Chemical modification of human α_1 -proteinase inhibitor by tetranitromethane

Structure–function relationship

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Nitration of tyrosine residues of α_1 -proteinase inhibitor (α_1 -PI) by tetranitromethane yielded a product that maintained its inhibitory activity against trypsin but lost most of its inhibitory activity against elastase. Chemical analysis of the product showed that four out of the six tyrosine residues in α_1 -PI had been nitrated to various degrees: Tyr-38 and Tyr-297 were not nitrated, whereas Tyr-138, Tyr-160, Tyr-187 and Tyr-244 were nitrated to extents in the range 40–80%. We interpreted these data to mean that modification of these tyrosine residues decreased the association constant between α_1 -PI and the proteinases and that the decrease differs from one proteinase to the other. When either α_1 -PI–trypsin or α_1 -PI–elastase complex was nitrated, nitration took place only to a very slight extent at these latter four tyrosine residues. On the other hand, Tyr-38 and Tyr-297 underwent nitration to about 20%. We concluded that Tyr-138, Tyr-160, Tyr-187 and Tyr-244 were located on the surface of α_1 -PI that interacts with either trypsin or elastase in the formation of complexes, and were therefore protected from nitration.

INTRODUCTION

α_1 -Proteinase inhibitor (α_1 -PI) is a glycoprotein present in human plasma at a concentration of approx. 1.5 g/l. It is responsible for over 90% of the proteinase-inhibitory activity in human plasma. Experiments *in vitro* demonstrated that α_1 -PI inhibits many serine proteinases, including trypsin, chymotrypsin and elastase, by forming an inhibitor–proteinase complex at a 1:1 molar ratio (Cohen, 1973; Pannell *et al.*, 1974; Travis *et al.*, 1978). Interest in this protein heightened when it was shown that genetic deficiency of α_1 -PI is frequently accompanied by the early onset of pulmonary emphysema (Laurell & Erickson, 1963). It has been hypothesized that the major physiological function of α_1 -PI is to inhibit leucocytic elastase activity and thereby protect the lung tissues from proteolysis.

The chemical structure of this protein has been extensively investigated. The primary structure of α_1 -PI both in terms of amino acid sequence (Chan, 1982; Carrell *et al.*, 1982; Zhu *et al.*, 1987) and of the oligosaccharide structure (Hodges *et al.*, 1979; Mega *et al.*, 1980; Vaughan & Carrell, 1981; Hodges & Chan, 1982) has been reported. In addition, the gene for α_1 -PI from the baboon has been cloned and the nucleotide sequence elucidated (Kurachi *et al.*, 1981). With this structural information available, it was appropriate to inquire further into the mechanism of inhibition of proteinases by α_1 -PI and the chemistry of the complex-formation between them. By correlating the modification of specific amino acid residues at known locations in the sequence with alterations in chemical activities, it should be possible to identify those amino acid residues responsible for maintaining the biological activities of the protein molecule and the region of the protein that interacts with proteinases.

Previously it had been reported (Feste & Gan, 1981) that nitration of the tyrosine residues in α_1 -PI by tetranitromethane yielded a product that maintained its inhibitory activity against trypsin but lost its inhibitory activity against elastase. Studies described in the present paper identified four specific tyrosine residues that were located on the surface of α_1 -PI, and appeared to reside in the area where α_1 -PI interacted with either trypsin or elastase.

EXPERIMENTAL

Chemicals and reagents

The following materials were purchased from commercial sources: 3-nitro-L-tyrosine, 3-amino-L-tyrosine dihydrochloride, tetranitromethane, *N*-t-butoxycarbonyl-L-alanine *p*-nitrophenyl ester and pig pancreatic elastase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), bovine pancreatic trypsin was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), $\text{Na}_2\text{S}_2\text{O}_4$ was from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) and *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride was from Cyclo Chemical Co. (Los Angeles, CA, U.S.A.). α_1 -PI was prepared from human plasma as described previously (Shochat *et al.*, 1978). All other chemicals were of reagent-grade quality.

Enzyme assays

The concentration of active trypsin was determined by active-site titration (Chase & Shaw, 1967) with *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride as the active-site titrant. Elastase activity was determined with *N*-t-butoxycarbonyl-L-alanine *p*-nitrophenyl ester as the

Abbreviation used: α_1 -PI, α_1 -proteinase inhibitor (formerly α_1 -antitrypsin).

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substrate (Visser & Blout, 1972). The rate of release of *p*-nitrophenol was measured at 347.5 nm.

