THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 35, Issue of September 1, pp. 27258–27265, 2000
Printed in U.S.A.

Oxidation of either Methionine 351 or Methionine 358 in α_1-Antitrypsin Causes Loss of Anti-neutrophil Elastase Activity*

Received for publication, June 5, 2000
Published, JBC Papers in Press, June 23, 2000, DOI 10.1074/jbc.M004850200

Clifford Taggart‡§¶, Daniel Cervantes-Laurean¶‖, Geumsoo Kim, Noel G. McElvaney‡‖, Nancy Wehr**, Joel Moss‖, and Rodney L. Levine**‡‡**

*From the ‡Pulmonary Division, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland and the ‖Pulmonary-Critical Care Medicine Branch and **Laboratory of Biochemistry, NHLBI, National Institutes of Health, Bethesda, Maryland 20892*

Hydrogen peroxide is a component of cigarette smoke known to be essential for inactivation of α_1-antitrypsin, the primary inhibitor of neutrophil elastase. To establish the molecular basis of the inactivation of α_1-antitrypsin, we determined the sites oxidized by hydrogen peroxide. Two of the nine methionines were particularly susceptible to oxidation. One was methionine 358, whose oxidation was known to cause loss of anti-elastase activity. The other, methionine 351, was as susceptible to oxidation as methionine 358. Its oxidation also resulted in loss of anti-elastase activity, an effect not previously recognized. The equal susceptibility of methionine 358 and methionine 351 to oxidation was confirmed by mass spectrometry. To verify this finding, we produced recombinant α_1-antitrypsins in which one or both of the susceptible methionines were mutated to valine. M351V and M358V were not as rapidly inactivated as wild-type α1-antitrypsin, but only the double mutant M351V/M358V was markedly resistant to oxidative inactivation. We suggest that inactivation of α_1-antitrypsin by oxidation of either methionine 351 or 358 provides a mechanism for regulation of its activity at sites of inflammation.

α_1-Antitrypsin (α_1-AT)[1] is a 52-kDa glycoprotein synthesized mainly by hepatocytes (1). Its primary function, however, is in the lung parenchyma, where it protects the alveolar matrix from destruction by neutrophil elastase (NE), a serine protease capable of destroying most of the structural components of the alveolar wall (2, 3). In addition to its presence in the lung, α_1-AT is abundant in human plasma with concentrations in the 20–53 μM range (4). When plasma levels of α_1-AT are below a protective threshold of approximately 11 μM, due to mutations of the α_1-AT gene, the anti-NE protection on the alveolar surface is inadequate, resulting in unopposed proteolytic activity, eventually leading to lung destruction and the development of emphysema by the third or fourth decade of life (5). These and other data have led to the protease-antiprotease hypothesis to explain the pathogenesis of emphysema (2, 6).

While there is compelling evidence for this hypothesis in explaining the pathogenesis of emphysema associated with α_1-AT deficiency, it is less clear why emphysema develops in cigarette smokers with normal levels of α_1-AT. In this regard, it has been suggested that lung destruction in smokers results from a functional defect in α_1-AT in the lower respiratory tract secondary to oxidative inactivation (7, 8). A similar mechanism has been suggested for other oxidant-mediated lung disorders such as hyperoxia-induced lung damage (9), adult respiratory distress syndrome (10), cystic fibrosis (11), and bronchopulmonary dysplasia (12); in these diseases, a combination of an increased protease burden and an oxidant-inactivated antiprotease defense exposes the lung to attack.

α_1-Antitrypsin binds to NE in a 1:1 molar ratio with an association constant of 10^7 M^{-1} s^{-1} (13). Based on structural similarities with legume protease inhibitors (14) and effects of oxidation of methionine residues, it was proposed that methionine 358 is located at the reactive site of α_1-AT. For example, oxidation of methionine 358 and possibly other methionines in α_1-AT to methionine sulfoxide by oxidants (*e.g.* myeloperoxidase (15), *N*-chlorosuccinimide (16), chloramine-T (17), peroxynitrite (18), or hydrogen peroxide generated by glycation with glucose and cupric ion (19)) greatly decreases its activity. Treatment of α_1-AT with *cis*-dichlorodiammineplatinum (II) (20), a square-planar antineoplastic agent, which reacts with DNA and nucleophile side groups of amino acids such as methionine and cysteine, protected methionine 358 against oxidation to its sulfoxide by hydrogen peroxide.

Human α_1-AT contains nine methionines, and the seminal studies of Johnson and Travis established that two are readily oxidized by chemical and enzymatic systems (16), methionine 358 and methionine 351. More recent studies of the oxidation of methionine residues in α_2-macroglobulin and glutamine synthetase led us to hypothesize that readily oxidizable, surface-exposed methionine residues may function as endogenous antioxidants whose purpose is to protect essential residues from oxidation (21). We hypothesized that the oxidation of methionine 351 in α_1-AT was another example, serving to protect the critical methionine 358 from oxidation. To clarify the role of methionine oxidation in α_1-AT activity, we investigated the site, sequence, and extent of methionine oxidation by hydrogen peroxide, a major oxidizing agent in cigarette smoke (22), and the effect of oxidation on α_1-AT inhibition of NE. Contradicting our hypothesis, we found that oxidation of methionine 351 actually causes inactivation of α_1-AT rather than preventing it.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of travel grants from the Higher Education Authority of Ireland and the Charitable Infirmary Charitable Trust, Dublin, Ireland.

¶ These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: NIH, Bldg. 3, Rm. 106, MSC 0320, Bethesda, MD 20892-0320. Tel.: 301-496-2310; Fax: 301-496-0599; E-mail: rlevine@nih.gov (for reprint requests).

[1] The abbreviations used are: α_1-AT, α_1-antitrypsin; NE, neutrophil elastase; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight.

EXPERIMENTAL PROCEDURES

N-Methoxysuccinyl-Ala-Ala-Pro-Val-nitroanilide, allatotropin, Chelex 100, and formic acid (99%) were obtained from Sigma. HEPES (free acid) was obtained from ICN Biomedicals, Inc. (Aurora, OH). CNBr-activated Sepharose 4B was obtained from Amersham Pharmacia Biotech. Ultrafiltration membranes were obtained from Amicon, Inc. (Beverly, MA). Neutrophil elastase was purchased from Elastin Products Company, Inc. (Owensville, MO) and Sigma. α_1-AT in the expression plasmid pFEAT30 was obtained from Myeong-Hee Yu (Korea Institute of Science and Technology, Taejon, South Korea). The QuikChange Mutagenesis Kit and BL21(DE3)pLysS-competent cells were obtained from Stratagene (La Jolla, CA). Mutagenic primers for the synthesis of M351V-, M358V- and M351V/M358V-α_1-AT were obtained from Biosynthesis Inc. (Lewisville, TX). Dialysis cassettes were from Pierce.

