The specificity and reactivity of human $\alpha_1$-proteinase inhibitor has been investigated by in vitro mutagenesis of the reactive site $P_1$, methionine 358 residue to alanine 358 and cysteine 358. A comparison of the second-order association rates of both uncharged mutants with 9 serine proteinases indicated that each reacted similarly to either the normal plasma inhibitor or to a mutant containing valine in this position (Travis, J., Owen, M., George, P., Carrell, R., Rosenberg, S., Hallewell, R. A., and Barr, P. J. (1985) J. Biol. Chem. 260, 4384–4389) when tested against either neutrophil or pancreatic elastase. However, oxidation, carboxymethylation, or aminoethylation of the cysteine mutant to yield a charged $P_1$ residue resulted in a significant decrease in association rates with both elastolytic enzymes, and aminoethylation created an excellent trypsin and plasmin inhibitor. These results indicate that the specificity of $\alpha_1$-proteinase inhibitor is determined in a general manner by the class of amino acid residue in the $P_1$ position. Substitution within the same category, such as from valine to alanine or cysteine among the aliphatic hydrophobic residues, has little effect on association rates with the elastolytic enzymes tested. However, alteration from an uncharged to a charged residue may cause considerable change in both inhibitor specificity and reactivity as noted here with the cysteine derivatives and also previously with a natural variant in which methionine 358 to arginine 358 conversion resulted in the production of a potent thrombin inhibitor (Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) N. Engl. J. Med. 309, 694–698).

Human plasma $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI) is a major inhibitor of serine proteinases in tissues (1) and functions primarily in controlling the activity of human neutrophil elastase (2), an enzyme capable of degrading connective tissue components, including elastin, collagen, and proteoglycan (3). Under normal circumstances the $\alpha_1$-PI concentration in tissues is sufficiently high to control this enzyme and thus prevent connective tissue damage, especially in the lung; however, if the level of active $\alpha_1$-PI in the blood is reduced as occurs, for example, in individuals synthesizing mutant forms of the inhibitor, lung damage may occur resulting in the development of pulmonary emphysema (4).

In individuals with normal plasma protein levels of $\alpha_1$-PI there are probably several pathways by which functional activity is decreased. A major mechanism suggested involves oxidation of the critical reactive site methionine residue at position 358 in the primary structure of the protein to the sulfoxide derivative by chemicals present in cigarette smoke and by enzymatically derived oxidants produced by the neutrophil (5–7). This alteration not only results in a 2000-fold decrease in the second-order association rate with neutrophil elastase (2) but also renders the oxidized inhibitor ineffective in controlling elastin degradation by this enzyme (8).

Confirmation of the importance of methionine 358, referred to as the $P_1$ residue, in dictating specificity has been obtained through the isolation of a natural mutant form of the inhibitor, referred to as $\alpha_1$-PI (Pittsburgh), from a patient with a severe bleeding disorder (9). This protein was found to contain an arginine residue in the $P_1$ position with a resultant altered specificity. Indeed, its reactivity was directly opposite to that of normal plasma $\alpha_1$-PI in that it inactivated thrombin rapidly and elastase very slowly.

Using recombinant DNA technology it is now possible to produce human $\alpha_1$-PI mutants with amino acids other than methionine in the $P_1$ or $P_1'$ positions (10, 11), as well as substitutions elsewhere in the primary structure of this protein, and examine both the range of specificities and the mechanism by which enzyme-inhibitor complex formation occurs with this protein. Furthermore, since yeasts are able to produce large quantities of such inhibitors, it may also prove practical to utilize such mutant forms as therapeutic agents against a variety of serine proteinase-related diseases, including emphysema (10).

Two yeast-produced $\alpha_1$-PI mutants, with methionine or valine in the $P_1$ position, have previously been studied (10, 11). The methionine 358 protein appeared to be similar to normal plasma $\alpha_1$-PI except for decreased stability in the absence of reducing agents, while the valine mutant was both stable and oxidation-resistant. In a further effort to define the effects of various $P_1$ residue replacements on both the inhibitory activity and specificity of $\alpha_1$-PI we describe in this report the properties of both the structurally related alanine 358 and cysteine 358 mutants. The latter was chosen because it is amenable to chemical modification at the $P_1$-reactive site position.