Assay for inhibitory activity against trypsin

The protein concentration of native or modified α_1 -PI was determined by amino acid analysis. A trypsin solution was prepared by dissolving approx. 12 μ g of enzyme in 1 ml of 1 mM-HCl containing 20 mM- CaCl_2 . The precise concentration of active trypsin present in this solution was determined by the active-site titration method described above. Native α_1 -PI or modified α_1 -PI (approx. 1 mg/ml) was dissolved in 0.1 M-sodium barbital buffer, pH 8.3, containing 20 mM- CaCl_2 . To determine the inhibitory activity of native or modified α_1 -PI against trypsin, 100 μ l of the α_1 -PI solution was mixed with 20 μ l of the trypsin solution of known concentration, it being made certain that excess trypsin was always present in each case. After 10 min the amount of excess trypsin that did not form a complex with α_1 -PI in the mixture was measured by the active-site titration method. From these data the specific trypsin-inhibitory activity of the native or modified α_1 -PI could be calculated.

Assay for inhibitory activity against elastase

The protein concentration of native or modified α_1 -PI was determined by amino acid analysis. A solution of elastase (approx. 1 μ g/ μ l in 50 mM-sodium acetate buffer, pH 4.0) and a solution of α_1 -PI or modified α_1 -PI (approx. 1 μ g/ μ l in 10 mM-Tris/HCl buffer, pH 8.0) were prepared. The activity of the elastase solution was assayed before and after the addition of various amounts of native and modified α_1 -PI. From these data the percentage of elastase-inhibitory activity remaining in the modified α_1 -PI after nitration was calculated.

Nitration reaction

The nitration procedure followed an established procedure (Sokolovsky *et al.*, 1966). A 10 mg portion of α_1 -PI in 10 ml of 50 mM-Tris/HCl buffer, pH 8.0, was mixed with 23 μ l of 1 M-tetranitromethane in 95% (v/v) ethanol (molar ratio of tetranitromethane to tyrosine in α_1 -PI was 17.5:1) at room temperature. Then 3 h later the reaction mixture was dialysed overnight against 10 mM- NH_4HCO_3 and freeze-dried. The freeze-dried product was dissolved in 1 ml of 50 mM- NH_4HCO_3 , pH 7.8, and applied to a Sephadex G-100 gel-filtration column. In order to convert the nitrotyrosine residues into aminotyrosine, 10 mg of the monomeric nitrated α_1 -PI from the Sephadex G-100 column was dissolved in 10 ml of 50 mM-Tris/HCl buffer, pH 8.0, to which 9.7 mg of $\text{Na}_2\text{S}_2\text{O}_4$ was added. The reduction was allowed to take place at room temperature for 15 min. Afterwards the protein solution was dialysed against 50 mM- NH_4HCO_3 at 4 °C for 2 h and freeze-dried.

Formation of α_1 -PI-trypsin and α_1 -PI-elastase complexes, and the nitration of the complexes

α_1 -PI (100 mg) and either trypsin (30 mg) or elastase (30 mg) (α_1 -PI/proteinase molar ratio was 1.5:1) were mixed in 100 ml of 50 mM-Tris/HCl buffer, pH 8.0, and incubated at room temperature for 2 min. Afterwards 1.25 ml of 0.1 M-phenylmethane + sulphonyl fluoride, a serine-proteinase inhibitor, was added, followed by the addition of 0.38 ml of 1 M-tetranitromethane in 95% ethanol. After 3 h the mixture was dialysed overnight

against 10 mM- NH_4HCO_3 containing 1 mM-phenylmethanesulphonyl fluoride, freeze-dried and chromatographed on a DEAE-cellulose (Whatman DE-52) anion-exchange column (3 cm $\times$ 30 cm).

Dissociation of the nitrated complexes

Each of the isolated nitrated complexes was dissolved in 15 ml of 5 mM-sodium phosphate buffer, pH 6.5, to which 96 μ l of 10 mM-phenylmethanesulphonyl fluoride had been added. The pH of the solution containing the complex was raised to pH 12 with 20% (w/v) NaOH solution. The dissociation of the complex was left to proceed at room temperature for 2 h. Afterwards the solution containing the dissociated products including nitrated α_1 -PI* (the * indicates nitrated α_1 -PI that was derived from the complex) was adjusted to pH 7.0 by the addition of conc. H_3PO_4 . The solution was then dialysed overnight against 10 mM- NH_4HCO_3 containing 1 mM-phenylmethanesulphonyl fluoride and freeze-dried. The freeze-dried sample was dissolved in 5 ml of 5 mM-sodium phosphate buffer, pH 6.5, and chromatographed on DEAE-cellulose (Whatman DE-52).

