HUMAN ALPHA-1-PROTEINASE INHIBITOR MECHANISM OF ACTION: EVIDENCE FOR ACTIVATION BY LIMITED PROTEOLYSIS

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Summary: Human plasma alpha-1-proteinase inhibitor (α_1-antitrypsin) has been re-isolated from its complex with porcine trypsin. The re-isolated protein (α_1-PI*) was found to be non-inhibitory and 8,000 lower in molecular weight than the native inhibitor. Sequence analysis of α_1-PI* showed that an amino terminal peptide had been lost, apparently the result of cleavage at a Lys-Thr bond. These data indicate that limited proteolysis is the first step in the inhibitory mechanism.

INTRODUCTION

The role of human α_1-proteinase inhibitor (α_1-PI), formerly called α_1-antitrypsin, in controlling tissue destruction by endogenous serine proteinases is well-known (1). The mechanism by which proteinase inactivation occurs is, however, not yet understood, although stable complexes between inhibitor and trypsin, chymotrypsin, or elastase are readily detected (2,3,4). In an attempt to clarify the steps involved during inactivation of serine proteinases we have re-isolated the inhibitor (α_1-PI*) from complexes formed with porcine trypsin. In this report we present certain of the properties of α_1-PI* which suggest a possible mechanism for the initial interactions of inhibitor with serine proteinases.

Abbreviations: α_1-PI, α_1-proteinase inhibitor; α_1-PI*, re-isolated post-complex α_1-proteinase inhibitor; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Sephadex G-75 was the product of Pharmacia Fine Chemicals. Benzamidine hydrochloride was purchased from Pfaltz and Bauer, while hydroxylamine hydrochloride was obtained from Fisher. Bio-Rad acrylamide reagents were used in electrophoresis. Carboxypeptidases A and B, which had been treated with diisopropyl fluorophosphate, were supplied by Worthington. The trypsin substrate Bz-L-Arg-OEt was purchased from Sigma. All other chemicals were of reagent grade or equivalent.

Human α_1-PI was prepared and assayed as previously described (5). Porcine trypsin was isolated by affinity chromatography on Sepharose-Trasylol (6). Electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborn (7). The method of Ambler (8) was followed for digestions with carboxypeptidases. Sequential degradation of α_1-PI and α_1-PI* was performed in a Beckman Model 890C sequencer, using the 0.1 M Quadrol program of Brauer et al. (9). The residues were identified by amino acid analysis after back hydrolysis of the thiazolinones to the free amino acids at 150° with 6 N HCl, 0.1% $SnCl_2$ for 4 hr in vacuo according to Mendez and Lai (10). We found this method superior to HI hydrolysis in that only Cys is destroyed. The residues of the native inhibitor were additionally identified by mass spectrometry of the PTH-amino acids (11).

RESULTS

Trypsin-inhibitor complex was prepared by the addition of 22.9 mg porcine trypsin (65 ml, 1 mM HCl) to 30 mg α_1-PI (31.3 ml 0.03 M sodium phosphate buffer pH 6.5 to which 4 ml of 1 M Tris-HCl pH 8.0 had been added) with rapid stirring at 20°C. Assay of the inhibitor and trypsin solutions prior to mixing had shown that this would result in a slight excess of inhibitor (288 inhibitory units vs 284 trypsin units). After the trypsin was added 0.2 ml of the mixture was assayed and found to be free of trypsin

esterase activity. One minute after the addition of trypsin solid benzamidine HCl was added to make the solution 0.25 M with respect to this strong competitive inhibitor of trypsin. The pH of this solution was 7.9. Previous studies had shown that benzamidine and other nucleophiles such as hydroxylamine would dissociate the complex, but benzamidine was chosen to ensure against proteolysis. The benzamidine treated complex was incubated overnight at 37°C. The mixture was then concentrated to 10 ml using an Amicon UM-10 membrane, before it was chromatographed on Sephadex G-75 equilibrated with 0.05 M Tris-HCl pH 8.0 at 4°C. The first peak of the chromatographic profile shown in Figure 1 is α_1-PI* and the second peak is porcine trypsin. Although α_1-PI* was no longer inhibitory, even when pre-incubated with porcine trypsin for 18 hours, the latter had not changed in specific activity during the interaction with and dissociation from the inhibitor.