Purification of Plasma α_1-AT—The order of oxidation of α_1-AT methionine residues following oxidative exposure was evaluated using α_1-AT purified from plasma of an individual with the MM α_1-AT phenotype. Blood was collected in heparinized tubes on ice. Plasma, isolated by centrifugation, was then applied to an affinity column of anti-α_1-AT polyclonal antibody (DAKO Co., Carpinteria, CA) linked to CNBr-activated Sepharose 4B. α_1-AT was eluted with a solution of 100 mM sodium carbonate, 500 mM sodium chloride, 5 mM EDTA, and 0.1% NaN_3 at pH 10. The eluate was concentrated by pressure filtration (YM 10) (Amicon) and applied to a Sephadex G-100 gel permeation column. The α_1-AT peak was collected and applied to an ion exchange column (Mono Q). Purified α_1-AT from this column was concentrated by pressure filtration (YM-10), and the final α_1-AT concentration was determined by radial immunodiffusion. The protocol was approved by the NHLBI Institutional Review Board.

Treatment of α_1-AT with H_2O_2—Twenty μg of purified human α_1-AT were incubated for 2 h at room temperature in 1 ml of reaction mixture containing 50 mM potassium phosphate, 100 mM potassium chloride, 1 mM magnesium chloride at pH 5.0 and the indicated concentrations of hydrogen peroxide (30%; Fisher). The concentration of H_2O_2 was measured at 240 nm ($\epsilon = 39.4 \pm 0.2\ M^{-1}\ cm^{-1}$) (23). At this pH, methionine is oxidized selectively to methionine sulfoxide (24). At the end of the reaction, a sample was taken to measure anti-NE activity. The activity of α_1-AT was measured at 410 nm (Beckman DU-70) by titrating increasing amounts of the oxidized sample against a fixed amount of purified human NE (50 nM) of known activity. The proteins were incubated for 30 min, and then residual NE was quantified by assay with the NE-specific substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-nitroanilide (change of absorbance was measured every 20 s for 2 min) (25). The remainder of the sample was then dialyzed at 4 °C, using dialysis cassettes (Pierce), against 5 mM HEPES, 10 mM sodium chloride at pH 7.4, with six changes of buffer, and then dried by vacuum centrifugation (Savant). To measure tryptophan by spectral deconvolution (26), 350 μg of α_1-AT were used; following reaction, α_1-AT was dialyzed against 5 mM potassium phosphate, pH 5.0, with six changes of buffer.

CNBr Cleavage and Amino Acid Analysis—CNBr cleaves peptide bonds on the carboxyl side of methionine, yielding homoserine; it does not cleave at methionine sulfoxide (27). CNBr cleavage was carried out on 20 μg of protein dried by vacuum centrifugation (Savant) in 4-ml glass vials (Wheaton) fitted with Teflon-lined caps. CNBr was prepared as a 10 M stock solution in acetonitrile and then diluted to 100 mM with 70% formic acid just before use. One hundred μl were added to the vial, which was capped, protected from light by wrapping with aluminum foil, and incubated overnight at room temperature in a hood. These samples were used for peptide sequencing. Amino acid analyses were carried out on 20 μg of α_1-AT with and without CNBr treatment (28). The model substrate (allatotropin) was incubated at 70 °C for 1 h for the CNBr treatment, equivalent to an overnight incubation at room temperature (27).

Formation of α_1-AT·NE Complexes—Formation of the α_1-AT·NE complexes occurred in a 1-ml reaction mixture containing 38.5 μM purified α_1-AT and 34.5 μM NE, 500 mM NaCl, 0.1% Brij, and 100 mM HEPES, pH 7.5, which was incubated at room temperature for 30 min. A sample was taken to quantify for complex formation in 4–20% SDS-polyacrylamide gradient gels. Ninety-five percent of α_1-AT migrated as a complex with NE, while 5% of α_1-AT was cleaved.

Simultaneous Sequencing of Peptide Mixtures—Fractional modification of each methionine residue was quantified in the oxidatively modified proteins. While this might have been accomplished by reverse phase HPLC mapping of proteins cleaved by specific proteases, it is a challenging process complicated by varying recoveries and changing retention times caused by oxidation of the methionine residues. Instead, the technique of simultaneous sequencing of a peptide collection by an automated Edman sequencer was used (21). This procedure generates quantitative results from which the location and extent of covalent modifications can be determined, provided that the sequence of the protein is known and the cleavage methods are selected to minimize or eliminate ambiguities. The technique has been used to identify the histidine residues modified by metal-catalyzed oxidation of glutamine synthetase, the cysteine residue in carbonic anhydrase modified by glutathiolation, and oxidized methionine residues in glutamine synthetase (21, 29, 30).

CNBr treatment of α_1-AT should yield 10 peptides. Sequencing of this collection of peptides will thus generate multiple phenylthiohydantoin-derivatives in each cycle of Edman degradation. The peptide collection and resultant phenylthiohydantoin-derivative patterns will change upon formation of methionine sulfoxide, since CNBr does not cleave these residues. Ambiguities may result from multiple oxidations, but whether this actually occurs can only be determined experimentally for each protein.

The CNBr peptide collection of each of the α_1-AT preparations was loaded onto the sequencing column of a Hewlett-Packard G1005A automated sequencer equipped with a model 1040 diode array spectrophotometer. Five cycles were run for each protein, providing an overdetermination useful for confirming the status of each methionine.

Peptide Mapping by HPLC-Mass Spectrometry—To provide an independent analytical assessment of the status of Met-351 and Met-358, we prepared a Lys-C digest. α_1-AT (25–50 μg in 50 mM HEPES, 50 mM NaCl, pH 7.0) was dried by vacuum centrifugation in a glass autosampler vial (catalog no. 79909-C, Scientific Resources, Eatontown, NJ). Then 25 μl of 200 mM Tris, 2 mM EDTA, pH 8.5, were added followed by 25 μl of hexafluoroisopropanol as a denaturant. The sample was again dried and then taken up in 50 μl of water. One μg of lysyl endopeptidase (catalog no. 125-02543; Wako Chemicals USA, Richmond, VA) was added, and the solution was incubated at 37 °C overnight. The peptide mixture was separated by reverse phase HPLC with both spectrophotometric and mass spectrometric detection (model 1100; Hewlett-Packard, Palo Alto, CA) using a Vydac narrow bore C_{18} column (catalog no. 218TP5205; Vydac, Hesperia, CA). The initial solvent was 0.05% trifluoroacetic acid with gradient elution by acetonitrile, 0.05% trifluoroacetic acid increasing at 1%/min with a flow rate of 0.2 ml/min. The effluent from the spectrophotometric detector was mixed in a tee with 100 μl/min acetic acid pumped by another model 1100 pump, and the mixture was introduced into the mass spectrometer (31). The capillary voltage was 4500 V, and the fragmentor was programmed to ramp from 50 V at 50 mass units, 80 V at 1500 mass units, and 140 V at 2500 mass units. Data were collected from 550 to 2000 mass units during development of the method and then narrowed to 900–1300 for routine analyses.

Under the gradient used, the native peptide containing intact Met-351 and Met-358 eluted at ~30% acetonitrile, the peptides with either Met-351 or Met-358 oxidized eluted as a doublet at ~28% acetonitrile, and the peptide with both methionines oxidized eluted at ~26% acetonitrile. The two peaks in the doublet were collected separately, dried, and further digested by dissolving in 50 μl of 50 mM Tris, 5 mM $CaCl_2$, pH 7.8, and adding 0.2 μg of chymotrypsin (catalog no. 1418467; Roche Molecular Biochemicals). The products were analyzed with the same elution program on the HPLC-mass spectrometer. These analyses established that the peptide with oxidized Met-358 eluted first, followed by the peptide with oxidized Met-351. Quantitation of the four peptides of interest was accomplished by integrating the area of the extracted ion chromatogram for each peptide, with a mass/charge ratio of 2 (native = 1130.8, monooxidized = 1138.8, dioxidized = 1146.8). Since the shapes of the peaks were nongaussian, the area of the doublet peak was allocated to the Met-351- and Met-358-oxidized peptides in proportion to their peak heights.