Isolation and purification of tyrosine-containing peptides from nitrated α_1 -PI and nitrated α_1 -PI*

There are six tyrosine and nine methionine residues in α_1 -PI. An adjacent pair of methionine residues occurs at positions 220 and 221 (Chan, 1982; Carrell *et al.*, 1982; Kurachi *et al.*, 1981). CNBr cleavage of α_1 -PI after carboxyamidomethylation yielded three large and six small fragments in addition to free homoserine (Zhu *et al.*, 1987). All tyrosine residues were contained in the three large CNBr-cleavage fragments, I (residues 64–206), II (residues 243–351) and III (residues 1–63). CNBr-cleavage fragment I was digested with *Staphylococcus aureus* proteinase, CNBr-cleavage fragment III was digested with trypsin, and CNBr-cleavage fragment II was digested with either trypsin or *S. aureus* proteinase. Procedures and results for isolation and purification of the CNBr-cleavage fragments I, II and III and the peptides from CNBr-cleavage fragment II have been published (Shochat *et al.*, 1978). Similar procedures were used for obtaining peptides derived from CNBr-cleavage fragments I and III (Zhu *et al.*, 1987). With the separation methods employed, nitrated peptides and the corresponding non-nitrated peptides were not resolved and were isolated together. Those peptide fractions that contained nitrated products were yellow in colour and could be monitored spectrophotometrically at 410 nm. From amino acid analysis of these peptides the ratio of nitrotyrosine to tyrosine could be obtained and the percentage of nitration of a specific tyrosine residue could be calculated.

Amino acid analysis

Proteins and peptides were hydrolysed in constant-boiling HCl at 110 °C *in vacuo* for 24 h. Norleucine was used as the internal standard in all analyses. Amino acid analysis was performed by using an automated amino acid analyser with a stepwise gradient of citrate buffers (Liao *et al.*, 1973). In this system both 3-nitrotyrosine and 3-aminotyrosine were eluted between phenylalanine and histidine, with the nitro derivative emerging ahead of the amino derivative. Determination of methionine sulfoxide in protein samples was carried out by the method of Shecter *et al.* (1973).

Fig. 1. Elution profile of nitrated α_1 -PI chromatographed on Sephadex G-100

The column (1 cm $\times$ 100 cm) was developed with 50 mM- NH_4HCO_3 , pH 7.8. The flow rate was 4.5 ml/h and the fraction size was 1 ml. The fractions pooled are indicated by a bar.

RESULTS

Nitration of α_1 -PI

After α_1 -PI had been allowed to react with tetranitromethane the nitrated α_1 -PI was purified by gel filtration as described in the Experimental section. The elution profile is shown in Fig. 1. Two peaks emerged, and proteinase-inhibitory activities were determined for both fractions. The sample from the first peak was devoid of inhibitory activity against either trypsin or elastase, whereas the sample from the second peak was a potent inhibitor of trypsin but inhibited elastase only minimally. Compared with non-nitrated α_1 -PI, 95% of antitryptic activity was retained upon nitration, whereas less than 5% antielastase activity could be detected. SDS/polyacrylamide-gel electrophoresis of the sample from the second peak showed that it had a similar molecular mass to the non-nitrated protein, whereas the sample from the first peak appeared to be composed of aggregated polymeric proteins. The protein in the second peak was used as the nitrated α_1 -PI for the experiments described below.

Amino acid composition of nitrated α_1 -PI revealed no significant differences in the content of any of the amino acid residues compared with the native protein except for tyrosine (cysteine and tryptophan were not determined). Approx. 50% of the tyrosine residues in the nitrated α_1 -PI appeared as 3-nitrotyrosine. Methionine sulfoxide determinations were made in the nitrated α_1 -PI sample, but none was found. When nitrated α_1 -PI was reduced

with $\text{Na}_2\text{S}_2\text{O}_4$, the product maintained its inhibitory activity against trypsin, but did not recover its inhibitory activity against elastase. The amino acid composition of the reduced product showed little change in the content of any of the amino acids except for 3-nitrotyrosine. The majority of the 3-nitrotyrosine residues (over 90%; results not shown) disappeared after reduction, with the concomitant appearance of the equivalent amount of 3-aminotyrosine.