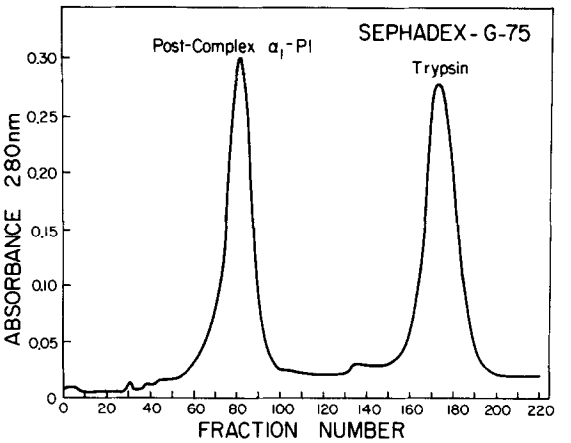

Fig. 1. Chromatographic separation of α_1-PI* and porcine trypsin on Sephadex G-75 (4 x 90 cm; 0.05 M Tris-HCl pH 8.0), after dissociation of the complex with benzamidine. The first 200 ml of effluent was collected batch-wise prior to fraction No. 1 and 4 ml fractions were taken at 10 min. intervals.

Gel electrophoresis of both α_1-PI and α_1-PI* after SDS treatment either in the presence or absence of reducing agents (Fig. 2) indicated a significantly lower molecular weight for α_1-PI* (45,000 vs 53,000 for α_1-PI).