Site-specific Mutants—α_1-AT mutants were produced as recombinant protein in *Escherichia coli* using the expression vector pFEAT30, containing the α_1-AT gene with the sequence of the M2 variant and V68L (32, 33). Mutant α_1-AT DNA constructs were designed using the QuikChange Mutagenesis kit. Briefly, mutagenic oligonucleotides containing the desired mutation, and each complementary to opposite strands of the plasmid, were extended by polymerase chain reaction using the *Pfu* DNA polymerase. Following polymerase chain reaction, the parental DNA containing the wild type α_1-AT cDNA was degraded using the *Dpn*I restriction enzyme. The synthesized DNA containing the desired mutation, which remained intact in the presence of *Dpn*I, was transformed into *Escherichia coli* XL-1 supercompetent cells. Transformants were obtained and grown in Luria medium with ampicillin. Plasmid DNA was isolated from cultures containing the putative M351V-, M358V- and M351V,M358V-α_1-AT mutations and trans-

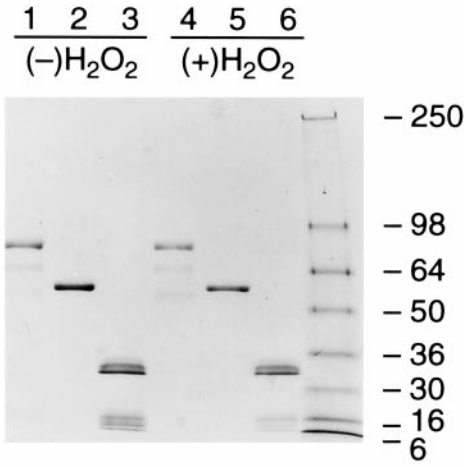

FIG. 1. **SDS-PAGE of α_1-AT, NE, and α_1-AT·NE complexes.** α_1-AT·NE, (*lanes 1* and *4*), α_1-AT (*lanes 2* and *5*), and NE (*lanes 3* and *6*) were incubated without or with 10 mM H_2O_2 as described under "Experimental Procedures." One μg of total protein was loaded in each lane of the SDS-PAGE 4–20% gradient gel, run under nonreducing conditions.

formed into *E. coli* BL21(DE3)pLysS cells for expression. Cultures were grown to A_{600} of 0.8 and induced with 1 mM isopropyl-thio-β-D-galactopyranoside for 3 h, the optimal time for maximal α_1-AT production (34). Cells were lysed by sonication and spun at 10,000 × *g* for 20 min. The supernatant was discarded, and the pellet, constituting the inclusion bodies in which α_1-AT was present, was washed with 20 mM HEPES, pH 7.4. The presence of α_1-AT in each sample was confirmed by SDS-PAGE and Western blotting (not shown).

The inclusion bodies were solubilized with 6 M guanidine-HCl in 50 mM potassium phosphate, pH 2.5. Solutions were centrifuged at 10,000 × *g* for 10 min, and the supernatant was retained. α_1-AT was purified from the supernatant samples by reverse phase HPLC using a Hewlett-Packard diode array-equipped system, model 1050 (Palo Alto, CA). 200-μl samples of supernatant were loaded onto a Vydac C_{18} column that had been equilibrated with 0.05% trifluoroacetic acid. A gradient was generated using acetonitrile with 0.05% trifluoroacetic acid, ramping rapidly to 40% in 7 min, followed by an increase to 60% over the next 20 min (1%/min). α_1-AT eluted at approximately 53% acetonitrile.

To refold the protein (34), fractions containing α_1-AT were dried in a vacuum centrifuge and then dissolved in 8 M urea, 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.05% Triton X-100. The sample was then diluted 10-fold with 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, 1 mM dithiothreitol. The samples were loaded into dialysis cassettes and dialyzed for 24 h with three changes of buffer. Protein concentration was determined by the Coomassie Blue dye binding method (35).

RESULTS

Effect of Methionine Oxidation on Activity—As shown by SDS-PAGE (Fig. 1), treatment with H_2O_2 did not cleave or cross-link α_1-AT, NE, or α_1-AT·NE complexes. Methionine sulfoxide content of α_1-AT following incubation for 2 h at pH 5.0 with increasing concentrations of H_2O_2 plateaued after three methionines were oxidized (Fig. 2*A*). A similar oxidation in α_2-macroglobulin has been reported, but a more detailed investigation correlated loss of activity to oxidation of a single tryptophan residue (28). Therefore, the method of spectral deconvolution was used to quantify aromatic residues in α_1-AT. No change in tyrosine, phenylalanine, or tryptophan was found (Fig. 2*B*). α_1-AT contains one cysteine, and measurement of that residue, by Ellman's reagent, demonstrated that it was not oxidized by hydrogen peroxide treatment. This was confirmed by SDS-PAGE under nonreducing conditions, in which higher molecular weight aggregates would have appeared if cysteines had been oxidized to cystine (Fig. 1).

Identification of Oxidizable Methionines in α_1-AT and an Artifact from Vicinal Methionines—Simultaneous sequencing

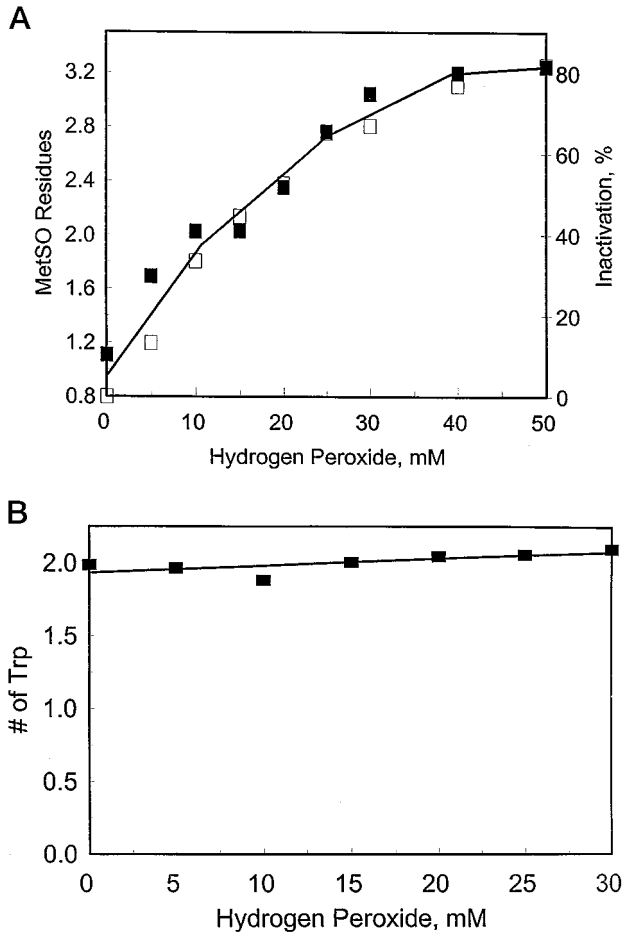

FIG. 2. **Oxidation of methionine residues in α_1-AT by H_2O_2 as determined by amino acid analysis.** *A*, α_1-AT (20 μg) was incubated with the indicated concentration of H_2O_2 at pH 5.0 for 2 h. A sample was taken to measure activity (*open squares*), and the remainder was incubated with or without CNBr and subjected to amino analysis to measure methionine sulfoxide (*closed squares*). *B*, α_1-AT (350 μg) was incubated with 0, 5, 10, 15, 20, 25, or 30 mM H_2O_2 at pH 5.0 for 2 h at room temperature before quantitation of aromatic residues. The line was fit by the method of least squares. These experiments were repeated three times, and the results were averaged.