Distribution of nitrotyrosine in nitrated α_1 -PI

The six tyrosine residues in α_1 -PI are located at positions 38, 138, 160, 187, 244 and 297 (Chan, 1982; Carrell *et al.*, 1982; Zhu *et al.*, 1987). The tyrosine-containing peptides were obtained by fragmentation of the nitrated α_1 -PI molecule with CNBr, purification of the CNBr-cleavage fragments, enzymic digestion of the CNBr-cleavage fragments with either trypsin or *S. aureus* proteinase, and subsequent purification of the peptides from each proteolytic digest. Altogether, six peptides that contained tyrosine and/or nitrotyrosine were isolated and purified. The amino acid compositions of these peptides were obtained and are shown in Table 1. From the unique compositions and the known sequence of α_1 -PI (Chan, 1982; Carrell *et al.*, 1982; Zhu *et al.*, 1987) these six peptides were identified and their putative amino acid sequences are shown below.

Residues 36–39: Ser-Leu-Tyr-Arg, a peptide isolated from CNBr-cleavage fragment III after tryptic digestion.

Residues 133–141: Asp-Val-Lys-Lys-Leu-Tyr-His-Ser-Glu, a peptide isolated from CNBr-cleavage fragment I after *S. aureus*-proteinase digestion.

Residues 153–162: Ala-Lys-Lys-Gln-Ile-Asn-Asp-Tyr-Val-Glu, a peptide isolated from CNBr-cleavage fragment I after *S. aureus*-proteinase digestion.

Residues 176–199: Leu-Asp-Arg-Asp-Ile-Val-Phe-Ala-Leu-Val-Asn-Tyr-Ile-Phe-Phe-Lys-Gly-Lys-Trp-Glu-Arg-Pro-Phe-Glu-Val-Lys-Asp-Thr-Glu-Glu-Glu, a peptide isolated from CNBr-cleavage fragment I after *S. aureus*-proteinase digestion.

Residues 243–257: Lys-Tyr-Leu-Gly-Asn-Ala-Ile-Ala-Ile-Phe-Phe-Leu-Pro-Asp-Glu, a peptide isolated from CNBr-cleavage fragment II after *S. aureus*-proteinase digestion.

Residues 291–300: Leu-Ser-Ile-Thr-Gly-Thr-Tyr-Asp-Leu-Lys, a peptide isolated from CNBr-cleavage fragment II after tryptic digestion.

Since the nitrated peptides and the corresponding non-nitrated peptides were not resolved in the separation procedures used in these experiments, the isolated peptides as shown above contained both the nitrated products and their corresponding non-nitrated counterparts. Therefore it is possible to calculate the percentage of nitration at each tyrosine position, as shown in Table 2. It is clear that the tyrosine residues in positions 38 and 297 were not nitrated at all, whereas in positions 138, 160, 187 and 244 the degree of nitration varied from 40 to 81%.

Protection from nitration in the α_1 -PI-proteinase complex

α_1 -PI formed a complex with either trypsin or elastase. In each case the reaction mixture was nitrated with tetranitromethane. Both complexes were isolated as described in the Experimental section. The elution profile

Table 1. Amino acid compositions of the six tyrosine-containing peptides of α_1 -PI

The values given are those calculated from the amino acid analysis of the peptides, with in parentheses the number of residues present in the peptides. Abbreviation: N.D., not determined.

Amino acid	Residue nos. ...	Composition (mol of residue/mol)					
		36-39	133-141	153-162	176-199	243-257	291-300
Asp			0.97 (1)	1.92 (2)	3.85 (4)	1.92 (2)	1.01 (1)
Thr					1.74 (2)	0.82 (1)	1.75 (2)
Ser	0.81 (1)	0.84 (1)					0.85 (1)
Glu		1.03 (1)	2.11 (2)	5.10 (5)	1.01 (1)		
Pro					1.01 (1)	1.02 (1)	
Gly					1.21 (1)	1.22 (1)	1.24 (1)
Ala				0.89 (1)	1.11 (1)	2.10 (2)	
Val			1.14 (1)	1.11 (1)	2.97 (3)		
Ile				0.99 (1)	0.94 (1)	0.98 (1)	1.12 (1)
Leu	1.18 (1)	1.02 (1)		1.89 (2)	1.98 (2)	1.94 (2)	
Tyr	0.99 (1)	0.32	0.21	0.42	0.58	0.99 (1)	
NO ₂ -Tyr		0.68	0.79	0.58	0.42		
Phe				3.74 (4)	1.88 (2)		
His			0.98 (1)				
Lys			2.02 (2)	2.01 (2)	3.24 (3)	1.10 (1)	1.08 (1)
Arg	1.02 (1)	2.10 (2)					
Trp	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Table 2. Nitration of tyrosine residues in α_1 -PI and in α_1 -PI-proteinase complexes

