritin.

In the studies reported here we have investigated the ability of aqueous solutions of cigarette smoke to release iron from ferritin, and the effects of SOD and catalase on this release. We also report the effect of aqueous solutions of cigarette smoke on the structure of ferritin.
EXPERIMENTAL

Chemicals. Bathophenanthroline disulfonic acid, 4,7-diphenyl-1,10-phenanthroline disulfonic acid, ortho-phthalaldehyde, 1,4-benzenediol (hydroquinone), 1,2-benzenediol (catechol), diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), mannitol, amino acid standard solution (2.5 mM, 0.1 N HCl), D-glucose, and 1,4-dithiothreitol were all purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Hydrochloric acid (6N) was purchased from Pierce (Rockford, IL). Research cigarettes (1R1 and 2R1F) were purchased from the University of Kentucky, Tobacco and Health Research Institute, stored at -20°C, and humidified in a desiccator over saturated ammonium nitrate at room temperature for at least 24 hr prior to use. Chelex-100® resin was purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of reagent grade, and used without further purification.

Enzymes. Bovine liver catalase (EC 1.11.1.6), bovine erythrocyte copper-zinc superoxide dismutase (EC 1.15.1.1), and glucose oxidase (EC 1.1.3.4) were all purchased from Sigma Co. In order to remove adventitious iron, glucose oxidase was incubated with 10 mM DTPA for 1 hr in ice, ultrafiltered and washed with two volumes of 250 μL of deionized distilled water in a Millipore Ultrafree-PF filter unit with a molecular weight cutoff of 10,000.
Ferritin Preparations. Aliquots of type I horse spleen ferritin, purchased from Sigma, were ultrafiltered, washed with two volumes of deionized distilled water, and redissolved in 0.1 M phosphate buffer (pH 7.4, Chelex-treated) to give a final concentration of 30 mg/mL. This solution was stored at 4°C for periods not longer than 72 hr. The purpose of this treatment is to eliminate unconfined iron from ferritin solutions. A more rigorous purification of ferritin was not pursued since the aqueous solutions of cigarette smoke contain traces of iron themselves. Total iron content of ferritin, determined as described by Brumby and Massey (135), was found to be 4.69 μmol Fe/mg protein.

Preparation of Cigarette Smoke Aqueous Solutions. Using the puff protocol described elsewhere (53), the smoke from 1R1 or 2R1F research cigarettes was bubbled through phosphate buffer (0.1 M, pH 7.4, Chelex-treated) contained in a test-tube immersed in an ice-water bath. No filter was used to prepare solutions termed "whole-smoke solution", while those termed "filtered-smoke solution" were prepared by passing the smoke through the filter tip that 2R1F cigarettes contain. Solutions termed "gas-phase solutions" were prepared by smoking 1R1 cigarettes through two Cambridge filters. All solutions were prepared by bubbling the smoke of 1 cigarette (ten 35-mL puffs) per 100 μL of buffer. All smoke solutions used were prepared immediately before experiments unless otherwise indicated.

Gel Electrophoresis. Aliquots (25 μg) of ferritin solutions similar to
those used for iron release experiments were subjected to native
electrophoresis on a 5.5% acrylamide gel at 30 mA for approximately 3 hr at
25 °C. After electrophoresis, gels were removed and stained with
Coomasie brilliant blue R-250 for one hour, and then allowed to destain for
24 hours in destaining solution (5% methanol, 7% glacial acetic acid, and
2% glycerol).

**Amino Acid Analysis.** Ferritin samples (125 µg aliquots) were
incubated with solutions of cigarette smoke for 12 hr at 37°C and
hydrolyzed with 6 N HCl at 110°C for 24 hr *in vacuo*. Amino acids were
separated and quantitated by HPLC in an AminoQuant® column (Hewlett-
Packard) using o-phthalaldehyde precolumn derivatization according to
Schuster and Apfel (78). Analysis of proline, cysteine, and tryptophan was
not performed. Oxidation of methionine to methionine sulfoxide was
determined by the method described by Shechter *et al.* (79). Briefly,
lyophilized protein samples were dissolved in 80% formic acid and allowed
to react with cyanogen bromide (100 mM) for 24 hr at room temperature.
The reaction was stopped by addition of an equal volume of water, frozen
and lyophilized. The cyanogen bromide peptides were hydrolyzed as
described above but in the presence of 13 mM 1,4-dithiothreitol.

**Iron Release.** Initial rates of iron release were measured
spectrophotometrically using bathophenanthroline disulfonate as metal
chelator essentially as described by Thomas and Aust (136). Incubation
mixtures (1 mL final volume) contained 1 mM bathophenanthroline
disulfonate and 750 μg of ferritin in 0.1 M phosphate buffer (pH 7.4,
15 mM NaCl, Chelex-100® treated). Catalase and SOD solutions were
added as indicated in figure legends. Reactions were started by the addition
of 100 μL of smoke solution or benzenediol. The change in absorbance was
measured continuously at 530 nm for the first 20 minutes using a double
beam spectrophotometer. A cuvette containing ferritin, bathophenanthroline
and added enzymes (catalase or SOD) but lacking cigarette smoke solution
or hydroquinones, was used as reference. The amount of ferrous iron
released from ferritin was calculated using a molar extinction coefficient of
20.66 mM⁻¹ cm⁻¹ obtained from a calibration curve using ferric sulfate
reduced with a 10% solution of thioglycolic acid. For anaerobic
measurements all solutions were purged with argon, and, before addition of
the smoke solution, incubation mixtures were purged again with argon
through a capillary glass tube inserted in the UV cell. In order to scavenge
remaining oxygen, glucose oxidase and glucose were always added along
with catalase to both sample and reference cuvettes.