permitted assessment of the status of each methionine residue following the analysis shown in Table I. As discussed below, methionines 351 and 358 were readily and equally oxidized, while methionine 226 was substantially less sensitive. None of the other six methionines were significantly oxidized by exposure to H_2O_2 (Fig. 3). Methionine 221 appeared to be ~40% oxidized in the control protein not exposed to H_2O_2. If such an oxidation actually occurred *in vivo* or during *in vitro* purification, we would expect the residue to be especially sensitive to oxidation by peroxide. However, the apparent 40% oxidation did not increase even following treatment with very high concentrations of H_2O_2 (Fig. 3). Methionine 221 forms a vicinal pair with methionine 220. In a recombinant prion protein (36), we have observed a similar apparent partial oxidation of the carboxyl residue of vicinal methionines.[2] Further, the original studies of CNBr cleavage of methionine-containing dipeptides reported yields of about 75% (37). Each of these observations is consistent with the hypothesis that amino-terminal methionine residues do not react with CNBr as efficiently as methionines at other positions in peptides. Cleavage of methionine

[2] C. Taggart, D. Cervantes-Laurean, G. Kim, N. G. McElvaney, N. Wehr, J. Moss, and R. L. Levine, unpublished observations.

TABLE I

Susceptibility of methionine residues in α_1-AT to oxidation by hydrogen peroxide and the expected decrease in residue yields during simultaneous peptide sequencing

Oxidation of a methionine residue prevents cleavage by CNBr, thus causing a decrease in yield of residues in the following peptide. Assessment of the status of Met-220 and Met-221 can be made in several ways, including examination of the recovery of Arg-223. If both Met-220 and Met-221 are oxidized, then Arg-223 will not appear in cycles 1–5 because it was fused to the peptide beginning at residue 64. If only Met-221 is oxidized, Arg-223 will run in cycle 3 instead of cycle 2. Since there was no change in the recovery of Arg in cycles 2 and 3, Met-220 remained intact. One can also take advantage of the fact that methionine sulfoxide was reduced back to methionine under conditions of Edman sequencing. Thus, yields of methionine during sequencing increased as residues were oxidized. In particular, the yield of Met in cycle 1 was a measure of oxidation of Met-221, and the results agreed with monitoring of Arg-223. The second column refers to oxidation by exposure to hydrogen peroxide.

Met no.	Oxidized by H_2O_2	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
63	No	Leu	Ser	Leu	Gly	Thr
220	No	Met				
221	No	Lys	Arg	Leu	Gly	Met
226	Later	Phe	Asn	Ile	Gln	His
242	No	Lys	Tyr	Leu	Gly	Asn
351	Yes	Phe	Leu	Glu	Ala	Ile
358	Yes	Ser	Ile	Pro	Pro	Glu
374	No	Ile	Glu	Gln	Asn	Thr
385	No	Gly	Lys	Val	Val	Asn

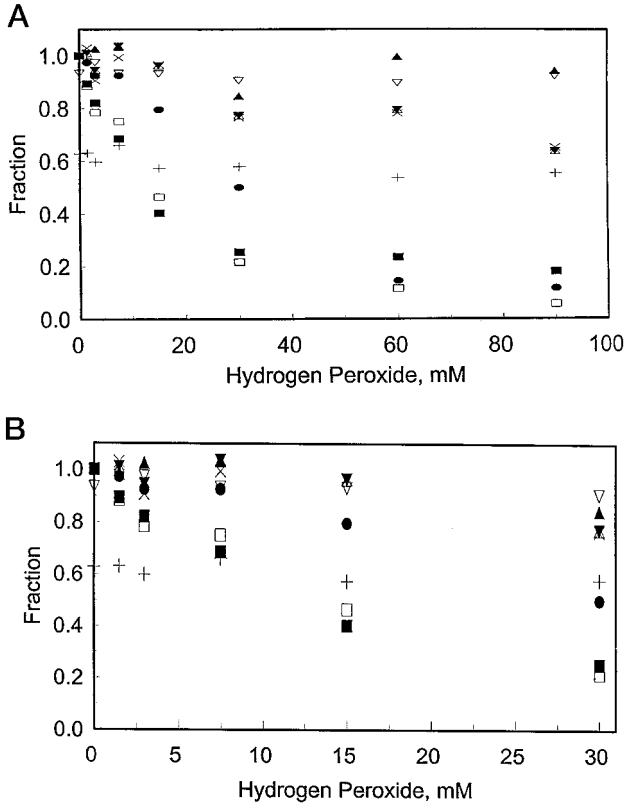

FIG. 3. **Susceptibility to oxidation of methionines in H_2O_2-treated α_1-AT.** α_1-AT (20 μg) was treated with the indicated concentration of H_2O_2 at pH 5.0 for 2 h at room temperature and subjected, after CNBr treatment, to Edman amino sequencing to determine the location and extent of oxidation of each methionine. Methionine 358 (■), methionine 374 (▲), methionine 63 (△), methionine 385 (×), methionine 226 (●), methionine 221 (+), methionine 242 (▼), methionine 220 (▽), and methionine 351 (□). *A* gives the results over the entire concentration of H_2O_2, while *B* focuses on the lower concentration to facilitate assessment of the changes associated with inactivation. This experiment was performed twice, and the results were averaged.

220 by CNBr would release a peptide with methionine 221 at its amino terminus.

To test this possibility, we studied a purified model peptide containing vicinal methionines. Allatotropin is an insect hormone consisting of 13 residues with the sequence Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe-amide, placing the vicinal methionines at positions 7 and 8. The supplier's claim of >97% purity was confirmed by reverse phase chromatography and amino acid analysis (not shown). Its mass was confirmed to be that expected by MALDI-TOF mass spectrometry (m/z = 1,487.8; expected = 1,487.8), with no detectable contaminants. Therefore, the model peptide did not contain methionine sulfoxide. Following CNBr treatment, amino acid analysis demonstrated one-third of a residue of methionine sulfoxide. Simultaneous sequencing detected the same amount in cycle 1, indicating that it derived from Met-8, the carboxy-sited residue of the vicinal methionines. Unlike amino acid analysis and simultaneous sequencing, MALDI-TOF mass spectrometry cannot provide quantitation of yields, but it does provide qualitative identification by virtue of its high mass accuracy. The mass spectrum of the CNBr-treated peptide is shown in Fig. 4. Three products are expected: 1) residues 1–7 with homoserine at residue 7; 2) homoserine from residue 8, which will not be detected in this analysis; and 3) residues 9–13. The two expected peptides are present in the spectrum, but two additional peaks are also readily identified. The peak with m/z = 682.0 contains residues 8–13 with methionine intact (expected m/z = 681.8), and the peak with m/z = 697.9 is the same peptide with the methionine oxidized to methionine sulfoxide (expected m/z = 697.8). These results establish that CNBr does not efficiently react with amino-terminal methionines, leading to the generation of peptides with either methionine or methionine sulfoxide at the amino terminus. Thus, the apparent presence of ~40% methionine sulfoxide at position 221 in control α_1-AT was an artifact.