Tyrosine residue no.	Degree of nitration (%)		
	α_1 -PI	α_1 -PI-trypsin complex	α_1 -PI-elastase complex
38	0	22	22.5
138	69	0	18
160	81	12	18
187	64	0	4
244	40	0	5
297	0	22.5	21

Fig. 2. Elution profile of nitrated α_1 -PI-trypsin complex chromatographed on DEAE-cellulose (DE-52)

The column (3 cm $\times$ 30 cm) was equilibrated in 5 mM-phosphate buffer, pH 6.5, and developed with a linear 0–0.2 M-NaCl gradient. The flow rate was 42 ml/h and the fraction size was 4.5 ml. The fractions pooled are indicated by a bar.

for the isolation of the α_1 -PI-trypsin complex is illustrated in Fig. 2. A sample from the pooled fractions containing the complex was subjected to SDS/poly-

Fig. 3. Elution profile of nitrated α_1 -PI-trypsin complex after dissociation by alkali, chromatographed on DEAE-cellulose (DE-52)

The column (3 cm $\times$ 30 cm) was equilibrated in 5 mM-phosphate buffer, pH 6.5, and developed with a linear 0–0.2 M-NaCl gradient. The flow rate was 42 ml/h and the fraction size was 4.5 ml. The fractions pooled are indicated by a bar.

acrylamide-gel electrophoresis and confirmed to have a molecular mass of 75000 Da, as expected. After dissociation in alkali, nitrated α_1 -PI* from each complex was obtained as described in the Experimental section. The elution profile of isolation of the nitrated α_1 -PI* from nitrated α_1 -PI-trypsin complex is shown in Fig. 3. A sample from the pooled fractions that were presumed to contain nitrated α_1 -PI* was subjected to SDS/polyacrylamide-gel electrophoresis and confirmed to have a molecular mass slightly smaller than that of native α_1 -PI. The amino acid compositions of nitrated α_1 -PI* derived from the α_1 -PI-trypsin and α_1 -PI-elastase complexes indicated that 16% and 23% respectively of the tyrosine residues appeared as 3-nitrotyrosine.

The distribution of nitrotyrosine in these samples was determined as in the case of the nitrated α_1 -PI, and the results are shown in Table 2. It is obvious that both trypsin and elastase afford great protection against

nitration of α_1 -PI in the complexes, as the four tyrosine residues positioned at 138, 160, 187 and 244, which were nitrated in the native α_1 -PI, revealed minimal nitration in α_1 -PI* derived from either the α_1 -PI-elastase or the α_1 -PI-trypsin complex. In contrast, at residues 38 and 297, where no nitration was observed in nitrated native α_1 -PI, approx. 20% nitration was found.

DISCUSSION

The reaction of α_1 -PI with tetranitromethane under the prescribed experimental conditions resulted in the nitration of approx. 50% of the tyrosine residues of the protein. The protein α_1 -PI consists of a single polypeptide chain with six tyrosine residues, located at positions 38, 138, 160, 187, 244 and 297. Nitrotyrosine was present in four of these positions, but the residues located closest to the C- and N-termini of the protein were not modified. These data indicated that the tyrosine residues in positions 138, 160, 187 and 244 were exposed and accessible to nitration, whereas the tyrosine residues in positions 38 and 297 probably were less exposed and thus protected from nitration.