Experimental Procedures

Materials

Human neutrophil elastase and human neutrophil cathespin G were prepared as described previously (12, 13). Human plasminogen...
Reactive Site Mutations in Human α₁-Proteinase Inhibitor

Polycrylamide Gel Electrophoresis—NaDodSO₄—polycrylamide gel electrophoresis was performed in the 2-amino-2-methyl-1,3-propane diol (Ammediol)/glycine/chloride buffer system of Wyckoff et al. (16), as described by Barrett et al. (17). Samples of proteinase and α₁-PI mutant were incubated together for 1 min at room temperature, made 2% in NaDodSO₄ with or without 1% 2-mercaptoethanol, boiled for 2 min, and run in 8% gels. Staining was then performed using 0.1% Coomassie Brilliant Blue G.

RESULTS

Expression of Inhibitor Mutants in Yeast—The α₁-PI coding sequence was derivatized by silent third position mutagenesis to form a unique Psfl site close to the active center methionine 358 residue (10). By using this restriction site together with the natural AuaI site unique in the cDNA for α₁-PI, synthetic DNA coding for alanine 358 or cysteine 358 mutants was inserted directly into the yeast expression vector pC1/1. Using identical conditions to those described previously for the production of wild-type and valine 358 α₁-PI (11), high level expression of both the alanine and cysteine mutants was achieved in yeast.

Isolation and Properties of Alanine 358 and Cysteine 358 Inhibitor Mutants—Both of the mutants studied were isolated exactly as described previously (11). Each migrated as a single band with a M₀ of 46,000 after NaDodSO₄ electrophoresis with reduction (Fig. 1, lane 1; Fig. 2, lane 4). This M₀ is lower than that of the native plasma inhibitor (M₀ = 52,000) because of the lack of glycosylation in the yeast system utilized. Initially, both were found to inhibit porcine pancreatic and human neutrophil elastase. However, the cysteine mutant rapidly lost this activity upon storage, presumably due to dimer formation involving not only cysteine 358 but also possibly the other cysteine residue known to be present at position 232 (18, 19) (Fig. 2, lane 1). The dimerized protein did not form complexes with either neutrophil or pancreatic elastases (Fig. 2, lanes 2 and 3), instead being degraded by each of these enzymes. However, if the cysteine mutant was preincubated with 0.001 M diethiothreitol only the monomeric

Determination of Second-order Association Rate Constants—The rates of association of the α₁-PI variants with several proteinases were determined as previously described (2, 11). Briefly, human plasma α₁-PI was standardized against porcine pancreatic trypsin which had been active site-titivated with nitrophenyl p'-guanidino-benzoate. This inhibitor was then utilized as a secondary standard for determining the activity of all other proteinases utilized in this study. The activities of the yeast alanine and cysteine α₁-PI mutants were determined against human neutrophil elastase while the carbomethoxy cysteine and aminoethylcysteine derivatives were titrated against bovine chymotrypsin.

For the determination of association rates, equimolar amounts of the α₁-PI variants and proteinases were incubated at room temperature (20°C) in 0.05 M sodium phosphate, pH 7.4, 0.15 M NaCl, for appropriate time periods followed by the addition of saturating concentrations of substrate specific for each proteinase. The residual enzyme activity was then determined and compared to a control sample containing enzyme alone. The final molarity of substrates was 2.0 mM for human neutrophil elastase, human plasmin, S. aureus V8 proteinase, and porcine pancreatic elastase, 1.0 mM for bovine chymotrypsin and human cathepsin G, and 0.9 mM for porcine pancreatic trypsin and papain. All of the association rates reported are average values with standard deviations of 20% or less.
Effect of Modification of Cysteine Residues on Complex Formation—The reason for modifying the cysteine mutant was to determine whether changing the charge on the P₁ residue would affect inhibitor specificity. Carboxymethylation of cysteine with iodoacetic acid introduced an acidic side chain reminiscent of glutamic acid while aminoethylation with 2-chloroethylamine yielded a basic side chain with a structure analogous to lysine. The carboxymethylated derivative was used to examine interactions with S. aureus V8 proteinase, an enzyme which accommodates a glutamic acid residue in its S₁ pocket (21).

After reductive carboxymethylation, the protein migrated as a single band (Fig. 4, lane 1) in a nonreducing system and could inactivate a number of proteinases tested. However, complex formation with the V8 proteinase was not detected (Fig. 4, lane 2) and the inhibitor was, instead, converted to a modified form of lower Mr. Since a glutamic acid residue is known to occur in the P₁ position it is possible that the enzyme cleaved the modified mutant inhibitor either at this position or at the modified P₁ position, thus inactivating the inhibitor.