Iron Incorporation. All studies were conducted at pH 7.0 in
0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] buffer
according to the method of Collawn et al. (137) with only minor changes.
Briefly, 4.5 mL of a solution of ferritin (0.1 mg/mL) in buffer was placed in a
constant temperature water bath at 14°C, and allowed to equilibrate. A
solution of ferrous ammonium sulfate (0.5 mL, 10 mM) dissolved in deairated water was added and mixed vigorously. Aliquots (1 mL) were taken at 1 minute intervals and added to Eppendorf tubes containing 0.3 g of Chelex-100®. Samples were vortexed for 4 minutes and needle holes were punched in the bottom of the tubes. The perforated Eppendorfs were placed in larger test tubes and centrifuged in a bench-top centrifuge for 4 minutes. The eluted ferritin solution, free of Chelex-100®, was then collected and its absorbance measured at 310 nm in Hewlett Packard 8451A Diode Array spectrophotometer; HEPES buffer was used as reference. The time of reaction was taken as the period elapsed between the addition of iron and the addition of the reaction mixture to Chelex-100®.

**HPLC.** A) By electrochemical detection: Aqueous solutions of cigarette smoke were separated using a Hewlett-Packard ODS (5 µm, 200x2.1 mm) (Hewlett-Packard Co., Avondale, PA) reverse phase column. The electrochemical detector consisted of a Model 5100A Coulochem® electrochemical detector (ESA, INC. Bedford, MA), a dual electrode analytical cell Model 5010 (ESA) consisting of two porous graphite in-line working electrodes with associated reference and counter electrodes, and a guard cell Model 5020 (ESA) placed before the column. Constant potentials of +350 mV and +400 mV were applied to the working electrodes of the analytical and guard cells, respectively. The mobile phase consisted of a binary system of water and methanol; both solvents contained 10 mM
NaClO₄. With a flow rate of 0.50 mL/min, 5 μL aliquots of smoke solution were injected onto the column and eluted with 10% MeOH. After 6 min the percentage of methanol was linearly increased to 100% over 35 min. Response factors used for quantitative analysis of hydroquinone and catechol were calculated from calibration curves obtained using authentic compounds.

B) By UV detection: Aqueous solutions of cigarette smoke were separated in a Hypersil ODS column (200 x 4.6 mm) connected to a Hewlett Packard HPLC model 1090M equipped with a diode array detector and an automatic injector. The eluent consisted of water and acetonitrile with an initial composition of 3% acetonitrile which was linearly increased to 50% acetonitrile over 12 min at a constant flow rate of 1.0 mL/min. The column was washed with 100% acetonitrile for 20 min after each injection (15 μL). After separation components were detected using four wavelengths (230, 254, 280 and 300 nm), and the UV spectra of peaks in smoke solutions were compared to those obtained in chromatograms of authentic compounds. All solvents used were HPLC grade and filtered through a 0.3 μm filter prior to use.
RESULTS

Release of Iron from Ferritin by Aqueous Solutions of Cigarette Smoke. When ferritin is incubated with aqueous solutions of cigarette smoke, iron is reduced and mobilized from the protein core. Table III.I shows initial rates of iron release, measured spectrophotometrically under aerobic conditions. In the absence of ferritin none of the cigarette smoke solutions form significant amounts of bathophenanthroline-Fe complex (data not shown). Lines 1, 4 and 7 suggest the amounts of ferrous iron released increase as the smoke is subjected to less filtration (see experimental for smoke solution preparations). The rates of iron release depend on the amounts of all three types of smoke solution (Figure III.1A), and on the concentration of ferritin (Figure III.1B; data only shown for filtered-smoke solution). Figure III.1A shows that the rates of iron release for the different solutions of cigarette smoke increase over the range of smoke solution concentrations used in the order gas-phase < filtered < whole smoke.

To determine whether superoxide participates in the reductive mobilization of ferric iron by cigarette smoke solutions, SOD was included in the incubation mixtures. SOD does not affect the rate of iron release when the reaction mixture contains gas-phase solution (line 3, Table III.I). However, for both filtered and whole smoke solutions, rates of iron release
## TABLE III.I. Aerobic Release of Iron from Ferritin by Aqueous Solutions of Cigarette Smoke

