# Z-Type α1-Antitrypsin Is Less Competent Than M1-Type α1-Antitrypsin as an Inhibitor of Neutrophil Elastase

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## Abstract

**Alpha 1-antitrypsin (α1AT) deficiency resulting from homozygous inheritance of the Z-type α1AT gene is associated with serum α1AT levels of < 50 mg/dl and the development of emphysema in the third to fourth decades. Despite the overwhelming evidence that the emphysema of PiZZ individuals develops because of a "deficiency" of α1AT and hence an insufficient antineutrophil elastase defense of the lung, epidemiologic evidence has shown that levels of α1AT of only 80 mg/dl protect the lung from an increased risk of emphysema. With this background, we hypothesized that homozygous inheritance of the Z-type may confer an added risk beyond a simple deficiency of α1AT by virtue of an inability of the Z-type α1AT molecule to inhibit neutrophil elastase as effectively as the common M1-type molecule. To evaluate this hypothesis, the functional status of α1AT from PiZZ individuals ($n$ = 10) was compared with that of α1AT from PiM1M1 individuals ($n$ = 7) for its ability to inhibit neutrophil elastase (percent inhibition) as well as its association rate constant for neutrophil elastase (K association). Plasma α1AT concentration, measured by radial immunodiffusion, was 34±1 mg/dl in PiZZ patients vs. 237±14 mg/dl for PiM1M1 plasma, a sevenfold difference. When titrated against neutrophil elastase, the present inhibition of PiZZ plasma was significantly less than Pi M1M1 plasma (ZZ 78±1% vs. M1M1 95±1%, $P < 0.001$) as was purified Z type α1AT (ZZ, 63±2% vs. M1M1 86±2%, $P < 0.001$). Sodium dodecyl sulfate (SDS) gel comparisons of the complexes formed with M1-type α1AT and Z-type α1AT with elastase demonstrated the Z α1AT-elastase complexes were less stable than the M1 α1AT-elastase complexes, thus releasing some of the enzyme to continue to function as a protease. Consistent with these observations, the $K$ association of purified Z-type α1AT for neutrophil elastase was lower than that of M1-type α1AT (ZZ 4.5±0.3 × $10^6$ $M^{-1}s^{-1}$ vs. M1M1 9.7±0.4 × $10^6$ $M^{-1}s^{-1}$, $P < 0.001$), suggesting that for the population of α1AT molecules, the active Z-type molecules take more than twice as long as the active M1-type α1AT to inhibit neutrophil elastase. Consequently, not only is there less α1AT in PiZZ individuals, but the population of Z-type α1AT molecules is less competent as an inhibitor of neutrophil elastase than M1-type α1AT molecules. This combination of defects suggests that PiZZ individuals have far less functional antielastase protection than suggested by the reduced concentrations of α1AT alone, further explaining their profound risk for development of emphysema.**

## Introduction

Alpha 1-antitrypsin (α1AT),[1] a 52-kD glycoprotein produced by hepatocytes and mononuclear phagocytes, serves as the major inhibitor of neutrophil elastase, an omnivorous protease capable of destroying elastin as well as at least some part of all protein components of connective tissue (1–5). The critical importance of this role for α1AT is highlighted by the hereditary disease α1AT deficiency, an autosomal recessive disorder associated with the development of emphysema by ages 20 to 40 yr (1–9). Since α1AT serves to provide the major antineutrophil elastase protection for the lower respiratory tract (3, 8), and because experimental animal studies have shown that excess amounts of neutrophil elastase instilled into the lung causes lesions similar to human emphysema (10–16), the concept has evolved that the emphysema associated with α1AT deficiency develops because there are insufficient amounts of α1AT in the lower respiratory tract to inhibit neutrophil elastase released in the local milieu, allowing unopposed destruction of the connective tissue framework of the lung parenchyma (3, 8, 17).

In normal individuals with the homozygous inheritance of the common M1-type α1AT gene, the serum α1AT levels are 150 to 350 mg/dl (3). In contrast, in the common form of α1AT deficiency associated with homozygous inheritance of the Z-type α1AT gene, serum α1AT levels are invariably < 50 mg/dl (1–3, 5–9, 18). In this context, it is accepted that the reduction of α1AT levels to < 50 mg/dl is sufficient to permit the burden of neutrophils in the lower respiratory tract to slowly destroy the lung parenchyma (1, 3, 7–9, 17).

However, while this logic gives a compelling basis for understanding the pathogenesis of the emphysema associated with α1AT deficiency, it ignores the data from epidemiologic studies that has convincingly shown that while serum α1AT levels of 150 mg/dl are the lower limit of normal individuals, individuals with levels of ≥ 80 mg/dl are at no increased risk for the development of emphysema beyond the risk for the general population (3, 19–24). Thus, while individuals with homozygous inheritance of the Z gene commonly have α1AT levels in the 25 to 45 mg/dl range, those with only 2- to 2.5-fold more α1AT have no increased risk for emphysema, i.e., the epidemiologic data leads to the conclusion that a relatively small reduction in α1AT levels is sufficient to place the individual at high risk for the development of emphysema.

While such a reduction in the antineutrophil elastase screen for the lower respiratory tract could be sufficient to cause such a high risk for disease, we have hypothesized that the mutations in the coding sequence for the α1AT gene may

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1. *Abbreviations used in this paper:* α1AT, alpha 1-antitrypsin.