While reduction and/or carboxymethylation of the air-oxidized cysteine inhibitor caused restoration of inhibitory activity, it should be noted that stronger oxidizing agents, such as N-chlorosuccinimide, caused the conversion of the protein into a non-dimerizable and inactive form (Fig. 4, lane 3) which was degraded by neutrophil elastase in a manner similar to that of the air-oxidized protein (Fig. 4, lanes 4 and 6).

The aminoethylcysteine 358 derivative was used to examine the effect of placing a basic residue analogous to lysine (22) in the P₁ position on interactions with trypsin and plasmin. NaDodSO₄-gel electrophoresis patterns showed that most of the modified protein migrated as a monomer in a nonreducing system (Fig. 5, lane 4), indicating nearly complete conversion to the aminomethylcysteine derivative. However, traces of a dimer could also be detected which may represent unreacted protein or, possibly, products of the long reaction time with

form of the inhibitor was detectable (Fig. 2, lane 4) and inhibitory activity was restored.

Complex Demonstration between Alanine 358 or Cysteine 358 Mutants and Proteinases—The interaction of the alanine mutant of α₁-PI with human neutrophil elastase yielded NaDodSO₄-stable complexes as shown in Fig. 1. When the inhibitor and human neutrophil elastase (5:1 molar ratio) were incubated together for 1 min an NaDodSO₄-stable complex of Mr about 70,000 was formed, representing a 1:1 complex between the two proteins (Fig. 1, lane 2). As the proportion of neutrophil elastase relative to the alanine mutant was increased more complex formed until equivalence, after which excess elastase could be demonstrated and also slight degradation of the complex (Fig. 1, lane 4).

As described above, the cysteine mutant could only inactivate neutrophil or pancreatic elastase after reduction with 0.001 M dithiothreitol. As shown in Fig. 2, lanes 5 and 6, some complex formation could be detected at a 2:1 molar ratio of inhibitor to enzyme. However, degradation of inhibitor and/or complex was also apparent presumably because of slower inactivation of the enzymes tested.

Although papain is known to readily convert human plasma α₁-PI into a modified form by cleavage of the methionine 358/serine 359 peptide bond (20), the presence of a cysteine residue in the P₁ position of this inhibitor could have impaired papain function. However, as shown in Fig. 3, lanes 2 and 3, the cysteine mutant was converted into a lower Mr form at low enzyme:inhibitor molar ratios and completely degraded at higher molar ratios. This pattern was also observed with the plasma form of the inhibitor (Fig. 3, lanes 4 and 5).

Effect of Modification of Cysteine Residues on Complex Formation-The reason for modifying the cysteine mutant was to determine whether changing the charge on the P₁ residue would affect inhibitor specificity. Carboxymethylation of cysteine with iodoacetic acid introduced an acidic side chain reminiscent of glutamic acid while aminoethylation with 2-chloroethylamine yielded a basic side chain with a structure analogous to lysine. The carboxymethylated derivative was used to examine interactions with S. aureus V8 proteinase, an enzyme which accommodates a glutamic acid residue in its S₁ pocket (21).

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2-chloroethylamine. The former is not likely, however, since treatment of this protein with reducing agents had no effect on the migration pattern. After incubation with either porcine trypsin or plasmin (latter not shown), complex formation was readily detected with either the reduced cysteine or the aminoethylcysteine mutant (Fig. 5, lanes 3 and 5).

Association Rate Constants for Alanine, Cysteine, and Modified Cysteine Mutants with Serine Proteinases—The second-order association rates determined for the interaction of several proteinases with mutant forms of α1-PI utilized in the current study are given in Table I, together with those previously determined with the plasma form of this protein (2). Significantly, the alanine and cysteine mutants reacted rapidly with both elastases tested. However, neither was a good inhibitor of these two enzymes than the alanine mutant.

![Fig. 4. NaDodSO₄-polyacrylamide gel electrophoresis of carboxymethylcysteine 358 and oxidized cysteine 358 mutants of α1-proteinase inhibitor with S. aureus V8 protease and human neutrophil elastase. Samples of inhibitor (20 μg) were allowed to react with V8 protease or neutrophil elastase for 1 min at room temperature, and then treated as described in the legend to Fig. 2. Gel lanes are as follows: 1, carboxymethylated cysteine 358 mutant; 2, carboxymethylcysteine 358 mutant + V8 protease (81); 3, N-chlorosuccinimide-oxidized cysteine 358 mutant; 4, N-chlorosuccinimide-oxidized cysteine 358 mutant + human neutrophil elastase (5:1); 5, air-oxidized cysteine 358 mutant; 6, air-oxidized cysteine 358 mutant + human neutrophil elastase (5:1).]