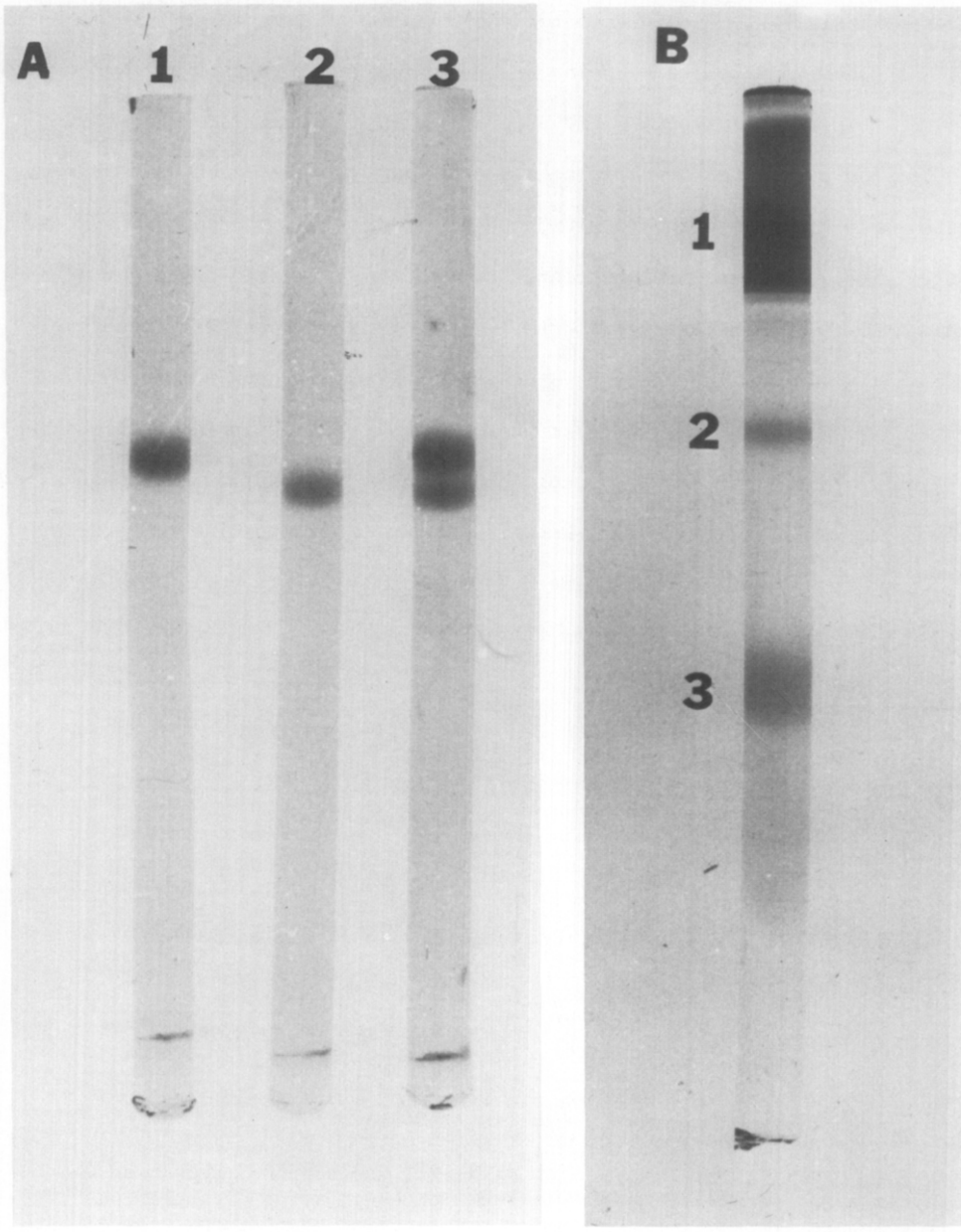

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (A) 7½% gels; (1) α_1-PI; (2) α_1-PI*; (3) α_1-PI and α_1-PI*. (B) 15% gel, complex was prepared with excess α_1-PI and treated with SDS prior to electrophoresis; (1) α_1-PI and complex; (2) fraction of trypsin that was inactive; (3) 8,000 molecular weight peptide.

Additionally, gel electrophoresis of the α_1-PI porcine trypsin mixture after SDS treatment, indicated a component with a molecular weight near 8,000. The latter was only demonstrable when 15% gels were used and the protein bands fixed in 20% sulfosalicylic acid prior to staining.

Since peptide cleavage in the inhibitor must have occurred during interaction with the enzyme, sequence studies were initiated on α_1-PI and α_1-PI* at both the amino and carboxy terminals. Digestions of the two proteins with either carboxypeptidase A or B as well as with mixtures of A and B indicated that both proteins had seryl-lysine at their carboxy terminus. Evidently, no alteration in structure had occurred in this area as a consequence of complex formation.

The amino terminal sequence of both proteins was determined by repetitive Edman degradation on 10 mg samples, and the sequence of the first ten residues of each protein is given in Table I. The dramatic difference in the sequences clearly demonstrates that an amino terminal peptide has been removed from the native inhibitor.

TABLE 1

AMINO TERMINAL SEQUENCE OF NATIVE AND POSTCOMPLEX α_1PI

	1	2	3	4	5	6	7	8	9	10
NATIVE	GLU	ASP	PRO	GLU	GLY	ASP	ALA	ALA	GLU	ASP
α_1-PI*	THR	ILE	THR	PRO	GLU	VAL	LYS	PHE	ASP	LYS

DISCUSSION

The evidence presented clearly demonstrates that a peptide bond in α_1-PI is hydrolyzed upon its interaction with trypsin. The bond cleaved is

on the amino terminal side of a Thr residue, and probably on the carbonyl side of a Lys residue since modification of the Lys residues of α_1-PI abolishes all inhibitory activities (12). Proof of this will lie in the isolation of the peptide and its carboxy terminal sequence.

The cleavage site apparently represents a trypsin sensitive bond and not the true inhibitory site, since the released peptide can be seen on a 15% SDS gel along with the inhibitor-trypsin complex. Recent experiments with human liver cathepsin B, a thiol- enzyme known not to be inactivated by α_1-PI (13) show that the enzyme does, however, cleave a peptide bond in this molecule, reducing its molecular weight and also inactivating the inhibitor (J. Travis and A. J. Barrett, unpublished observations).

From the results presented herein, it appears that α_1-PI exists as a pro-inhibitor which is activated by limited proteolysis. As a consequence of this activation the inhibitor "traps" the activating proteinase by forming a stable complex, presumably by acylation of the serine hydroxyl of the enzyme to a carboxyl group of the inhibitor. The latter is in complete agreement with the stability of the α_1-PI proteinase complex, which is stable to boiling in 1% SDS, but readily dissociates in the presence of strong nucleophilic agents such as benzamidine and hydroxylamine. However, it is possible that the complex exists as a tetrahedral intermediate which decays to an ester bond upon denaturation.

Several of the plasma proteinase inhibitors may function via similar mechanisms, because antithrombin III (14) and α_1-antichymotrypsin (D. Garner of this laboratory, unpublished results) also form SDS stable complexes, which dissociate when treated with hydroxylamine. However, α_1-PI is the first of these in which limited proteolysis is known to play a role.

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