*Table I. Clinical Data of Homozygous Z-Type Deficiency Patients*[*‡]*

| Patients | Age | Sex | Smoking status[§] | α1AT Level in plasma[‖] | | Pulmonary Function (% Predicted)[¶] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Commercial std | True std | VC | TLC | DLCO | $FEV_1$ |
| | | | | *mg/dl* | *μM* | | | | |
| S.G. | 32 | M | Ex (28) | 36 | 4.6 | 88 | 123 | 57 | 48 |
| H.J. | 43 | M | Ex (44) | 34 | 4.4 | 79 | 116 | 47 | 56 |
| F.S. | 53 | M | N | 37 | 4.8 | 90 | 127 | 29 | 23 |
| M.J. | 34 | M | N | 25 | 3.3 | 111 | 111 | 68 | 81 |
| G.E. | 43 | M | N | 34 | 4.3 | 90 | 102 | 69 | 64 |
| M.M. | 35 | M | N | 34 | 4.5 | 93 | 137 | 48 | 40 |
| H.K. | 35 | F | Ex (12) | 35 | 4.5 | 77 | 136 | 44 | 33 |
| A.F. | 34 | F | Ex (40) | 34 | 4.4 | 98 | 128 | 52 | 31 |
| W.A. | 26 | F | Ex (3) | 39 | 5.0 | 107 | 101 | 76 | 96 |
| O.R. | 36 | M | Ex (24) | 41 | 5.4 | 95 | 131 | 79 | 58 |
| Total | 37±7 | | | 35±1 | 4.5±0.2 | 93±3 | 121±4 | 63±10 | 53±7 |

* Error estimates are presented as mean±SEM. ‡ Abbreviations used in this table: Ex, ex-smoker; N, nonsmoker; std, standard; VC, vital capacity; TLC, total lung capacity; DLCO, diffusing capacity; $FEV_1$, forced expiratory volume in 1 s. § Number in parentheses = pack-yr of smoking. ‖ See Methods for a description of the α1AT standards. ¶ For methods relating to lung function tests see reference 74.

result, in addition to a reduction in the serum levels of α1AT, in a diminished ability of the α1AT molecule to work effectively as an inhibitor of neutrophil elastase. In this context, the present study was designed to evaluate the concept that, in addition to the Z-type α1AT protein being present in reduced amounts, the molecule itself is less able to inhibit neutrophil elastase compared to the normal M1 molecule. Interestingly, the data demonstrates that the population of α1AT molecules in the blood of individuals homozygous for Z-type α1AT is significantly less able to inhibit neutrophil elastase than normal M1-type α1AT molecules, suggesting that the emphysema associated with the Z-type α1AT deficiency results not only from the deficiency itself but also because the Z type α1AT is relatively impotent compared with the normal M1-type α1AT.

## Methods

*Study population.* The homozygous Z form of α1AT deficiency [referred to as Pi ("protease inhibitor") ZZ] was diagnosed in 10 individuals using criteria previously described (8, 25), including serum α1AT isoelectric focusing patterns, serum α1AT levels, and family studies (26). The average serum α1AT levels were 35±1 mg/dl. (All data are presented as mean±standard error of the mean and all statistical comparisons are by the two-tailed Student's *t* test.) All had clinical evidence of emphysema and all were nonsmokers or exsmokers (Table I). For controls, 7 normal individuals were evaluated. All were male; they had an average age of 35±2 yr. All were homozygous for the M1 form[2] of α1AT (PiM1M1), and they had average serum α1AT levels of 237±14 mg/dl. None had evidence of disease or were taking medications.

*Assessment of amounts of α1AT.* The quantification of amounts of α1AT is complicated by the fact that the commercially available standard (Calbiochem-Behring Corp., La Jolla, CA) commonly used for clinical studies yields values for amounts of α1AT that are higher than the true values (5, 27, 28). However, because clinical studies quantifying α1AT serum levels over the past two decades have used such commercially available standards, the α1AT values in the study will be presented based on both a commercial standard and on a true laboratory standard (25). The laboratory standard, a highly purified (> 99%) preparation of α1AT purified by the method of Laurell et al. (29), was isolated from serum of an individual homozygous for M1-type α1AT, and quantified by amino acid analysis (mean of three determinations); this value was within 2% of the concentration determined using an extinction coefficient for α1AT at 280 nm of 5.3 (27, 28). Values for the α1AT concentration in the text and figures presented as milligrams per deciliter are based on the commercial standard and those given as micromolars are based on the true laboratory standard (multiplying the commercial standard values by 0.71 corrects them to the true values, i.e., the commercial standard is 41% higher than the true standard, similar to that reported by other investigators) (5, 27, 28). All levels of α1AT were quantified in duplicate using radial immunodiffusion plates (Calbiochem-Behring Corp.) and the standards as described above. To insure that the M1- and Z-type α1AT were recognized equally by the polyclonal antibody used for quantification, the amount of protein in parallel samples of purified preparations of M1- and Z-type α1AT (see below) were assessed by a total protein assay based on the Biuret method (Bio-Rad Laboratories, Richmond, CA), absorption measurements using the extinction coefficient of α1AT ($E\ 1\%_{280} = 5.3$) (27, 28), and radial immunodiffusion. In all cases the three methods yielded the same values for the amount of M1- and Z-type α1AT, respectively.

*Isoelectric focusing.* α1AT phenotype determinations were made by isoelectric focusing of serum in polyacrylamide at pH 4–5 (Serva Fine Biochemicals, Inc., and Pharmacia Fine Chemicals, Piscataway, NJ) (30).

*Polyacrylamide gel electrophoresis and immunoblotting.* Proteins were prepared for electrophoresis by addition of sodium dodecyl sulfate (SDS; 2.0%) and heated (100°C, 5 min). The samples were then applied to a 7.5% SDS polyacrylamide gel in the presence of SDS (0.2%), electrophoresed (40 mA, 6 h), fixed in 50% methanol, 10% acetic acid, and stained with Coomassie Blue. Molecular weight estimates were made using standard markers (Bio-Rad Laboratories) including the α1AT standard (29). Immunoblot electrophoretic transfer of proteins onto nitrocellulose paper was carried out by the method of Towbin et al. (31) using an anti-α1AT antibody (Accurate Chemical

2. Identification of the M1 haplotype was accomplished by isoelectric focusing (IEF) of serum (26); it has been recently recognized that there are two forms of M1 [M1($Val^{213}$), M1($Ala^{213}$)] that comigrate on IEF, but these can be identified in genomic DNA by oligonucleotides or direct sequencing (48). The percent activity (time independent) and *K* association of M1($Val^{213}$) and M1($Ala^{213}$) are similar (48).

and Scientific Co., Westbury, NY) peroxidase conjugated anti IgG antibody and horseradish peroxidase (Bio-Rad Laboratories).