Oxidation of either Methionine 351 or 358 Inactivates α_1-AT—Oxidation of methionine 358 has been previously correlated with loss of α_1-AT activity (16). As shown in Fig. 3, methionine 351 is equally susceptible to oxidation, inviting investigation of its possible effect on activity. The percentage of oxidation of either methionine alone could not account for the corresponding loss of α_1-AT activity; however, a loss of activity caused by oxidation of either residue did account for the observed inactivation (Fig. 5).

While oxidation of Met-358 is well established to cause inactivation of α_1-AT, the effect of oxidation of Met-351 has not been reported. Thus, it seemed important to confirm the results obtained by simultaneous sequencing with an independent method of analysis. We therefore developed an HPLC-mass spectrometric method, which quantified the fraction of Met-351 and Met-358 that was oxidized. Analyses of the series of oxidatively modified α_1-AT confirmed the results obtained with simultaneous sequencing (Fig. 6). Thus, Met-351 is as susceptible to oxidation as Met-358.

Fig. 7 shows that only these two methionines are surface-

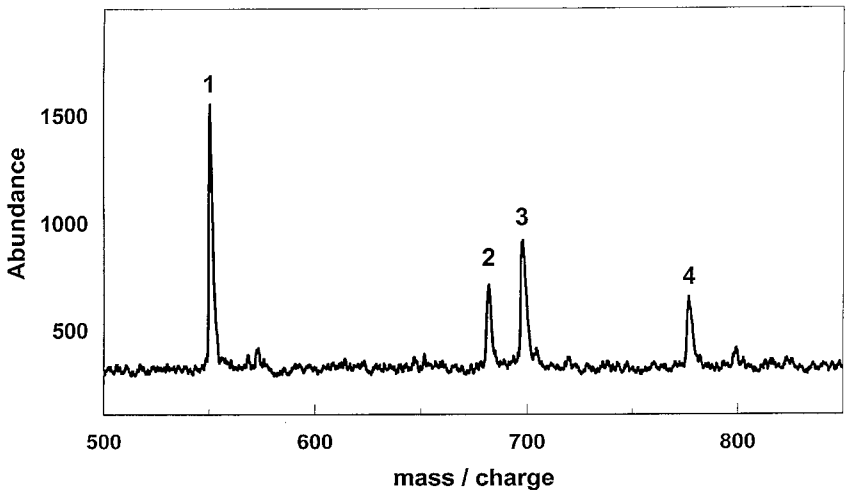

FIG. 4. **MALDI-TOF spectrum of allatotropin cleaved with CNBr.** The mass/charge (*m/z*) of each peak allows identification of the peptide. Peak 1 is the peptide containing the homoserine form of residues 9–13 (observed 550.6; calculated 550.6). Peak 2 is the intact methionine form of residues 8–13 (observed 682.0; calculated 681.8). Peak 3 is the methionine sulfoxide form of residues 8–13 (observed 697.9; calculated 697.8). Peak 4 is the homoserine lactone form of residues 1–8 (observed 777.0; calculated 776.9).

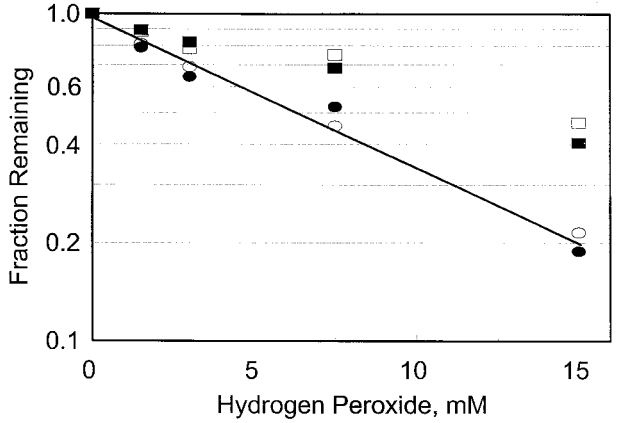

FIG. 5. **Effect of oxidation of methionines 351 and 358 by H_2O_2 on activity of α_1-AT.** Residual α_1-AT activity (○), intact methionine 351 (□), intact methionine 358 (■), and intact methionine 351 plus methionine 358 (●) were plotted *versus* the indicated H_2O_2 concentrations. This experiment was repeated twice, and the results were averaged.

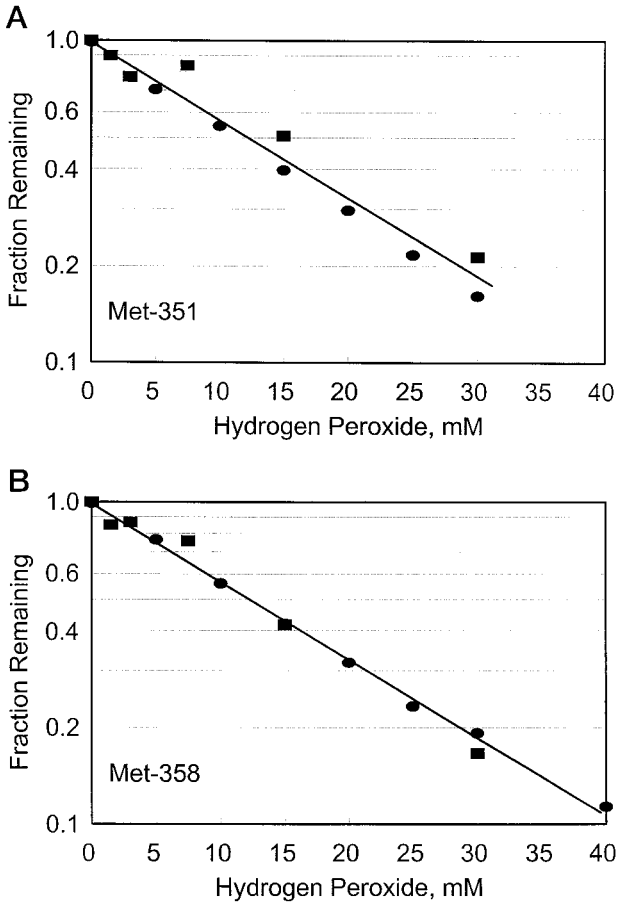

FIG. 6. **Susceptibility of methionines 351 and 358 to oxidation by H_2O_2 determined by simultaneous sequencing (●) or HPLC-mass spectrometry (■).** *A*, Met-351; *B*, Met-358. The data for simultaneous sequencing are from Fig. 5, and the data from mass spectrometry were obtained from two separate preparations of oxidatively modified α_1-AT.

exposed, in agreement with the suggestion that only those methionines on the surface of the molecule are susceptible to oxidation (21, 38). While the carbon side chain of methionine 226 is reasonably surface-exposed, the sulfur that is the target of oxidation is only partially exposed. The other six methionines are buried.