<table>
<thead>
<tr>
<th>Entry no.</th>
<th>Smoke Solution</th>
<th>Additions</th>
<th>nmol Fe Reduced/min per mg Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gas</td>
<td>None</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>Gas</td>
<td>Catalase</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>Gas</td>
<td>SOD</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>Filtered</td>
<td>None</td>
<td>0.89 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>Filtered</td>
<td>Catalase</td>
<td>1.15 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>Filtered</td>
<td>SOD</td>
<td>1.88 ± 0.07</td>
</tr>
<tr>
<td>7</td>
<td>Whole</td>
<td>None</td>
<td>1.95 ± 0.17</td>
</tr>
<tr>
<td>8</td>
<td>Whole</td>
<td>Catalase</td>
<td>2.56 ± 0.27</td>
</tr>
<tr>
<td>9</td>
<td>Whole</td>
<td>SOD</td>
<td>4.69 ± 0.26</td>
</tr>
</tbody>
</table>

* Reaction mixtures (final volume 1mL) contained ferritin (750 μg), the smoke from one 1R1 or 2R1F cigarette blown into 100 μL of buffer (see Experimental section), bathophenanthroline sulfonate (1 mM), SOD (500 units) or catalase (500 units) in 15 mM NaCl, pH 7.4. Reactions were initiated by addition of smoke solutions and absorbance was measured continuously at 530 nm during the first 20 min.

b "Gas" refers to solutions of cigarette smoke from 1R1 cigarettes passed through two Cambridge filters, "filtered" smoke solution from 2R1F cigarettes filtered with the self-contained filter tips, and "whole" to solutions of unfiltered smoke from 1R1 cigarettes.
FIGURE III.1. Initial Rates of Iron Release as a Function of the Concentration of Cigarette Smoke Solutions and Ferritin Concentration. Panel A shows initial rates of iron release from ferritin as a function of the concentration of the different cigarette smoke solutions. Reaction mixtures (final volume 1 mL) contained ferritin (750 μg) and bathophenanthroline sulfonate (1 mM) in 15 mM NaCl, pH 7.4. Reactions were started by addition of different volumes of smoke solution prepared by passing the smoke of one cigarette per 100 μL buffer. Whole smoke solution (■); filtered smoke solution (□); gas-phase smoke solution (▽). Panel B shows the initial rates of iron release as a function of the concentration of ferritin. Reaction mixtures contained bathophenanthroline sulfonate (1 mM), 100 μl of filtered smoke solution, and different amounts of ferritin.
increase when SOD is present in the reaction mixture (lines 6 and 9 in Table III.I).

The production of hydrogen peroxide by solutions of cigarette smoke (20,22) could have an inhibitory effect on the observed rates of iron release. Hydrogen peroxide can oxidize Fe(II) before complexation with bathophenanthroline sulfonate (138) but probably not after formation of the complex (139,140). In the presence of Fe (II), hydrogen peroxide can also oxidize species responsible for iron mobilization such as benzenediols present in cigarette smoke solutions. Both of these effects could cause a reduction in the amount of bathophenanthroline-Fe(II) complex formed. Inclusion of catalase in the reaction mixture with gas-phase smoke solution does not affect the rate of Fe(II)-bathophenanthroline complex formation (line 2 in Table III.I), while with filtered and whole smoke (lines 5 and 8 in Table III.I) it causes about 30% higher rates. To verify that these results are due to the catalytic activity of the enzymes, BSA was added to the incubation mixtures in concentrations equal to those of catalase and SOD and shown to have no effect in the rates of iron release (data not shown).

To investigate the role of oxygen in the rates of iron release, aerobic and anaerobic conditions were compared. The effect of oxygen on smoke was also investigated by measuring rates of iron release by filtered-smoke solutions incubated for 1 hr at room temperature in air or in argon. Figure III.2 shows typical curves obtained in these experiments with filtered-smoke
FIGURE III.2. Comparison of the Initial Rates of Iron Released by Filtered Smoke in the Presence and Absence of Air. Time-course formation of Fe(II)-bathophenanthroline disulfonate complex as iron is mobilized from ferritin by (1) filtered-smoke solution, release of iron measured under anaerobic conditions; (2) filtered-smoke solution incubated in argon for 1 hr, release of iron measured under aerobic conditions; and (3) filtered-smoke solution incubated in air for 1 hr, release of iron measured under aerobic conditions.
solutions. It is apparent from these curves that release of iron is faster in
the anaerobic system and that after 1 hr at room temperature, smoke
solutions incubated in argon release iron at a faster rate than its analogue
incubated in air. Table III.II summarizes these results and shows that initial
rates of iron release with filtered-smoke solutions under complete anaerobic
conditions (entry 1: buffer and solution in argon) are about 350% faster than
in air (entry 3), and also that smoke solutions release iron from ferritin, in the
presence of air, at rates about 150% faster when incubated in argon.