To characterize the M1 and Z molecules after interaction with neutrophil elastase, M1 or Z-type α1AT (10 μg each) were incubated (23°C, 15 min) with varying amounts of neutrophil elastase to yield α1AT to elastase molar ratios of 1 to 0.5, 1 to 1, and 1 to 2. At the end of the incubation, the mixtures were prepared and electrophoresed on SDS polyacrylamide gels as described above. Evaluation of the complexes of α1AT with elastase were based on the formulation of Beatty et al. (32). This is based on the concept that the reaction of α1AT with neutrophil elastase occurs as follows:

$$\mathrm{NE} + \alpha1\mathrm{AT} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} \mathrm{NE}\text{-}\alpha1\mathrm{AT} \xrightarrow{K_2} \mathrm{NE}\text{-}\alpha1\mathrm{AT}_{\mathrm{truncated}} + \alpha1\mathrm{AT}_{\mathrm{fragment}} \xrightarrow{K_3}$$

$$\mathrm{NE} + \alpha1\mathrm{AT}_{\mathrm{truncated}} + \alpha1\mathrm{AT}_{\mathrm{fragment}},$$

where NE = neutrophil elastase; NE-α1AT = elastase-α1AT complex; NE-$\alpha1\mathrm{AT}_{\mathrm{truncated}}$ = complex of elastase with a truncated form of α1AT ($\mathrm{Glu}^1$ to $\mathrm{Met}^{358}$); $\alpha1\mathrm{AT}_{\mathrm{fragment}}$ = C-terminal fragment ($\mathrm{Ser}^{359}$ to $\mathrm{Lys}^{394}$); $K_1$ = association rate constant of α1AT with elastase; $K_{-1}$ = dissociation rate constant of the elastase-α1AT complex; $K_2$ = rate constant of the initial cleavage of α1AT by elastase leaving a fragment of α1AT and a complex of elastase with a truncated form of α1AT; and $K_3$ = rate constant of the dissociation of the elastase-$\alpha1\mathrm{AT}_{\mathrm{truncated}}$ complex.

*Purification of M1 and Z-type α1AT.* M1 and Z-type α1AT were purified from plasma of individuals demonstrated to be homozygous for M1 or Z-type α1AT, respectively, using criteria described above. Venous blood was collected in heparinized glass tubes and immediately centrifuged (1,200 *g*, 15 min, 4°C). Purification of the α1AT was accomplished by positive selection affinity chromatography followed by molecular sieving and then negative selection affinity chromatography. To accomplish this, fresh plasma in 2-ml aliquots was applied to a 5-ml column of CNBr activated Sepharose 4B beads with attached anti-α1AT antibody (4 ml anti-α1AT antibody/1 g beads; beads from Pharmacia Fine Chemicals, anti-α1AT antibody from Accurate Chemical and Scientific Co.) and purified as described by Sugiura et al. (33). This partially purified (~ 80%) α1AT preparation was concentrated by pressure filtration (Amicon YM-10 membrane; Amicon Corp., Danvers, MA) and then applied to a Sephadex G-100 (Pharmacia Fine Chemicals) molecular sieve equilibrated in 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM EDTA, 0.01% $NaN_3$. The α1AT peak was collected and then circulated for 12 h (2 ml/min at 4°C) over a "negative selection" affinity column (prepared as described for the anti-α1AT affinity column) containing antibodies against human whole serum, albumin, prealbumin, α2-macroglobin, chymotrypsin, orosomucoid, IgG, IgA, IgM, C-1 esterase, and antithrombin III (all from Accurate Chemical and Scientific Co.) (29). Purified α1AT collected from this column was concentrated by pressure filtration (Amicon YM-10 membrane). The final concentration of purified α1AT was determined by radial immunodiffusion as described above and stored in aliquots in liquid nitrogen vapor until used. On the average, both the M1 and Z-type α1AT preparations were > 99% pure as assessed by SDS-polyacrylamide gel electrophoresis.

*Quantification of the neutrophil elastase inhibitory activity of M1 and Z-type α1AT.* The activities of M1 and Z-type α1AT as inhibitors of neutrophil elastase were determined in a titration assay by incubating increasing amounts of the purified α1AT preparation against a fixed amount (2 nM) of a standard active human neutrophil elastase (Elastin Products Co.) under conditions (23°C) and time (2 h) in which the inhibition of neutrophil elastase was complete. The activity of the neutrophil elastase was determined as described by Straus et al. (25). In brief, the activity of the neutrophil elastase was determined by titration of neutrophil elastase against a standard of purified α1AT that had been titrated against trypsin (Worthington Diagnostics, Inc.). The trypsin was active site titrated according to the method of Chase and Shaw (34) using *p*-nitrophenyl-*p*-guanidinobenzoate HCl (*p*-NPGB, Calbiochem-Behring Corp.). All time independent reactions were carried out in 1 ml containing 0.1 M Hepes, pH 7.5, 0.5 M NaCl, and 0.1% Brij 35 (to minimize the adsorption of elastase to the walls of assay tubes). Variable amounts of α1AT were added to the reaction mixture containing neutrophil elastase and the reaction was carried out for 2 h, 23°C. Specific neutrophil elastase substrate methoxy-succinyl-alanyl-alanyl-prolyl-valyl-nitroanalide (Me-S-AAPV-NA; 1 mM; Sigma Chemical Co.) (35) was added and the residual elastase activity quantified as a change in optical density at 410 nm/min using a spectrophotometer (DU-7, Beckman Instruments, Inc., Fullerton, CA). The activity of the α1AT preparation was calculated by plotting the percent elastase activity remaining versus the α1AT concentration, fitting the data by linear regression analysis and determining the concentration of α1AT of that α1AT preparation that would result in no elastase activity. In this context, the proportion of the α1AT preparation capable of inhibiting neutrophil elastase was determined as: [(concentration of active neutrophil elastase used in the assay) × 100]/[concentration of α1AT required to completely inhibit the elastase]. For example, if 2 nM active elastase was used in the assay and if 3 nM α1AT were required for complete inhibition, the α1AT preparation would be 67% active. For each α1AT preparation, each assay was performed in triplicates and within each assay, each concentration of α1AT was evaluated in duplicate.