Site-specific Mutants—Four recombinant α_1-ATs were purified from *E. coli*: wild-type, M351V, M358V, and M351V/M358V. The anti-NE specific activities of each were the same and equivalent to that of the plasma α_1-AT. Each recombinant protein was analyzed by electrospray-mass spectrometry to confirm that site-specific mutations were produced, and all masses were within 2 mass units of theoretical (wild-type, 44,294 (calculated) and 44,295 (observed); M351V, 44,262 (calculated) and 44,264 (observed); M358V, 44,262 (calculated) and 44,264 (observed); and M351V/M358V, 44,230 (calculated) and 44,228 (observed)). From the results described above, we predicted that M351V and M358V would be inactivated by hydrogen peroxide at approximately the same rate, with that rate being distinctly slower than the wild type. The double mutant M351V/M358V should be even more resistant than the single mutants. The time course and concentration dependences shown in Fig. 8 confirm these predictions.

DISCUSSION

Oxidation of either methionine 351 or 358 in α_1-AT caused loss of anti-NE activity. The partially exposed methionine 226 was less susceptible to oxidation. Although the importance of methionine 358 in the activity of α_1-AT is very well documented, the role of methionine 351 in α_1-AT activity is not well defined. Site-specific mutants in which methionine 358 was changed to valine were shown to be relatively resistant to oxidative inactivation by several laboratories (39, 40). These reports have understandably led to the impression that methionine 351 is not important in the oxidative inactivation of α_1-AT, but the issue had not been directly investigated. In this

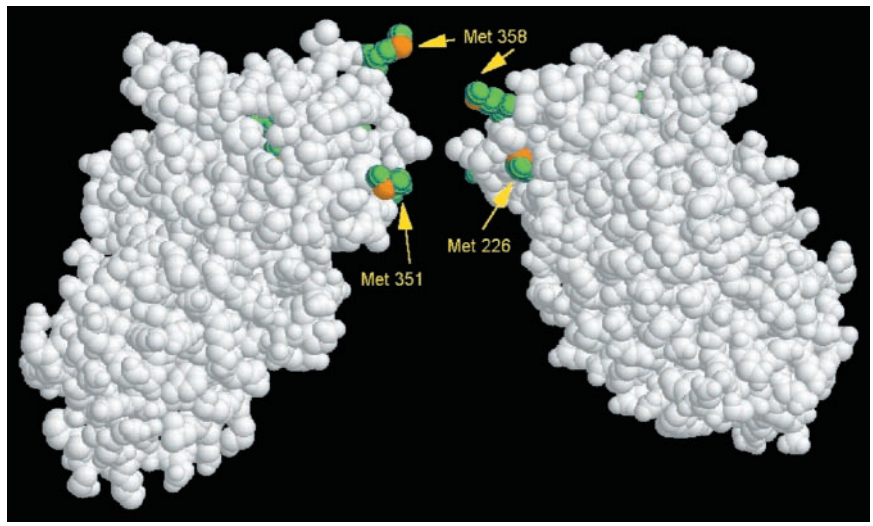

FIG. 7. **Space-filling model based on the x-ray crystal structure of human α_1-AT (34, 35).** The right view was generated by rotating that on the left by 180° about the vertical axis, displaying the "rear view." The carbon atoms of the side chains of the nine methionine residues are colored *green* and the sulfurs *orange*. These views were generated by the program Rasmol (53). The sulfurs of methionine 351 and 358 are completely exposed, that of methionine 226 is only partially exposed, and all others are not visible on the surface of the molecule.

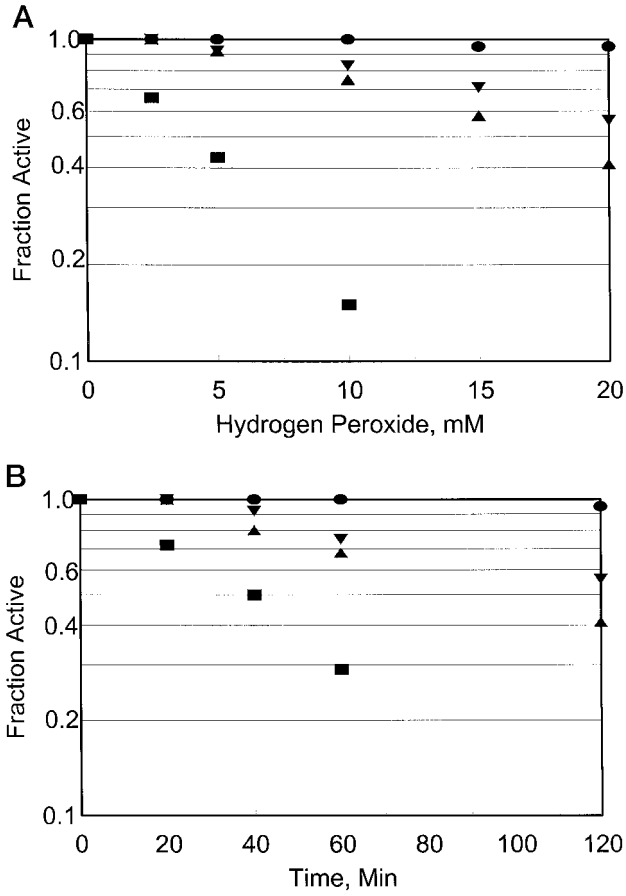

FIG. 8. **Time course of inactivation of recombinant α_1-AT by hydrogen peroxide.** Wild-type (■), M351V (▲), M358V (▼), M351V/M358V (●). *A*, concentration dependence, 120-min exposure to hydrogen peroxide. *B*, time course for 20 mM hydrogen peroxide.

regard, Luisetti and Travis (41) have pointed out that available data suggest that oxidation of other methionine residues may be important in the loss of α_1-AT activity. Our results establish that oxidation of methionine 351 occurs readily in the wild-type α_1-AT and causes loss of activity under our standard assay conditions. We then postulated that site-specific mutation of methionine 351 would also increase resistance to oxidative modification and that mutation of both methionine 351 and 358 could generate an α_1-AT even more resistant to oxidative inactivation than the single mutants. These predictions were confirmed with site-specific mutants produced in *E. coli*.

A recombinant mutant α_1-AT with only methionine 358 changed to valine was found by previous investigators to be resistant to oxidative inactivation by *N*-chlorosuccinimide, while the wild type was inactivated (42). These recombinants were produced in yeast, and the wild-type α_1-AT was quite labile, requiring mercaptoethanol to restore or prevent loss of activity. This is not the behavior of the human serum α_1-AT nor recombinant M358V that we have studied. In the human variant, α_1-AT-Pittsburgh, methionine 358 is changed to arginine, causing a severe bleeding diathesis because the variant α_1-AT is a potent inhibitor of thrombin, a property not possessed by wild type α_1-AT (43). Exposure of α_1-AT-Pittsburgh to *N*-chlorosuccinimide did not affect its ability to inhibit thrombin, but this finding may not be relevant to inhibition of elastase by wild type α-AT because unoxidized α_1-AT-Pittsburgh cannot inhibit elastase (44).