**HPLC Analysis of Aqueous Solutions of Cigarette Smoke.** Cigarette
smoke is an extremely complex mixture, with thousands of compounds
isolated and identified. The HPLC chromatogram obtained from a separation
of filtered smoke solution components is shown in Figure III.3. Catechol and
hydroquinone were positively identified by spiking the smoke solutions with
authentic compounds, and also by comparing the UV spectra recorded with
a diode array detector in chromatograms obtained from smoke solutions and
from authentic compounds (UV spectra not shown). Figure III.4 shows
typical HPLC-ECD chromatograms of all three aqueous solutions of cigarette
smoke obtained with oxidative-electrochemical detection. These traces
show that 1,4-dihydroxybenzene (hydroquinone) and 1,2-dihydroxybenzene
(catechol) are the major electrochemically-active components in the aqueous
solutions of cigarette smoke under the oxidative conditions used for this
### TABLE III.II. Effect of Oxygen in the Release of Iron from Ferritin by Aqueous Solution of Filtered-Smoke*

<table>
<thead>
<tr>
<th>Entry#</th>
<th>Anaerobic System</th>
<th>Smoke Solution</th>
<th>Incubation</th>
<th>nmol Fe Reduced/min per mg Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes Argon</td>
<td></td>
<td></td>
<td>4.23 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>No Argon</td>
<td></td>
<td></td>
<td>1.79 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>No Air</td>
<td></td>
<td></td>
<td>1.18 ± 0.09</td>
</tr>
</tbody>
</table>

* Reaction mixtures (final volume 1mL) contained ferritin (750 µg), 100 µL of filtered-smoke solution (one cig./100 µL), bathophenanthroline sulfonate (1 mM) and catalase (500 units) in 15 mM NaCl, pH 7.4. Anaerobic systems were purged with argon and contained glucose oxidase (10 units) and glucose (5 mM) in addition to the above components. Reactions were initiated by addition of filtered-smoke solutions (incubated for 1 hr in either air or argon at room temperature) and absorbance was measured continuously at 530 nm during the first 20 min.
FIGURE III.3. HPLC Separation of Filtered-Smoke Solution Components Detected at 254 nm. Separation was performed in a Hypersil ODS column (200 x 4.6 mm) with an eluent consisting of water and acetonitrile with an initial composition of 3% acetonitrile which was linearly increased to 50% acetonitrile over 12 min at a constant flow rate of 1.0 mL/min.
FIGURE III.4. HPLC with Electrochemical Detection of Aqueous Solutions of Cigarette Smoke. ECD-detection was carried out by applying a voltage of +350 mV in the detection cell, and +400 mV in the guard cell. Smoke solutions were diluted 1:100 before injection. Whole smoke solution (A), filtered smoke solution (B), and gas-phase smoke solution (C).
Quantitative analysis of both dihydroxybenzenes in the smoke solutions is shown in Table III.III. A comparison of these results with previously reported concentrations of catechol and hydroquinone in cigarette smoke condensates is difficult due to differences in the preparation of the condensates and the type of cigarettes used. Nanni et al. (75) recently reported concentrations of 38 to 58 μg for each catechol and hydroquinone per cigarette in electrostatic-precipitated condensates using commercially available and 1R4F cigarettes. Our determination shows that filtered and gas-phase smoke solutions contain concentrations of both dihydroxybenzenes below this range, and that hydroquinone is the major dihydroxybenzene in whole smoke solution with a concentration of 67 μg per 1R1 cigarette.

Release of Iron from Ferritin by Hydroquinone and Catechol.

Polyhydroxybenzenes (141,142) and more recently hydroquinone and catechol (143) have been shown to release iron from ferritin. Table III.IV shows rates of iron release obtained with catechol and hydroquinone under aerobic conditions in the presence of either catalase or SOD. Although the concentrations used for both benzenediols are equal to those found in whole smoke solution by HPLC-ECD (Table III.III), initial rates of iron release measured for a solution containing both diols (entry 3, Table III.IV) are about
TABLE III.III. Concentrations of Hydroquinone and Catechol in Aqueous Solutions of Cigarette Smoke\(^a\).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Catechol(^b)</th>
<th>Hydroquinone(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOLARITY (mM)</td>
<td>(\mu g/\text{cigarette})</td>
</tr>
<tr>
<td>Gas-Phase</td>
<td>0.52 ± 0.04</td>
<td>5.76 ± 0.46</td>
</tr>
<tr>
<td>Filtered</td>
<td>1.20 ± 0.10</td>
<td>13.16 ± 1.05</td>
</tr>
<tr>
<td>Whole</td>
<td>3.09 ± 0.25</td>
<td>33.97 ± 2.72</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations were determined by HPLC with electrochemical detection applying a constant voltage of +350 mV in the detection cell, and +400 mV in the guard cell. Response factors were calculated from calibration curves using pure compounds.

\(^b\) Concentrations of benzenediols in the aqueous solutions (1 cig/100 \(\mu L\)); they are expressed as mM (or \(\mu g\) grams per cigarette) ± S.D. (\(n=3\)).
TABLE III.IV. Aerobic Release of Iron from Ferritin by Benzenediols*.

<table>
<thead>
<tr>
<th>Entry no.</th>
<th>Benzenediol</th>
<th>Additions</th>
<th>nmol Fe Reduced/min per mg Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catechol</td>
<td>none</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>Hydroquinone</td>
<td>none</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>Catechol +</td>
<td>none</td>
<td>1.61 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Hydroquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Catechol +</td>
<td>Catalase</td>
<td>1.73 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Hydroquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Catechol +</td>
<td>SOD</td>
<td>1.76 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Hydroquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Whole smoke&lt;sup&gt;b&lt;/sup&gt;</td>
<td>none</td>
<td>1.95 ± 0.17</td>
</tr>
</tbody>
</table>

* Reaction mixtures (final volume 1 mL) contained ferritin (750 µg), bathophenanthroline sulfonate (1 mM), catechol (3.09 mM), hydroquinone (6.10 mM), catalase (500 units), and SOD (500 units) in 15 mM NaCl, pH 7.4. Reactions were initiated by addition of benzenediols and absorbance was measured continuously at 530 nm during the first 20 min.