As would be expected, N-chlorosuccinimide oxidation of the alanine variant had little effect on its interaction with either human neutrophil elastase or porcine pancreatic elastase while oxidation of the cysteine variant destroyed its inhibitory activity against all proteinases tested except human neutrophil elastase. Some slight inhibitory activity towards this enzyme could be measured, with the rate of interaction being approximately 3,000-fold less than the reduced cysteine variant and 34,000-fold less than that with human α1-PI.

Surprisingly, the rates of interaction of the carboxymethylcysteine 358 mutant with bovine chymotrypsin and porcine trypsin were not significantly altered by this modification. However, the modified inhibitor did react at a rate nearly 300-fold more slowly with human neutrophil elastase than the reduced cysteine 358 mutant and 3000-fold slower than human α1-PI. It also lost all of its activity towards cathepsin G, and only very slowly inactivated pancreatic elastase. Finally, it showed no effect as an inhibitor of the S. aureus V8 proteinase, as previously indicated by the gel electrophoresis profiles in Fig. 4.

Association rates of the aminoethylcysteine mutant with either human neutrophil elastase or porcine pancreatic elastase were considerably slower than those found with either the cysteine or alanine mutants, but chymotrypsin inhibition

![Fig. 5. NaDodSO₄-polyacrylamide gel electrophoresis of cysteine 358 and aminoethylcysteine 358 mutants of α1-PI with trypsin. Samples of inhibitor (20 μg) were allowed to react with porcine trypsin at a 2:1 molar ratio for 1 min at room temperature and then treated as described in the legend to Fig. 2. Gel lanes are as follows: 1, nonreduced cysteine 358 mutant; 2, reduced cysteine 358 mutant; 3, reduced cysteine 358 mutant + trypsin; 4, aminoethylcysteine 358 mutant; 5, aminoethylcysteine 358 mutant + trypsin; 6, M₉ standards.]

**Table I**  
Association rates of proteinases with α1-proteinase mutants (M⁻¹ s⁻¹)

| Enzyme                     | Plasmaᵃ | Ala-358 | Cys-358 |
|---------------------------|---------|---------|---------|
|                            | Native  | Oxidized | Native  | Oxidized | Native  | Oxidized | Carboxymethyl | Aminoethyl |
| Human neutrophil elastase | 6.5 × 10⁷ | 3.1 × 10⁴ | 6.5 × 10⁷ | 3.1 × 10⁴ | 1.2 × 10⁵ | 1.9 × 10⁵ | 2.1 × 10⁴ | 4.0 × 10⁴ |
| Porcine pancreatic elastase | 1.0 × 10⁶ | 0       | 1.3 × 10⁶ | 0       | 9.4 × 10⁶ | 1.9 × 10⁶ | 0       | 2.1 × 10⁴ |
| Bovine chymotrypsin       | 5.9 × 10⁶ | 2.4 × 10⁵ | 6.9 × 10⁶ | 2.4 × 10⁵ | 1.0 × 10⁶ | 0       | 8.5 × 10⁶ | 1.4 × 10⁶ |
| Porcine trypsin           | 4.2 × 10⁴ | 5.0 × 10³ | 6.9 × 10⁴ | 5.0 × 10³ | 5.3 × 10⁴ | 0       | 1.4 × 10³ | 4.0 × 10³ |
| Human cathepsin G         | 4.1 × 10⁵ | 6.4 × 10² | 3.5 × 10⁵ | 6.4 × 10² | 1.8 × 10⁵ | 0       | 4.2 × 10⁴ | 4.2 × 10⁴ |
| Human plasmin             | 1.9 × 10² | 0       | 1.9 × 10² | 0       | 8.1 × 10⁵ | 0       | 1.2 × 10⁵ | 1.2 × 10⁵ |

ᵃ Ref. 2.
ᵇ N-Chlorosuccinimide-treated.
rates were nearly equal. Significantly, the rates of inhibition of trypsin and plasmin were much higher than with any of the other mutants tested and far better than those obtained previously with plasma $\alpha_1$-PI. In this case the prediction that a change to a lysine-like amino acid residue in the P$_1$ position of $\alpha_1$-PI should yield a good inhibitor of trypsin-like proteinases was fulfilled.