There are several reports that only two methionines are oxidized to methionine sulfoxide when α_1-AT is exposed to oxidizing agents such as myeloperoxidase/H_2O_2 (15) or *N*-chlorosuccinimide (16). These were identified as methionine 358 and another residue thought to be within six residues. Janoff and Carp (17) confirmed that two methionines were oxidized when α_1-AT was treated with chloramine T. Maier and colleagues (45) showed that complete loss of activity of α_1-AT occurred when one methionine was oxidized, as determined by amino acid analysis of the total protein. They assumed that this was methionine 358, but their data are consistent with ~50% oxidation of methionines 351 and 358, as shown here.

In contrast to methionines in a number of other proteins (*e.g.* glutamine synthetase (21)), the other seven methionines in α_1-AT (63, 220, 221, 226, 242, 374, and 385) do not act as antioxidants or play a protective role in preventing oxidative inactivation of α_1-AT. In particular, Met-226 was oxidized to a significant extent only after methionines 351 and 358 were oxidized (Fig. 3). Carp *et al.* (8) reported that α_1-AT isolated from bronchoalveolar lavage fluid of smokers contained four residues of methionine sulfoxide with loss of only 40% of antiprotease activity. We observed, however, that a maximum of three methionines were oxidized under relatively harsh conditions, and loss of activity required only oxidation of two residues. Other investigators have also reported the oxidation of two methionines rather than four (15–17).

The susceptibility to oxidation of methionines 351 and 358, along with the crystallographic structure of uncleaved α_1-AT (46, 47), showing that these methionines are clearly surface-exposed, suggests strongly that the α_1-AT molecule is struc-

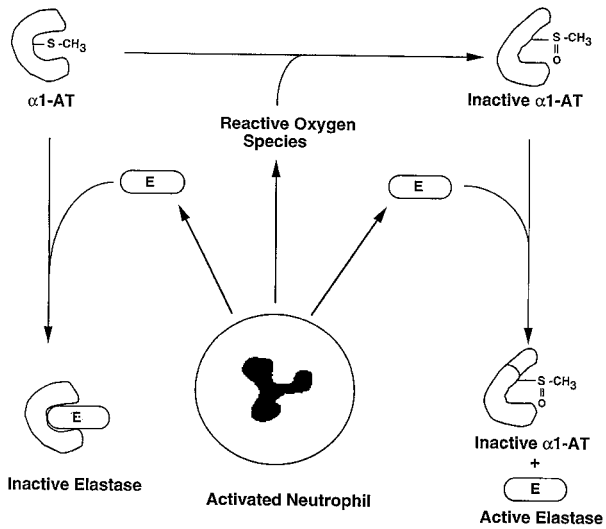

FIG. 9. **Proposed mechanism of action of α_1-AT when activated neutrophils release elastase.** In the immediate vicinity of activated neutrophils, the oxidizing species, which are released first, inactivate α_1-AT. Activity of NE, which is next secreted, is thereby protected. Farther from the neutrophil, where the short lived reactive oxygen species are nonexistent, α_1-AT can inactivate NE and thereby protect the lung from unrestricted protease action.

tured in such a way as to invite oxidative inactivation. The oxidation is probably an ongoing and necessary part of its *in vivo* function. This is in clear contrast to the lack of effect on biological activity of oxidation of surface-exposed methionines in at least eight proteins studied to date (21). Why might it be physiologically favorable for α_1-AT to have two methionines whose oxidation leads to inactivation? In the lung, α_1-AT functions mainly as an inhibitor of NE, which is released by neutrophils at sites of inflammation. Luisetti and Travis (41) postulated that a "halo" of oxidizing agents exists around the neutrophil during migration toward its target. During activation, there is an initial burst of oxidation, followed by release of NE. Close to the neutrophil, α_1-AT would be more efficiently inactivated by oxidation of either methionine 351 or 358 before NE is released (Fig. 9). Thus, the timing of the oxidative burst assures preservation of elastase activity when it is released. This hypothesis is consistent with the broad substrate specificity of NE and its central role in a number of disease states associated with proteolytic damage in the lung. Irreversible local inactivation of anti-proteases in the lung is potentially deleterious, but human methionine sulfoxide reductase can reduce α_1-AT and restore its activity (48). It has been postulated that NE may have other roles, such as modulating protease inhibitor turnover and the acute phase response and facilitating movement of neutrophils through tissue. Thus, after local inactivation of anti-proteases to ease passage of inflammatory cells through tissue, it is possible that anti-protease activity is restored locally to limit further tissue damage. However, when methionine sulfoxide reductase was added to α_1-AT containing four residues of methionine that had been oxidized with aqueous cigarette smoke and H_2O_2, the anti-NE activity of α_1-AT was not restored (17). Thus, homeostatic mechanisms involving local inactivation of anti-proteases followed by restoration of anti-protease activity during inflammatory cell migration may involve a fragile balance, easily upset in lung diseases associated with increased protease or oxidant burden.

One such condition is cystic fibrosis, which is characterized in the lung by markedly increased protease levels and increased amounts of oxidants released by inflammatory cells (49). The anti-protease defenses of the cystic fibrosis lung are inactivated because α_1-AT and secretory leukocyte protease inhibitor are complexed with or cleaved by NE or modified by oxidants released by inflammatory cells. This leaves active NE on the respiratory epithelial surface, which contributes directly to the lung destruction in cystic fibrosis. Pneumonia and adult respiratory distress syndrome are other disorders in which lung destruction is at least partly due to unopposed action of proteases (10, 50). In contrast, α_1-AT deficiency is characterized by a markedly decreased anti-protease capacity and associated lung destruction. Damage is accelerated by cigarette smoke (8), most likely due to inactivation of residual α_1-AT or secretory leukocyte protease inhibitor by oxidants in the smoke.

These findings have implications for the pathogenesis and treatment of lung conditions associated with increased protease activity (51). A number of studies have shown that aerosolized anti-proteases such as α_1-AT, purified from plasma (49), and recombinant secretory leukocyte protease inhibitor can inhibit NE *in vivo*, *e.g.* in cystic fibrosis (52). In these conditions, however, very high levels of anti-proteases are required to achieve complete inhibition of NE and restore anti-NE capacity at the respiratory epithelial surface. Since oxidative inactivation is common in an inflammatory milieu, the oxidant-resistant anti-protease M351V/M358V could circumvent this problem by maintaining its anti-protease activity, even in an oxidant-rich environment. If local inactivation of α_1-AT or secretory leukocyte protease inhibitor is required to allow protease-mediated movement of inflammatory cells to their targets, then oxidant-resistant antiproteases could actually impair host defense and tissue repair.