<sup>b</sup> Contains 3 mM catechol and 6 mM hydroquinone (Table III.III).
17% slower than those measured with the smoke solution (entry 6, Table III.IV). The effects of catalase and SOD (entries 4 and 5, Table III.IV) on these rates are not nearly as dramatic as are the effects of these enzymes on the rates obtained with filtered or whole smoke solutions. Both enzymes slightly increase the rates of iron release by a solution containing both benzenediols.

Structure of Smoke-Exposed Ferritin. The structure of ferritin after incubation with cigarette smoke solutions was studied by three methods: iron incorporation, native gel electrophoresis, and amino acid analysis.

Figure III.5 shows the uptake of ferrous iron by smoke-treated ferritin samples. After incubation with smoke solutions for 12 hr at 37 °C followed by exhaustive dialysis, all three smoke-exposed ferritin preparations exhibit rates of ferrous iron uptake comparable to that of the control. The choice of buffer in the Fe(II) uptake assay is of importance. When acetate buffer is used in place of HEPES considerably slower rates of iron incorporation are observed (144). Nonetheless, our results indicate that any damage suffered by the protein during the incubation with smoke solutions does not change its ability to reincorporate iron compared to native ferritin (control).

Smoke-exposed ferritin solutions were also subjected to non-denaturing gel electrophoresis; these results are shown in Figure III.6. Ferritin exposed to gas-phase solution (lane 5) does not exhibit a change in mobility compared to native ferritin (lanes 2, 4, and 6). On the other hand,
FIGURE III.5. Rates of Iron Uptake by Smoke-Treated Ferritin. Ferritin samples were incubated with cigarette smoke solutions for 12 hr at 37 °C, and then exhaustively dialyzed before iron incorporation assay. No ferritin (v); untreated ferritin control (●); exposed to gas-phase smoke (○); exposed to filtered smoke (□); exposed to whole smoke (◊).
FIGURE III.6. Non-Denaturing Polyacrylamide Gel Electrophoresis of Smoke-Treated Ferritin. Typical gels obtained after electrophoresis of ferritin samples exposed to different aqueous solutions of cigarette smoke for 12 hr at 37 °C, with lanes: 1, ferritin exposed to whole smoke solution; 2, 4, and 6, native ferritin; 3, ferritin exposed to filtered smoke solution; 5, ferritin exposed to gas-phase smoke solution.
filtered and whole smoke-treated ferritin samples (lanes 3 and 1 respectively) exhibit small changes in their mobility compared to the native protein, indicative of an increased anionic nature compared to native ferritin.

Table III.V shows the results of amino acid analysis of ferritin samples exposed to all three types of smoke solutions. Amino acid analysis obtained for native ferritin in this study compares very well with previously published amino acid compositions for horse spleen ferritin (145-147). Table III.V shows that no significant changes in amino acid composition are detected for ferritin exposed to gas-phase solution. However, ferritin exposed to filtered and whole smoke solutions shows significant changes in the levels of some amino acids. Histidine levels decrease by about 30% for filtered and 75% for whole smoke-treated ferritin. Lysine depletions of about 20 and 35% are observed for filtered and whole smoke solutions, respectively. Threonine, tyrosine, phenylalanine, and arginine levels decrease by approximately 60, 30, 28 and 20%, respectively, in whole smoke-exposed samples. On the other hand, levels of glycine, alanine and valine in ferritin exposed to whole smoke solutions increase.