To verify that the high salt concentration in the buffer did not differentially affect the M1 and Z types of α1AT, identical titration assays were carried out with the salt concentrations reduced 10-fold [i.e., 0.05 M NaCl in place of 0.5 M]. To insure that the differences observed between the M1 and Z proteins were not dependent on their differential interaction with the substrate, the titration assays were carried out using the neutrophil elastase specific substrate N-t-Boc-L-alanyl-L-prolyl-L-norvaline *p*-chlorothiobenzyl ester (Boc-Ala-Pro-Nva-SBzl; Sigma) as described by Harper et al. (36). To verify that the purification method used was not responsible for the observed differences in the function of M1 and Z type α1AT, a second method, described by Pannell et al. (28) was used to purify the proteins. Briefly, M1 or Z type α1AT was purified from plasma by passage over a blue Sepharose column (Pharmacia), followed by ammonium sulfate precipitation, DEAE cellulose ion-exchange chromatography in 50 mM M Tris-HCl, pH 8.8, 50 mM NaCl, and finally on DEAE-cellulose ion-exchange chromatography in 5 mM sodium phosphate, pH 6.5, 50 mM NaCl. The α1AT fraction was then circulated over a negative selection affinity column (as described above) to remove residual proteins and the purified α1AT (> 95% pure for both M1 and Z) was concentrated under nitrogen. Finally, to insure that the activity of the purified α1AT preparation had not been altered by the purification procedure, similar analyses were carried out using fresh M1 and Z plasma. All procedures were identical, except that the linear regression analysis ignored that portion of the curve contributed by α2-macroglobin (37).

*Quantification of the association rate constant of α1AT.* The time-dependent interaction of M1 and Z α1AT was carried out by measuring the association rate constant (*K* association) by the method of Beatty et al. (38) with minor modifications described by Straus et al. (25). These measurements were made only with the purified preparations of α1AT. In brief, the purified M1 and Z-type α1AT were titrated against neutrophil elastase to determine the percent active α1AT as described above. Equimolar amounts of neutrophil elastase and active α1AT (1 nM each) were then reacted at 23°C in a 1-ml reaction mixture containing 0.1 M Hepes, pH 7.5, 0.5 M NaCl, and 0.1% Brij 35. Residual elastase activity at 0 to 120 min was quantified by terminating the reaction with the Me-S-AAPV-NA elastase substrate (1 mM) as described above. At each time point, the percent inhibitory activity of the α1AT preparation against neutrophil elastase was determined as [(elastase activity without added α1AT − elastase activity with α1AT) × 100]/[elastase activity without added α1AT]. The *K* association of the α1AT preparation was quantified as described by Beatty et al. (38) by plotting [elastase activity at each time point]$^{-1}$ versus time; from the linear portion of the curve (the initial 4–5 min),

the $y$ intercept (elastase activity at 0 time) and the slope of the line were determined by least-squares analysis. From this data, the half-time of the reaction ($t_{1/2}$) was quantified at $t_{1/2}$ = ($y$ intercept)/(slope) and the $K$ association = [(concentration of active neutrophil elastase in the reaction) × $(t_{1/2})]^{-1}$ with $t_{1/2}$ in seconds and the concentration of active neutrophil elastase $10^{-9}$ M. Like the controls for the time independent assay, comparison of the $K$ association of the M1 and Z forms of α1AT was also carried out in 10-fold less salt, using a different elastase substrate, and with the M1 and Z proteins purified by a different method (see description of time independent assay above for details).

## Results

*Comparison of the antineutrophil elastase activity of M1 and Z-type α1AT.* The antineutrophil elastase activity of the population of purified M1-type α1AT molecules was higher than that of the population of purified Z-type α1AT molecules, i.e., a higher proportion of M1-type α1AT molecules was capable of inhibiting neutrophil elastase than Z-type α1AT molecules (Fig. 1). In this regard, complete inhibition of 2 nM of the neutrophil elastase standard required, on the average, 2.3±0.1 nM M1-type α1AT as compared to 3.2±0.1 nM for the Z-type α1AT ($P < 0.001$). Stated in terms of the proportion of α1AT molecules that were active (i.e., capable of inhibiting neutrophil elastase), there was a significantly greater proportion of M1-type α1AT molecules that were active than Z-type α1AT molecules (M1-type 86.4±2.1%, Z-type 63.3±2.2%, $P < 0.001$). When the M1 and Z molecules were evaluated after purification by an alternative method or when the assay was carried out in low salt concentration or with an alternative substrate (see Methods for details), the results confirmed the differences between the two forms of α1AT. Thus overall, a higher proportion of the population of M1-type α1AT molecules was more active against neutrophil elastase than the proportion of the population of Z-type α1AT molecules.

*Evaluation of the state of M1 and Z-type α1AT molecules in plasma.* To insure that the observed differences in the observed activity of the purified populations of M1 and Z-type α1AT molecules were not due to artifacts of sample preparation but rather represented true differences in the state of the molecules in vivo, the antineutrophil elastase activity in PiM1M1 and PiZZ plasma was compared by titrating fresh plasma against a fixed amount (2 nM) of neutrophil elastase (Fig. 2 *A*). Complete inhibition of 2 nM elastase activity occurred at a concentration of 2.1±0.1 nM for PiM1M1 plasma compared with 2.6±0.1 nM for PiZZ plasma ($P < 0.001$). At that concentration of plasma where equal amounts of elastase and α1AT were present, only 4±1% residual elastase activity was present with M1M1 plasma, while 24±1% neutrophil elastase activity remained with PiZZ plasma ($P < 0.001$). Thus, like the results obtained with purified M1-type and Z-type α1AT, the population of Z-type α1AT molecules in plasma was less active than the population of M1 type α1AT molecules in plasma (PiZZ plasma 78±1%, PiM1M1 plasma