Inhibition of serine proteinases by α_1 -PI is via a complex-formation between the proteinase and the inhibitor protein. The complex is quite stable even in the presence of denaturing agents (for review see Travis & Salvesen, 1983). Beatty *et al.* (1980) have investigated the association constant, K_A , of α_1 -PI with different proteinases and found that the rate of complex-formation varies more than a million-fold between the slowest and the most rapid combinations. The K_A values between α_1 -PI and pig elastase and pig trypsin respectively were reported to be $1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $4.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Upon oxidation with *N*-chlorosuccinimide, the K_A values between oxidized α_1 -PI and pig elastase and pig trypsin respectively were decreased to 0 and $5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. A previous report by Johnson & Travis (1979) has demonstrated that two specific methionine residues in α_1 -PI were oxidized in the *N*-chlorosuccinimide-oxidized α_1 -PI molecule and that the oxidized α_1 -PI molecule lost its antielastase activity but maintained essentially full antitryptic activity in an assay system (Chase & Shaw, 1967; Visser & Blout, 1972) where the small-molecular-mass substrate was introduced minutes after α_1 -PI and the proteinases had been mixed. These data amply demonstrated that oxidation of specific methionine residues of α_1 -PI affected the K_A of the inhibitor with both elastase and trypsin. In the former case the K_A was decreased to such a low value that little complex-formation could be achieved and thus no antielastase activity could be demonstrated. The results reported in the present paper showed that nitrated α_1 -PI maintained its inhibitory activity against trypsin but lost its activity against elastase, which is similar to the case of oxidized α_1 -PI. Even though K_A measurement was not made in the present study, it is reasonable to assume that nitration of the inhibitor molecule must have affected the K_A between the modified inhibitor and both the proteinases, as in the case of oxidized α_1 -PI. The basis of the differential effect probably is related to the differences in the specific amino acid residues present on the surface of the two proteinases to which the inhibitor molecule is bound.

It was necessary to confirm that the modification of biological activity of α_1 -PI by tetranitromethane was due

to nitration of tyrosine residues but not to oxidation of methionine residues. This possibility was eliminated by the following experimental evidence described in the Results section. First, methionine sulfoxide was not detected in the nitrated α_1 -PI sample. Secondly, when the nitrated α_1 -PI was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ the resulting product did not regain any of its elastase-inhibitory activity. In contrast, when α_1 -PI was oxidized with *N*-chlorosuccinimide, the oxidized α_1 -PI molecule was shown to regain over 80% of its antielastase activity when it was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ to regenerate methionine from methionine sulfoxide (S. Mierzwa & S. K. Chan, unpublished work). Another possibility that should be considered is whether nitration of α_1 -PI causes conformational changes of the protein, which, in turn, result in inactivation. This is considered unlikely, since nitrated α_1 -PI maintains almost full antitryptic activity. On the other hand, increasing the amount of tetranitromethane used in the nitration experiment 5-fold did result in a protein product without any inhibitory activity against either trypsin or elastase, indicating that extensive nitration could denature α_1 -PI (S. Mierzwa & S. K. Chan, unpublished work).

In order to obtain a nitrated product that would lose its inhibitory activity against elastase but maintain its inhibitory activity against trypsin, the experimental conditions reported in the present paper were chosen even though the nitrated product was not homogeneous and the four tyrosine residues were nitrated to different extents from 40 to 80%. Because of the heterogeneity of the products, it is not possible to ascertain whether only one or a combination of two or three of the four tyrosine residues was essential for α_1 -PI's interaction with the proteinase. However, if the four tyrosine residues were located in the area of α_1 -PI interacting with the proteinases, the modification of any of these residues might well interfere with the association to the proteinase molecules.

The hypothesis that these four specific tyrosine residues accessible to nitration were located in the area of the inhibitor molecule associating with the proteinase in a complex is consistent with the experimental results. When the inhibitor-trypsin and the inhibitor-elastase complexes were allowed to react with tetranitromethane, and the nitrated α_1 -PI* was recovered after the complex was dissociated in alkali, it was found that tyrosine residues 138, 160, 187 and 244 of the inhibitor protein were only slightly nitrated, suggesting that these residues were exposed in the native molecule but protected in the complex with either trypsin or elastase. These data demonstrate that the same or nearly the same surface of the inhibitor protein was involved in forming the complex with either proteinase. This interpretation is not necessarily in conflict with the data showing nitrated α_1 -PI possessing antitryptic activity but not antielastase activity. As pointed out earlier in this discussion, nitration of α_1 -PI decreased to a different degree the K_A between the inhibitor and the two proteinases. We believe that the differential effect is dictated by the differences in the chemical structure of the contact areas of the two proteinases.

It should be pointed out that our interpretation of these results is consistent with the three-dimensional structure of the proteolytically modified α_1 -PI (Löbermann *et al.*, 1984), where it is shown that Tyr-160 and Tyr-187 are very closely associated (O_{O} distance

0.49 nm) and they are also close to Tyr-138 ($C_{(\alpha)}$ -138- $C_{(\alpha)}$ -187 distance 1.3 nm), whereas Tyr-244 is farther away.

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