Cigarette smoke solutions produce hydrogen peroxide (20,22,23) which could react with ferrous iron released from ferritin to generate an activated oxygen species such as the hydroxyl radical or ferryl ion; these species could react with the side chains of amino acids in ferritin. The amino acid composition of ferritin incubated with whole smoke solution in
TABLE III.VI. Amino Acid Composition of Whole Smoke-Exposed Ferritin in the Presence of Metal Chelators or Mannitol*.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Control</th>
<th>Whole(^b)</th>
<th>Bathophe.(^c)</th>
<th>Mannitol(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASX</td>
<td>19.5 ± 2.1</td>
<td>18.6 ± 1.8</td>
<td>17.7 ± 0.2</td>
<td>18.2 ± 0.1</td>
</tr>
<tr>
<td>GLX</td>
<td>24.2 ± 2.0</td>
<td>25.4 ± 2.0</td>
<td>31.5 ± 1.5</td>
<td>32.2 ± 0.2</td>
</tr>
<tr>
<td>SER</td>
<td>8.1 ± 0.6</td>
<td>7.7 ± 1.8</td>
<td>6.7 ± 0.0</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>HIS</td>
<td>5.9 ± 0.1</td>
<td>1.3 ± 0.7</td>
<td>4.1 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>GLY</td>
<td>10.7 ± 0.7</td>
<td>19.4 ± 1.5</td>
<td>13.7 ± 0.3</td>
<td>13.7 ± 0.0</td>
</tr>
<tr>
<td>THR</td>
<td>5.1 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>4.4 ± 0.0</td>
<td>4.7 ± 0.0</td>
</tr>
<tr>
<td>ALA</td>
<td>15.1 ± 0.4</td>
<td>18.3 ± 0.2</td>
<td>17.1 ± 0.1</td>
<td>17.7 ± 0.0</td>
</tr>
<tr>
<td>ARG</td>
<td>10.1 ± 0.5</td>
<td>8.0 ± 0.4</td>
<td>7.3 ± 0.1</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>TYR</td>
<td>5.0 ± 0.5</td>
<td>3.5 ± 0.2</td>
<td>5.4 ± 0.1</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>VAL</td>
<td>7.8 ± 0.7</td>
<td>9.2 ± 0.5</td>
<td>9.8 ± 0.2</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>MET</td>
<td>3.0 ± 0.2</td>
<td>2.9 ± 0.5</td>
<td>3.1 ± 0.3</td>
<td>3.1 ± 0.0</td>
</tr>
<tr>
<td>METSO</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ILE</td>
<td>3.8 ± 0.3</td>
<td>3.9 ± 0.7</td>
<td>5.6 ± 0.3</td>
<td>4.3 ± 0.0</td>
</tr>
<tr>
<td>PHE</td>
<td>7.4 ± 0.4</td>
<td>5.3 ± 0.5</td>
<td>6.9 ± 0.1</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td>LEU</td>
<td>27.5 ± 0.7</td>
<td>28.5 ± 0.8</td>
<td>25.1 ± 0.1</td>
<td>25.1 ± 0.1</td>
</tr>
<tr>
<td>LYS</td>
<td>9.2 ± 0.7</td>
<td>6.1 ± 0.5</td>
<td>6.5 ± 0.1</td>
<td>6.1 ± 0.1</td>
</tr>
</tbody>
</table>

* Amino acid composition is given as number of residues per subunit of 22,500 D. Results are expressed as the mean ± standard deviation (n = 5).
\(^b\) Ferritin samples (750 µg) were incubated with whole smoke solutions (100 µL) in 100 mM phosphate buffer for 12 hr at 37 °C (from Table III.V).
\(^c\) In the presence of 1 mM bathophenanthroline sulfonate.
\(^d\) In the presence of 1 mM mannitol.
TABLE III.VI. Amino Acid Composition of Whole Smoke-Exposed Ferritin in the Presence of Metal Chelators or Mannitol.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Control</th>
<th>Whole&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Bathope.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mannitol&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASX</td>
<td>19.5 ± 2.1</td>
<td>18.6 ± 1.8</td>
<td>17.7 ± 0.2</td>
<td>18.2 ± 0.1</td>
</tr>
<tr>
<td>GLX</td>
<td>24.2 ± 2.0</td>
<td>25.4 ± 2.0</td>
<td>31.5 ± 1.5</td>
<td>32.2 ± 0.2</td>
</tr>
<tr>
<td>SER</td>
<td>8.1 ± 0.6</td>
<td>7.7 ± 1.8</td>
<td>6.7 ± 0.0</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td>HIS</td>
<td>5.9 ± 0.1</td>
<td>1.3 ± 0.7</td>
<td>4.1 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>GLY</td>
<td>10.7 ± 0.7</td>
<td>19.4 ± 1.5</td>
<td>13.7 ± 0.3</td>
<td>13.7 ± 0.0</td>
</tr>
<tr>
<td>THR</td>
<td>5.1 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>4.4 ± 0.0</td>
<td>4.7 ± 0.0</td>
</tr>
<tr>
<td>ALA</td>
<td>15.1 ± 0.4</td>
<td>18.3 ± 0.2</td>
<td>17.1 ± 0.1</td>
<td>17.7 ± 0.0</td>
</tr>
<tr>
<td>ARG</td>
<td>10.1 ± 0.5</td>
<td>8.0 ± 0.4</td>
<td>7.3 ± 0.1</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>TYR</td>
<td>5.0 ± 0.5</td>
<td>3.5 ± 0.2</td>
<td>5.4 ± 0.1</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>VAL</td>
<td>7.8 ± 0.7</td>
<td>9.2 ± 0.5</td>
<td>9.8 ± 0.2</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>MET</td>
<td>3.0 ± 0.2</td>
<td>2.9 ± 0.5</td>
<td>3.1 ± 0.3</td>
<td>3.1 ± 0.0</td>
</tr>
<tr>
<td>METSO</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ILE</td>
<td>3.8 ± 0.3</td>
<td>3.9 ± 0.7</td>
<td>5.6 ± 0.3</td>
<td>4.3 ± 0.0</td>
</tr>
<tr>
<td>PHE</td>
<td>7.4 ± 0.4</td>
<td>5.3 ± 0.5</td>
<td>6.9 ± 0.1</td>
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</tr>
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</tr>
</tbody>
</table>

<sup>a</sup> Amino acid composition is given as number of residues per subunit of 22,500 D. Results are expressed as the mean ± standard deviation (n = 5).