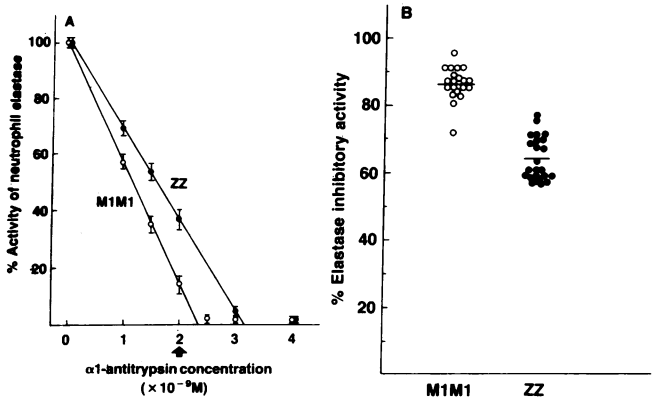


*Figure 1.* Time independent titration of purified M1-type and Z-type α1AT with human neutrophil elastase. The α1AT preparations were purified from M1M1 and ZZ homozygotes, respectively. Neutrophil elastase was incubated (2 h, 23°C) with various amounts of either M1-type or Z-type α1AT and the residual elastase activity quantified by addition of the elastase substrate. (*A*) Titration curves for purified M1-type (○) and Z-type (●) α1AT. Each data point represents the mean±standard error of triplicate determinations of 7 M1-type individuals and 10 Z-type individuals. The arrow below the abscissa indicates that concentration of 100% active α1AT molecules necessary to inhibit all of the neutrophil elastase in the reaction (2 nM). (*B*) Time independent neutrophil elastase inhibitory activity of purified M1-type and Z-type α1AT. Percent activity was determined from the evaluation of the time independent titration of human neutrophil elastase purified M1-type and Z-type α1AT as shown in panel *A*. The data shown represents 20 determinations of 7 PiM1M1 individuals and 24 determinations of 10 PiZZ individuals. The horizontal line represents the mean for each group.

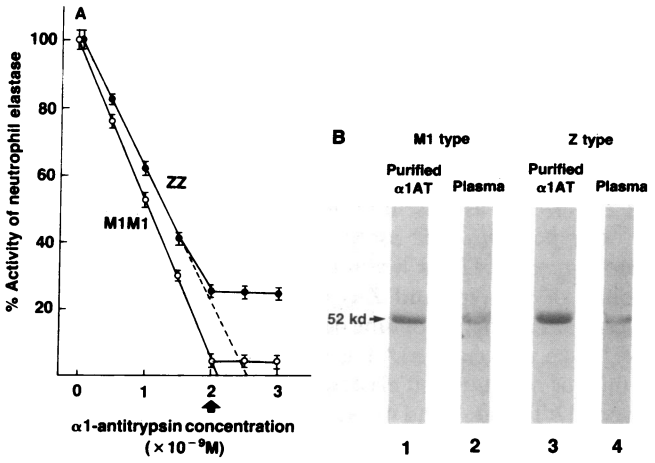


*Figure 2.* Evaluation of the relative function and form of M1-type and Z-type α1AT in fresh plasma prior to purification. (*A*) Time independent titration of human neutrophil elastase by fresh plasma of M1M1 and ZZ homozygotes. Elastase (2 nM) was incubated (2 h, 23°C) with increasing concentrations of either M1-type (○) or Z-type (●) plasma and the residual elastase activity quantified as in Fig. 1 *A* for the purified α1AT preparations. The data at each concentration represents the mean±standard error of the mean of triplicate determinations from four Z-type individuals and four M1-type individuals. The horizontal portion of the titration curves at the higher concentrations of α1AT results from the presence of α2-macroglobulin in the plasma (see text). The arrow below the abscissa indicates the point at which 100% active α1AT would reach the abscissa, i.e., 2 nM of 100% active α1AT would completely inhibit the 2 nM elastase used in the assay. (*B*) Apparent molecular weight of M1-type and Z-type α1AT while present in fresh plasma compared to purified M1 and Z α1AT. The samples were evaluated by PAGE and Western blotting using an antihuman α1AT antibody. (Lane *1*) SDS gel Coomassie Blue stain of purified M1-type α1AT. (Lane *2*) Western blot of PiM1M1 plasma. (Lane *3*) SDS gel Coomassie Blue stain of purified Z-type α1AT. (Lane *4*) Western blot of PiZZ plasma. The apparent molecular weight of 52 kD is indicated.

95±1% active, $P < 0.001$). These observations with plasma demonstrate that, for a purified population of α1AT molecules, a higher proportion of M1 molecules is capable of inhibiting neutrophil elastase than a purified population of Z-type α1AT molecules, and that this is not due to experimental artifacts resulting from the purification procedure.

One explanation for the observation that a lower proportion of Z-type molecules is capable of functioning as an inhibitor of neutrophil elastase is to hypothesize that a greater proportion of Z-type molecules is "used up" in vivo than are M1-type molecules, i.e., that some of the population of Z-type molecules in plasma (and hence in the subsequent purified protein) cannot inhibit neutrophil elastase in vitro because they have already done so in vivo. If this were the case, then since the interaction of α1AT with neutrophil elastase is almost entirely irreversible, we would expect that a proportion of the circulating Z-type α1AT molecules would be complexed with (or cleaved by) neutrophil elastase, and that some of the α1AT in the population of Z molecules would be a higher molecular weight (i.e., complexed) or lower molecular weight (i.e., cleaved), than the normal 52-kD α1AT molecule. However, SDS-PAGE and immunoblot analysis of M1-type and Z-type purified α1AT and of PiM1M1 and PiZZ plasma did not demonstrate differences in the molecular mass of the two types of α1AT (Fig. 2 *B*) for both the purified proteins (lanes *1, 3*) and the proteins in plasma (lanes *2, 4*); in all cases, each inhibitor migrated as a single 52-kD band. Thus, the hypothesis that the Z-type molecule has already formed α1AT-elastase complexes or been degraded in vivo is not valid and thus does not account for the difference in antielastase activity observed between the M1 and Z-type molecule, i.e., the decreased antielastase activity of Z-type α1AT compared with M1-type α1AT reflects an intrinsic property of the population of Z molecules as present in plasma in vivo.