**DISCUSSION**

Since it seems well established that: (a) the best inhibitors of serine proteinases are actually excellent substrates, the difference being in the fact that dissociation rates are extremely slow (23); (b) human $\alpha_1$-PI reacts most rapidly with neutrophil and pancreatic elastase (2); and (c) both enzymes cleave peptide bonds after methionine, valine, and alanine residues (24, 25) but not after methionine sulfoxide residues (26), the introduction of valine (11), alanine, or reduced cysteine into the P$_1$ position is not expected to have any significant effect on elastase inhibition, and this was indeed what was found. Similarly, oxidation of the valine or alanine mutants should not have affected inhibitory activity as is known to occur with either the recombinant methionine mutant or the natural plasma inhibitor (11), and this was also the case. Oxidative inactivation of the cysteine mutant could also have been predicted on the basis of dimer formation (air oxidation) or probable conversion to a cysteic acid mutant (N-chlorosuccinimide oxidation), and a marked reduction in association rates with neutrophil elastase in both cases was observed. The carboxymethylcysteine and aminoethylcysteine mutants, both of which contained charged groups, were equally ineffective as neutrophil elastase inhibitors, again in agreement with the defined substrate specificity of this enzyme.

Porcine pancreatic elastase appeared to be more readily affected by a change in the P$_1$ position than the neutrophil enzyme. Although any of these mutants in their native configuration were effective pancreatic elastase inhibitors, the effect of oxidation, carboxymethylation, or aminoethylation of the cysteine variant was to substantially reduce or eliminate $\alpha_1$-PI inhibitory activity towards this enzyme. Bovine chymotrypsin and porcine trypsin, on the other hand, were quite nonspecific in their requirements since all the mutants were reasonably effective as inhibitors of these enzymes with the exception of the oxidized cysteine mutant which was completely inactive against either enzyme.

Perhaps the most unexpected finding of this study involved the reaction between trypsin and the carboxymethylcysteine 358 mutant. Trypsin is regarded as selective for substrates and inhibitors with basic side chains in the P$_1$ site. Although there are exceptions to this rule, most notably with methionine in the P$_1$ position, we believe this is the first report of a substrate or inhibitor with an acidic side chain in P$_1$ reacting at a significant rate with trypsin. Presumably, the interaction between the carboxyl group of carboxymethylcysteine 358 in the mutant inhibitor and the carboxyl group of aspartyl 189 in trypsin which comprises the bottom of the S$_1$ pocket of this enzyme, although unfavorable, is not so great as to overcome other interactions occurring between the reactive site of the modified inhibitor and the substrate binding region of trypsin. Thus, although the P$_1$ residue usually dictates the specificity of a proteinase inhibitor other interactions can compensate for an unfavorable interaction.

None of the mutants tested were good inhibitors of cathepsin G. This enzyme appears to be controlled by the plasma inhibitor $\alpha_1$-antichymotrypsin, indicating that methionyl residues are not well tolerated by this enzyme. Furthermore, when valine, alanine, cysteine, carboxymethylcysteine, or aminoethylcysteine are present in the P$_1$ position, association rates are decreased even further. While it is certainly likely that other amino acids in $\alpha_1$-PI make important contributions to its function, the results presented here would clearly support the premise that the P$_1$ residue dictates the primary specificity of this inhibitor.

The most significant changes in inhibitor specificity were obtained when the aminoethylcysteine variant was prepared. This mutant became an excellent inhibitor of both trypsin and plasmin, relative to all of the other mutants examined and to the natural inhibitor and behaved in a similar manner to the Pittsburgh mutant (9), further supporting the importance of the P$_1$ residue in $\alpha_1$-PI. Unfortunately, the use of the carboxymethylcysteine mutant as an inhibitor of the S. aureus V8 proteinase was unsuccessful. This is probably because the carboxymethylcysteine residue was too large to fit into the binding pocket of the enzyme but also could be due to the fact that the V8 proteinase is of bacterial origin and only very distantly related to members of the chymotrypsin family (28).

Clearly, it would be important to test a mutant with glutamic acid in the P$_1$ position against this enzyme not only for the further understanding of the mechanism of action of this inhibitor but also for potential therapeutic use against S. aureus infection. Should such an alteration in specificity be successful in controlling this bacterial proteinase it may be possible to develop other mutant inhibitors directed specifically against serine proteinases elicited from pathogenic organisms, some of which may play a direct role in the development of disease states, such as septicemia.

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Reactive Site Mutations in Human α1-Proteinase Inhibitor

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