<sup>b</sup> Ferritin samples (750 µg) were incubated with whole smoke solutions (100 µL) in 100 mM phosphate buffer for 12 hr at 37 °C (from Table III.V).

<sup>c</sup> In the presence of 1 mM bathophenanthroline sulfonate.

<sup>d</sup> In the presence of 1 mM mannitol.
the presence of bathophenanthroline sulfonate or the hydroxyl radical scavenger mannitol was studied in an effort to determine the origin of the damage to the protein; these results are shown in Table III.VI. Both bathophenanthroline and mannitol give similar results; they afford almost complete protection to threonyl, tyrosyl, and phenylalanyl residues. Histidine is partially protected (ca. 61%)\(^9\) by bathophenanthroline and less efficiently by mannitol (ca. 43%). Lysine and arginine depletions are not prevented by the metal chelator or by mannitol. Alanine and valine levels remain higher than in the control in the presence of bathophenanthroline sulfonate or mannitol, while levels of glycine are increased by 30% compared to an increase of 80% in the absence of bathophenanthroline or mannitol. No significant methionine oxidation was detected in either the presence or absence of bathophenanthroline sulfonate or mannitol.

**DISCUSSION**

The data presented above demonstrate that aqueous solutions of cigarette smoke are capable of reducing and mobilizing iron from ferritin. Two possible mechanisms for this release are: reduction of ferritin iron by superoxide ion generated during the autoxidation of polyhydroxybenzenes

\[9\] Percent protection is defined as 100 times the difference: 100-[(control -experiment with additive)/(control -smoke experiment)].
present in smoke solutions (1,23); or direct reduction of iron by smoke components. Our data on the effect of SOD suggest that the second mechanism is more likely.

Superoxide ion and hydrogen peroxide are formed in aqueous tar solutions of cigarette smoke from the autoxidation of smoke components (22,23). The results obtained here with SOD indicate that superoxide ion, which is known to release iron from ferritin (138,148), does not contribute significantly to the release of iron caused by cigarette smoke. In fact, SOD enhances the rate of formation of the Fe(II)-bathophenanthroline complex in the presence of filtered and whole smoke, and has no effect with gas-phase smoke solutions. Addition of catalase gives similar results, enhancing rates of Fe(II) complex formation with filtered and whole smoke solutions but showing no effect in the presence of gas-phase smoke. The effects of these two enzymes indicate that superoxide ion and hydrogen peroxide formation is only relevant in filtered and whole smoke preparations, and that both species inhibit the rate of formation of the Fe(II)-bathophenanthroline complex. The effect of catalase is most probably due to preventing the oxidation of Fe(II) released from ferritin by hydrogen peroxide and consequently causing an increased recovery of reduced iron by bathophenanthroline (138). The effect of SOD is discussed below.

Analysis by HPLC using electrochemical detection shows the presence of hydroquinone and catechol in all our smoke solutions, along with other
unidentified minor components (Figure III.4). Recently Leanderson and Tagesson (29) also detected hydroquinone and catechol, using HPLC-ECD under oxidative conditions, as the major electrochemically-active components in aqueous solutions of cigarette smoke.

The concentration of these phenols increases as the smoke is subjected to less filtration and the sum of their amounts in all smoke solutions correlates with the rates of iron release (see Figure III.7). The difference in concentration of phenols in filtered and non-filtered smoke solutions could in part explain the differing rates of iron release observed. Although these benzenediols are abundant in cigarette smoke (75) and they are probably important contributors in the reduction of ferritin, our data do not allow us to discard other smoke components also intervening in the observed release of iron. For example, cigarette smoke contains high concentrations of nitric oxide which has been shown to release iron from ferritin (149). Our results also suggest that cigarette smoke is a much more complex system than a mixture of hydroquinone and catechol. The rates of iron release measured with a mixture of both benzenediols, in concentrations comparable to those determined in whole smoke, are not increased by catalase and SOD as much as with the smoke solutions (Table III.IV).

**Protein Damage.** Amino acid analysis (Tables III.V and III.VI) along with the results obtained from native gel electrophoresis indicate that both filtered and whole smoke solutions are capable of changing the chemical
FIGURE III.7: Initial Rates of Iron Release as a Function of the Combined Concentrations of Hydroquinone and Catechol Found in the Three Different Solutions of Cigarette Smoke. Abscissa values obtained from Table III.I, ordinate values obtained from Table III.II ($R^2 = 0.992$). A: gas-phase smoke solution; B: filtered-smoke solution; C: whole-smoke solution.
composition of ferritin. These modifications, however, do not seem to underline the ability of ferritin to reincorporate iron. The changes in the levels of histidine, lysine, and arginine for both filtered- and whole smoke-exposed ferritin samples could explain the changed electrophoretic mobilities observed for these two treated ferritin samples in native gels. Modification of these basic amino acids could result in a net decrease in positive charge, making the protein more anionic than untreated ferritin. Damage to lysine, arginine and about 30% of total histidine is not protected by either bathophenanthroline or mannitol, indicating that not all amino acid damage involves ferrous iron or an active oxygen species. These results suggest that cigarette smoke components react with histidyl, arginyl and lysyl residues in ferritin. We have previously observed depletions of lysyl and histidyl residues in \( \alpha \)-1-proteinase inhibitor after incubation with cigarette smoke aqueous solutions\(^{10}\). Also, the formation of cyanomethyl derivatives of lysyl and arginyl residues upon reaction with cigarette smoke has been reported (72). The lack of methionine oxidation by smoke solutions even in the absence of bathophenanthroline or mannitol is unexpected, but our present data do not allow us to afford a proper explanation for this observation.