*Comparison of the association rate constants of M1-type and Z-type α1AT for neutrophil elastase.* Evaluation of the ability of M1-type and Z-type α1AT to inhibit neutrophil elastase as a function of time demonstrated that the population of Z-type molecules α1AT is significantly less effective as an inhibitor of neutrophil elastase than is the population of M1-type α1AT (Figs. 3 and 4). At all incubation periods up to 1 h, the Z-type α1AT inhibited less elastase activity than did the M1-type protein. This difference was most pronounced at incubation time intervals of < 15 min (Fig. 3). The calculated half-times ($t_{1/2}$) of the reaction between α1AT and neutrophil elastase were 1.7±0.1 min for M1-type α1AT and 3.6±0.2 min for Z-type α1AT ($P < 0.001$). Comparison of the *K* association of α1AT for neutrophil elastase for M1-type and Z-type α1AT demonstrated that the Z-type α1AT had a lower *K* association (Fig. 4, M1-type $9.7\pm0.4 \times 10^6$ $M^{-1}s^{-1}$, Z-type $4.5\pm0.3 \times 10^6$ $M^{-1}s^{-1}$, $P < 0.001$). Thus, on the average, the Z-type protein had a *K* association for neutrophil elastase that was 46% less than that of the M1-type α1AT. Significantly, among the 44 samples tested, there was no overlap among the M1 and Z samples, strongly supporting the concept that the observed differences in the interaction of each of these types of α1AT with neutrophil elastase was due to a fundamental difference in the function of the population of Z α1AT molecules and not due to individual variations among subjects tested. Like the time-independent assay, when the *K* association of the M1 and Z molecules was evaluated after purification by an alternative method or where the assay was carried out in low salt concentration or with an alternative substrate (see Methods for details), the results confirmed the differences between the two forms of α1AT.

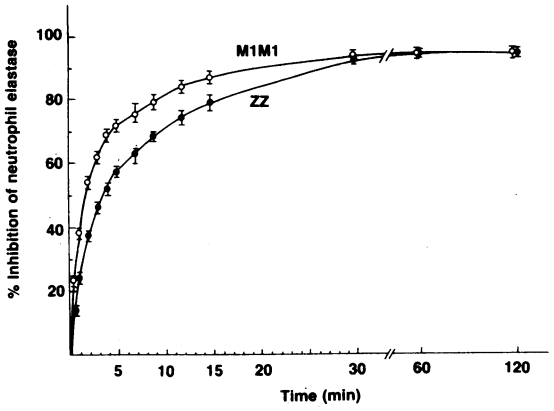


*Figure 3.* Comparison of the time dependent inhibition of neutrophil elastase by M1-type and Z-type α1AT. Purified M1-type (○) or Z-type (●) α1AT (1 nM active α1AT each) were incubated (23°C) with 1 nM neutrophil elastase for the indicated times and the residual elastase activity quantified by the addition of the neutrophil elastase specific substrate. The data shown represents the mean±SEM for triplicate determinations each of 20 samples from 7 M1-type individuals and 24 samples of 10 Z-type α1AT individuals.

*Comparison of the forms of M1 and Z α1AT after interaction with neutrophil elastase.* Using SDS gels to evaluate the form of the M1 and Z-type molecules after interaction with neutrophil elastase demonstrated that the Z-type molecule was less able to maintain a stable complex with elastase (Fig. 5). In

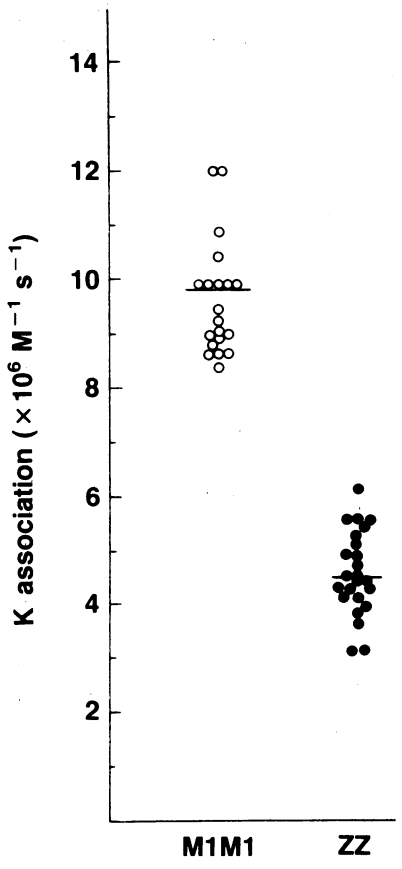


*Figure 4.* Association rate constants (*K* association) of M1-type and Z-type α1AT for human neutrophil elastase. Neutrophil elastase and the M1 or Z-type α1AT were incubated for various times as shown in Fig. 3, the half time of the reaction was determined, and the *K* association calculated. Each data point represents the *K* association calculated from individual samples of PiM1M1 and PiZZ individuals; there were 20 samples from 7 PiM1M1 individuals and 24 samples from 10 PiZZ individuals. For each sample, each time point was determined in triplicate. The horizontal line represents the mean for each group.