Acknowledgments—We are indebted to Myeong-Hee Yu (Korea Research Institute of Bioscience and Biotechnology) and her colleagues for providing the pFEAT30 plasmid containing the wild-type α_1-AT. We thank Martha Vaughan (National Institutes of Health) for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Alper, C. A., Raum, D., Awdeh, Z. L., Petersen, B. H., Taylor, P. D., and Starzl, T. E. (1980) *Clin. Immunol. Immunopathol.* **16,** 84–89
2. Laurell, C. B., and Eriksson, S. (1963) *Scand. J. Clin. Invest.* **15,** 132–140
3. Gadek, J. E., Fells, G. A., Zimmerman, R. L., Rennard, S. I., and Crystal, R. G. (1981) *J. Clin. Invest.* **68,** 889–898
4. Brantly, M. L., Wittes, J. T., Vogelmeier, C. F., Hubbard, R. C., Fells, G. A., and Crystal, R. G. (1991) *Chest* **100,** 703–708
5. Gadek, J. E., and Crystal, R. G. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrikson, D. S., Goldstein, J. L., and Brown, M. S., eds) pp. 1450–1467, McGraw-Hill Book Co., New York
6. Stone, P. J. (1983) *Clin. Chest Med.* **4,** 405–412
7. Gadek, J. E., Fells, G. A., and Crystal, R. G. (1979) *Science* **206,** 1315–1316
8. Carp, H., Miller, F., Hoidal, J. R., and Janoff, A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2041–2045
9. McElvaney, N. G., Cervantes-Laurean, D., Wehr, N., Gabrielle, K., Barnes, P., Robinson, W., Moss, J., and Levine, R. L. (1996) *Am. J. Res. Crit. Care Med.* **153,** 737 (abstr.)
10. McGuire, W. W., Spragg, R. G., Cohen, A. B., and Cochrane, C. G. (1982) *J. Clin. Invest.* **69,** 543–553
11. Roum, J. H., Buhl, R., McElvaney, N. G., Borok, Z., and Crystal, R. G. (1993) *J. Appl. Physiol.* **75,** 2419–2424
12. Ogden, B. E., Murphy, S. A., Saunders, G. C., Pathak, D., and Johnson, J. D. (1984) *Am. Rev. Respir. Dis.* **130,** 817–821
13. Beatty, K., Bieth, J., and Travis, J. (1980) *J. Biol. Chem.* **255,** 3931–3934
14. Johnson, D., and Travis, J. (1978) *J. Biol. Chem.* **253,** 7142–7144
15. Matheson, N. R., Wong, P. S., Schuyler, M., and Travis, J. (1981) *Biochemistry* **20,** 331–336
16. Johnson, D., and Travis, J. (1979) *J. Biol. Chem.* **254,** 4022–4026
17. Janoff, A., and Carp, H. (1977) *Am. Rev. Respir. Dis.* **116,** 65–72
18. Moreno, J. J., and Pryor, W. A. (1992) *Chem. Res. Toxicol.* **5,** 425–431
19. Hall, P. K., and Roberts, R. C. (1992) *Biochim. Biophys. Acta* **1121,** 325–330
20. Gonias, S. L., Swaim, M. W., Massey, M. F., and Pizzo, S. V. (1988) *J. Biol. Chem.* **263,** 393–397
21. Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15036–15040
22. Evans, M. D., Church, D. F., and Pryor, W. A. (1991) *Chem. Biol. Interact.* **79,** 151–164
23. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem.* **49,** 474–478
24. Brot, N., and Weissbach, H. (1983) *Arch. Biochem. Biophys.* **223,** 271–281
25. Wewers, M. D., Casolaro, M. A., Sellers, S. E., Swayze, S. C., McPhaul, K. M., Wittes, J. T., and Crystal, R. G. (1987) *N. Engl. J. Med.* **316,** 1055–1062

26. Levine, R. L., and Federici, M. M. (1982) *Biochemistry* **21,** 2600–2606
27. Fliss, H., Weissbach, H., and Brot, N. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7160–7164
28. Reddy, V. Y., Desorchers, P. E., Pizzo, S. V., Gonias, S. L., Sahakian, J. A., Levine, R. L., and Weiss, S. J. (1994) *J. Biol. Chem.* **269,** 4683–4691
29. Apffel, A., Sahakian, J., and Levine, R. L. (1994) *Protein Sci.* **3,** 99 (abstr.)
30. Cabiscol, E., and Levine, R. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4170–4174
31. Apffel, A., Fischer, S., Goldberg, G., Goodley, P. C., and Kuhlmann, F. E. (1995) *J. Chromatogr. A.* **712,** 177–190
32. Kwon, K. S., Kim, J., Shin, H. S., and Yu, M. H. (1994) *J. Biol. Chem.* **269,** 9627–9631
33. Lee, K. N., Park, S. D., and Yu, M. H. (1996) *Nat. Struct. Biol.* **3,** 497–500
34. Kwon, K. S., Lee, S., and Yu, M. H. (1995) *Biochim. Biophys. Acta* **1247,** 179–184
35. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
36. Mehlhorn, I., Groth, D., Stockel, J., Moffat, B., Reilly, D., Yansura, D., Willett, W. S., Baldwin, M., Fletterick, R., Cohen, F. E., Vandlen, R., Henner, D., and Prusiner, S. B. (1996) *Biochemistry* **35,** 5528–5537
37. Gross, E. (1967) *Methods Enzymol.* **11,** 238–255
38. Shechter, Y., Burstein, Y., and Patchornik, A. (1975) *Biochemistry* **14,** 4497–4503
39. Courtney, M., Jallat, S., Tessier, L. H., Benavente, A., Crystal, R. G., and Lecocq, J. P. (1985) *Nature* **313,** 149–151
40. Rosenberg, S., Barr, P. J., Najarian, R. C., and Hallewell, R. A. (1984) *Nature* **312,** 77–80
41. Luisetti, M., and Travis, J. (1996) *Chest* **110,** 278S-283S
42. Travis, J., Owen, M., George, P., Carrell, R., Rosenberg, S., Hallewell, R. A., and Barr, P. J. (1985) *J. Biol. Chem.* **260,** 4384–4389
43. Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) *N. Engl. J. Med.* **309,** 694–698
44. Travis, J., Matheson, N. R., George, P. M., and Carrell, R. W. (1986) *Biol. Chem. Hoppe Seyler* **367,** 853–859
45. Maier, K. L., Matejkova, E., Hinze, H., Leuschel, L., Weber, H., and Beck-Speier, I. (1989) *FEBS Lett.* **250,** 221–226
46. Ryu, S. E., Choi, H. J., Kwon, K. S., Lee, K. N., and Yu, M. H. (1996) *Structure.* **4,** 1181–1192
47. Elliott, P. R., Stein, P. E., Bilton, D., Carrell, R. W., and Lomas, D. A. (1996) *Nat. Struct. Biol.* **3,** 910–911
48. Carp, H., Janoff, A., Abrams, W., Weinbaum, G., Drew, R. T., Weissbach, H., and Brot, N. (1983) *Am. Rev. Respir. Dis.* **127,** 301–305
49. McElvaney, N. G., Hubbard, R. C., Birrer, P., Chernick, M. S., Caplan, D. B., Frank, M. M., and Crystal, R. G. (1991) *Lancet* **337,** 392–394
50. Braun, J., Dalhoff, K., Schaaf, B., Wood, W. G., and Wiessmann, K. J. (1994) *Eur. Respir. J.* **7,** 127–133
51. McElvaney, N. G., and Crystal, R. G. (1997) in *The Lung*(Crystal, R. G., West, J. B., Weibel, E. R., and Barnes, P. J., eds) pp. 2205–2218, Raven, Philadelphia
52. McElvaney, N. G., Nakamura, H., Birrer, P., Hebert, C. A., Wong, W. L., Alphonso, M., Baker, J. B., Catalano, M. A., and Crystal, R. G. (1992) *J. Clin. Invest.* **90,** 1296–1301
53. Sayle, R. A., and Milner-White, E. J. (1995) *Trends. Biochem. Sci.* **20,** 374–374