The reductive release of iron from ferritin has been well documented

\(^{10}\) See Tables I.II and I.IV in Chapter I.
to cause oxidative damage, principally by initiating lipid peroxidation (138,141,142,150,151). We suggest that release of iron from ferritin caused by solutions of cigarette smoke in the presence of hydrogen peroxide, also produced by smoke solutions, could also catalyze the oxidation of proteins.

The Effect of SOD. The release of iron is faster under argon than under air, and filtered-smoke solutions release iron faster when incubated in argon than when incubated in air. It was also observed that filtered and whole smoke solutions release iron from ferritin more efficiently when SOD is added to incubation mixture. This suggests that under aerobic conditions autoxidation of species responsible for the release of iron from ferritin occurs with the effect of diminishing the reducing capability of the smoke solutions, and that SOD prolongs the life of these reducing species. Oxygen uptake experiments with smoke solutions and ferritin (data not shown) suggest that SOD and catalase inhibit initial rates of oxygen consumption of both filtered and whole smoke solutions, confirming this hypothesis.

A possible mechanism for the release of iron from ferritin by cigarette smoke solutions which could explain the effect of SOD is shown in eqs 1 through 6. In eq 1, hydroquinone and catechol (QH$_2$), along with other electrochemically-active compounds present in lower concentrations in cigarette smoke solutions, are proposed to be the major contributors in the release of iron from ferritin. Nitric oxide is probably not a major contributor.
in the release of iron since solutions purged with argon, which are not likely
to contain high concentrations of this gas, release iron from ferritin most
efficiently. A contribution to the release of iron from semiquinones (QH\(^{+}\)),
generated in eqs 1 and 4, cannot be eliminated by the data presented here.
Semiquinones have been shown to release iron effectively from ferritin
principally under anaerobic or hypoxic conditions (152). However, Monteiro
et al. (153) observed that benzosemiquinone does not cause the release of
iron under aerobic conditions, and that oxygen causes a marked inhibition in
iron release by other semiquinones. Other studies (142,151) have shown
enhancement of iron release from ferritin in the presence of SOD using
dialuric acid, acid-hydrolyzed divicine and 6-hydroxydopamine as reducing
species, and a rationale similar to that proposed here was given.

\[
\begin{align*}
QH_2 + \text{Fe(III)} & \rightarrow QH^+ + \text{Fe(II)} + H^+ & (1) \\
QH^+ + \text{Fe(III)} & \rightarrow \text{Fe(II)} + Q + H^+ & (2) \\
QH^+ + O_2 & \rightarrow Q + O_2^- + H^+ & (3) \\
QH_2 + O_2^- + H^+ & \rightarrow QH^+ + H_2O_2 & (4) \\
QH^+ + O_2^- + H^+ & \rightarrow Q + H_2O_2 & (5) \\
2 QH^+ & \leftrightarrow QH_2 + Q & (6)
\end{align*}
\]
CONCLUSION

Our in vitro observations indicate that cigarette smoke releases iron from ferritin and that this release is accompanied by damage of the protein which is prevented in part by a metal chelator and a hydroxyl radical scavenger. Iron is known to catalyze the formation of reactive oxygen species (58) which have been implicated in a variety of deleterious oxidative processes (59). We suggest the release of iron from ferritin by cigarette smoke may alter iron metabolism in the lungs of smokers. Also, cigarette smoke in the presence of biological forms of iron could constitute an oxidation system capable of causing oxidative damage to proteins. These findings provide possible explanations for the observed inflammation of the lower respiratory tract and increased concentrations of iron found in PAM of tobacco users.

Acknowledgement. This work was supported in part by a grant from NIH and a contract from the National Foundation for Cancer Research. Helpful discussions with Professors C.C. Winterbourn and S.D. Aust are greatly appreciated.


Juan José Moreno Rodriguez was born in Quito, Ecuador on April 15, 1959. He attended the Colegio Nacional de Buenos Aires in Buenos Aires, Argentina and the Instituto Argentino de Enseñanza Secundaria, where he completed his high school studies. Later on, he attended the Universidad de Buenos Aires for a period of one year before his family moved back to Ecuador. Once in Ecuador, he applied for admission to Saint Louis University in Missouri where he was admitted and where he obtained the degree of B.S. in Chemistry *cum laude*. He then came to Louisiana State University and joined Dr. W.A. Pryor’s research group. Juan J. Moreno R. is currently a candidate for the degree of Doctor of Philosophy in the Department of Chemistry.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Juan J. Moreno R.

Major Field: Chemistry (Organic)


Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

EXAMINATION COMMITTEE:

June 21, 1991