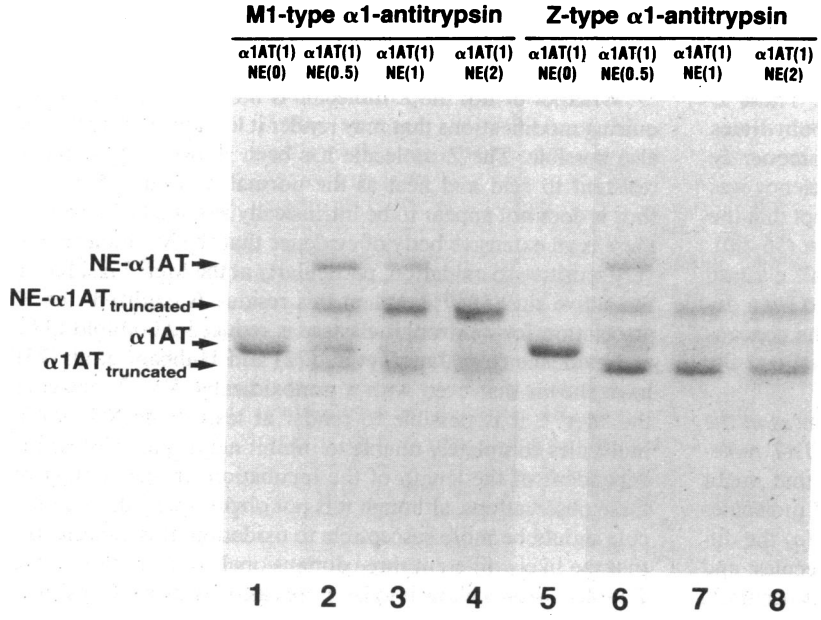


*Figure 5.* Characterization of the form of M1 and Z-type α1AT following interaction with neutrophil elastase. Native M1 and Z-type α1AT (10 μg each) was incubated with neutrophil elastase (NE) at varying molar ratios (α1AT to NE ratios 1:0.5, 1:1, 1:2) at 23°C for 15 min. The reaction mixtures were then evaluated by SDS-PAGE, fixed and stained with Coomassie Blue. (Lane *1*) M1-type α1AT, no elastase; (lane *2*) M1-type α1AT to elastase ratio 1:0.5; (lane *3*) M1-type α1AT to elastase 1:1; (lane *4*) M1-type α1AT to elastase 1:2, (lane *5*) Z-type α1AT, no elastase; (lane *6*) Z-type α1AT to elastase ratio 1:0.5; (lane *7*) Z-type α1AT to elastase 1:1; (lane *8*) Z-type α1AT to elastase 1:2. Arrows indicate various forms of α1AT: NE-α1AT, α1AT complexed with elastase; NE-$\alpha 1AT_{truncated}$, complex of elastase and truncated form of α1AT; α1AT, α1-antitrypsin; $\alpha 1AT_{truncated}$, free truncated form of α1AT.

this regard, when varying ratios of M1-type α1AT and elastase were incubated and then displayed on SDS gels, it was apparent that almost all of the α1AT formed a complex with the elastase. Specifically, with M1-type α1AT in a 2 to 1 ratio with elastase, the α1AT either was in the form of a complex with elastase or a complex in which the C-terminal portion of the α1AT was lost (i.e., a truncated form of α1AT complexed with elastase) (Fig. 5, lane *2*). As the molar ratio of elastase increased relative to the M1-type α1AT, a similar pattern was observed, except that at a ratio of α1AT to elastase of 1 to 2, most of the complex was in the truncated form (Fig. 5, lanes *3* and *4*). For all ratios of M1-type α1AT to elastase, most of the α1AT interacted with elastase (i.e., there was no free intact α1AT observed on the gels) and there was a small amount of the α1AT in a truncated form alone (i.e., no longer complexed with elastase). These observations are consistent with the observation that almost all of the M1-type α1AT was active as an inhibitor of neutrophil elastase (see Fig. 1 *A* and *B*).

In marked contrast to the M1 form of α1AT, a similar analysis with the Z form of α1AT showed that a greater proportion of the Z molecules was unable to maintain a stable complex with the neutrophil elastase (Fig. 5, lanes *5–8*). Like the M1 form of α1AT, the Z molecules were capable of interacting with elastase such that little of the 52-kD native form of the Z molecule remained (lanes *6–8*). Interestingly, compared to the M1-type α1AT, at all ratios of Z-type α1AT to elastase, there was less of the elastase-α1AT complex or the elastase-truncated α1AT complex, but more of the truncated form of α1AT alone. Put in the context of the observation that the activity titration assays comparing the population of Z-type α1AT molecules to the population of M1-type α1AT showed lower elastase inhibitory activity of the Z-type compared with the M1-type molecules, the observations of the form of the Z and M1 molecules after being incubated with the elastase suggest that some of the Z molecules cannot maintain a stable complex with the elastase, i.e., some of the Z-type α1AT-elastase complex is unstable such that the elastase is released to again function, leaving a truncated form of α1AT that is unable to again interact with the enzyme.

## Discussion

By evaluating the characteristics of the population of Z-type α1AT molecules in comparison to the population of normal M1-type of α1AT, the present study extends the concept of the pathogenesis of emphysema associated with the homozygous form of α1AT deficiency by demonstrating that a population of Z-type molecules is less competent as an inhibitor of neutrophil elastase than a comparable population of M1 molecules. These observations suggest that the relative lack of protection of the Z homozygote lung against neutrophil elastase stems not only from a "deficiency" of α1AT, but also from a relative impotence of those α1AT molecules that are present.

*Differences in the M1- and Z-type α1AT molecules.* The normal M1 protein purified from plasma is comprised of a single polypeptide chain of 394 amino acids together with three complex carbohydrate side chains N-linked to asparaginyl residues at $Asn^{46}$, $Asn^{83}$, and $Asn^{247}$ (4, 39, 40). The M1 protein is synthesized and secreted as a typical secretory glycoprotein; the mRNA is translated on the rough endoplasmic reticulum (RER), the high mannose form of N-linked carbohydrate side chains are added in the RER (41–44), the molecule is translocated to the Golgi where the carbohydrates are trimmed, and the molecule is secreted (39, 43–45). The active site of the M1-type α1AT is centered at $Met^{358}$-$Ser^{359}$ (4, 41, 46, 47). There are two M1 haplotypes: [M1($Val^{213}$) and M1($Ala^{213}$)]. The two molecules are identical except for the difference at residue 213. Their function and associated plasma levels are also similar (48).

The Z-type protein purified from plasma is also comprised of 394 amino acids and has carbohydrate side chains similar to that of the M1 molecule (4, 27, 49). The primary structure of the polypeptide differs from the M1($Val^{213}$) at two residues ($Val^{213}$ to $Ala^{213}$; $Glu^{342}$ to $Lys^{342}$) and from M1($Ala^{213}$) at one residue ($Glu^{342}$ to $Lys^{342}$) (48, 50–52). Like the M1 molecule, the active site of the Z-type α1AT is centered at $Met^{358}$-$Ser^{359}$. The serum level of Z homozygotes is < 50 mg/dl and usually ranges from 15 to 45 mg/dl. The reduced serum levels of the Z protein result from aggregation of the newly synthesized mole-

cules in the RER of the α1AT producing cells (hepatocytes and mononuclear phagocytes) (53, 54). The newly synthesized Z molecules reach the cisternae of the RER and have high mannose carbohydrates attached, but because the molecules aggregate, most are not translocated to the Golgi (55). Those Z molecules that do reach the Golgi have their carbohydrates trimmed appropriately and the molecules are subsequently secreted in a normal fashion (55). Since α1AT deficiency was discovered, there has been some interest in the concept that the Z molecule might not function in a normal fashion (56–60). However, none of these studies utilized neutrophil elastase standards for which the activity of the molecule had been determined by titration, α1AT standards in which the concentration was known accurately, or attempted to evaluate the time-dependent function of the molecule.

*Possible mechanisms responsible for the differences in the function of the population of the M1- and Z-type α1AT molecules.* A priori there are two general mechanisms that might explain the reduced function of the population of Z molecules in comparison to the population of M1 molecule: (*a*) the differences in the primary sequences of the two molecules; and (*b*) differential modification of the Z molecule that occurs in vivo after translation of the Z mRNA.

In regards to the question whether primary sequence differences among α1AT molecules can result in alteration of its function as an inhibitor for neutrophil elastase, it is known that changes in the region of the $Met^{358}$-$Ser^{359}$ active site have been shown to modify the function of the molecule (61). There is one human example of this: α1AT Pittsburgh, a molecule identical to M1 except for a substitution of $Met^{358}$ to $Arg^{358}$, a substitution that not only renders the molecule inefficient as an inhibitor of neutrophil elastase but also converts it into an efficient equivalent of antithrombin III (62). In addition, while recombinant α1AT molecules with residue 358 substitutions to Leu, Phe, Ala, or Ile all show a *K* association for neutrophil elastase below that of the normal molecule (63–66).

Whether or not the amino acid substitutions in the Z molecule are responsible for at least some of the reduction in the function of the Z molecule is a matter of conjecture. Since the substitutions that make up the Z mutation are not close to the active site at $Met^{358}$-$Ser^{359}$, and since small peptides with a normal sequence in this region are capable of inhibiting neutrophil elastase in a normal fashion, it is unlikely that the effect of the 213 and 342 substitutions have a direct effect on the interaction of the Z protein with neutrophil elastase. Furthermore, it is unlikely that the $Ala^{213}$ substitution is alone responsible, since the M1($Val^{213}$) and M1($Ala^{213}$) proteins behave similarly as inhibitors of neutrophil elastase (48). However, crystallographic studies by Huber et al. (67) suggested that in the M1 protein, the $Glu^{342}$ residue is involved in a critical salt bridge with $Lys^{290}$, and they have hypothesized that the $Lys^{342}$ substitution obviates this interaction, thus changing the three dimensional configuration of the molecule. This concept has become central to explaining why the Z protein aggregates in the RER, i.e., the loss of the salt bridge slows the rate of folding of the molecule into its three dimensional form, allowing hydrophobic residues in adjacent α1AT molecules to interact, causing aggregation (67). In concert with this concept, it is possible the loss of this salt bridge also effects the ability of the molecule maintain a stable complex with neutrophil elastase, particularly the complex of a truncated form of α1AT with elastase (32). If this occurs, the elastase would be released and thus continue to function as an active protease. A possible role for the 213 substitution in such a model is not as apparent, but the crystallographic structure suggests the 213 residue is close to a carbohydrate attachment site ($Asn^{247}$), and it is conceivable that it also affects the ability of the molecule to function normally.

Whether or not the Z molecule is more susceptible to acquiring modifications that may render it less able to function is also possible. The Z molecule has been shown to be equally resistant to acid and heat as the normal molecule (56), and thus is does not appear to be intrinsically less stable. However, there is an extensive body of evidence that the M1 molecule is very sensitive to oxidation, particularly at the $Met^{358}$ residue at the active site (68–71). When this residue is oxidized, the *K* association for neutrophil elastase is reduced 2,000-fold (38), and evidence from Janoff et al. (72) and Hubbard et al. (73) have shown that even with a nonoxidizable $Val^{358}$ replacing the $Met^{358}$, it is possible to render at least some M1 α1AT molecules completely unable to inhibit neutrophil elastase independent of the length of the incubation. In the context of these observations, although it is not obvious why the Z molecule should be more susceptible to oxidation, it is conceivable that the likely different three dimensional configuration of the Z molecule may allow it to be more easily oxidized. Consistent with this concept, it is known that the microorganism produced recombinant M1 molecule, a form of α1AT without carbohydrate side chains, and likely of a different three dimensional configuration than the normal M1 molecule, is much more susceptible to oxidation that the naturally occurring M1 molecule (unpublished observation).

An alternative hypothesis to explain why the Z molecule found in the plasma may be less able to function is to assume that a certain number of α1AT molecules in plasma are "used up" by virtue of a small burden of neutrophil elastase that is released in blood by effete or damaged neutrophils. In this context, since the Z homozygote has ~ 15% of the number of α1AT molecules in plasma as the M1M1 homozygote, it would follow that a higher proportion of the Z molecules would be relatively impotent. However, while this scenario is conceivable, it cannot explain the majority of the observations in the present study, since evaluation of the Z molecules in fresh plasma demonstrated that the molecule was neither complexed to elastase nor fragmented at the $Met^{358}$-$Ser^{359}$ bond.

*Likely physiologic consequences of the differences in the function of the M1- and Z-type α1AT molecules.* Independent of how it occurs, the observation that a population of Z-type α1AT molecules is less able to inhibit neutrophil elastase than a population of M1-type α1AT molecules has profound implications for the pathogenesis of emphysema in the ZZ homozygote form of α1AT deficiency and for the therapy of this disorder.

First, not only are the levels of α1AT reduced to 15% of normal because the Z molecule aggregates in the α1AT synthesizing cells, but also the fact that the the Z molecules are less active means that essentially these individuals are even more "deficient" than implied by the reduced plasma levels of this critical molecule. In this context, it is reasonable to conclude that in vivo, given an equivalent number of α1AT molecules in the local milieu, that a population of Z molecules will be less likely than a population of M1 molecules to prevent neutrophil elastase from interacting with its natural connective tissue substrates, thus increasing the likelihood of developing emphysema for a given burden of elastase.

Second, these observations also lead to the conclusion that therapy of the Z homozygote form of α1AT deficiency would

best not be approached by attempting to increase the synthesis and/or secretion of $\alpha$1AT by the $\alpha$1AT producing cells of the affected individual, since the molecules secreted by these cells are, at least in part, ineffective as inhibitors of neutrophil elastase. Likewise, in designing therapies in which the deficiency state is corrected by augmenting the $\alpha$1AT levels by administering purified $\alpha$1AT, it would be best to use M1-type $\alpha$1AT or $\alpha$1AT purified from the pooled plasma of normals. Alternatively, when it becomes practical to use the recombinant forms of $\alpha$1AT for augmentation therapy, it may be possible to design an $\alpha$1AT molecule that has a higher K association for neutrophil elastase than the naturally occurring normal M1 molecule.

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