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ON THE PROPERTIES OF PORCINE ELASTASE RELEASED FROM ITS COMPLEX WITH HUMAN ALPHA-1-ANTITRYPSIN BY ALKALINE CLEAVAGE

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SUMMARY

These investigations were made to determine how the elastase released from its complex with alpha-1-antitrypsin at high pH is modified. Most of the elastase component precipitates on returning the pH to neutral. The elastase component and the native enzyme subjected to the same conditions of pH and temperature reacted to approximately the same extent with radioactively labeled diisopropyl fluorophosphate. There were about two moles of dehydroalanine per mole of enzyme either in the presence or absence of complex formation. Thus, the enzyme is either still capable of reacting with diisopropyl fluorophosphate or it is denatured and thus inactivated by partial conversion of cystine residues to dehydroalanine. Anhydroelastase is apparently not formed during cleavage of the complex.

INTRODUCTION

Data from this and other laboratories suggest that alpha-1-antitrypsin combines with serine proteases during the inhibitory process to form stable complexes which may be tetrahedral adducts or acyl esters representing a 1:1 molar combination between specific aminoacyl carbonyl moieties of the inhibitor and the active site serine hydroxyl group of the enzymes (1-4).

The experimental techniques used in the present study were carried out to investigate the chemical state of the active site serine moiety of the enzyme after alkaline cleavage of the purified alpha-1-antitrypsin-elastase complex (3). The released elastase component was isolated and compared with the native and DFP-inhibited enzyme. Since the alkaline conditions utilized for cleavage of the complex are analogous to the cleavage of

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Abbreviations used are: DFP, diisopropyl fluorophosphate; [^{32}P]-DFP, [^{32}P]-labeled diisopropyl fluorophosphate; β-NADH, β-nicotinamide-adenine dinucleotide, reduced form; LDH, lactate dehydrogenase; SDS, sodium dodecyl sulfate.

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tosylated or phenyl methane sulfonylated serine proteases at high pH (5, 6) with resultant conversion of the serine moiety at the active site of the enzyme to dehydroalanine, we examined the possibility that the elastase component released from the complex is "anhydroelastase". The data indicate that release of the elastase at high pH entails modification of amino acids other than the serine residue at the active site, resulting in sufficient change in properties to bring about its denaturation.

METHODS

Purified human plasma alpha-1-antitrypsin of normal phenotype MM was obtained as previously described (7). The purified inhibitor exhibited a single band (54,000 daltons) on polyacrylamide gel electrophoresis in SDS , formed a single precipitin arc in double immunodiffusion against antisera (Behring Diagnostics, Somerville, N.J.) to human alphal-antitrypsin or whole human serum, and completely inhibited porcine pancreatic elastase at a molar ratio of inhibitor to enzyme of 2.2:1 or greater (3) when tested in the pH stat (Radiometer Co., Copenhagen, Denmark) using N-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Calbiochem, San Diego, Cal.) as substrate (8). Porcine pancreatic elastase was isolated from an acetone powder (Sigma Chemical Co., St. Louis, Mo.) by the method of Shotton (9), and was treated for removal of contaminating tryptic and chymotryptic activities (10). The purified enzyme preparation was 94.5% active by active site titration using p-nitrophenyl-3-(N-acetyl-L-alanyl-L-alanyl)-2-methylcarbazate (11) and exhibited a single band (26,400 daltons) during SDS gel electrophoresis.

The complex between alpha-1-antitrypsin and elastase with a molecular weight of 74,100 was formed, utilizing a 3-fold molar excess of inhibitor, and the purified complex was subjected to alkaline cleavage (pH 12, 2 hr, room temp.) to obtain the free inhibitor and elastase components (3). Thus, the pH of a solution of 13.6 mg of complex in 1 ml of 0.1 M Tris-HCl, pH 7.6, containing 3 mM sodium azide (to which had been added a 100-fold molar excess of DFP over the enzyme in the complex), was raised to pH 12 using 10 N NaOH. After 2 hr at room temperature DFP was again added in the same amount and the pH was returned to 7.6 using concentrated HCl. Most of the released enzyme precipitated on return of the pH to 7.6. An aliquot of the washed, uniformly suspended precipitate was dissolved in 0.1 M Tris-HCl, pH 7.6, containing 8 M urea, and the absorbance at 280 nm was measured. The amount of protein thus determined was 3.1 mg, representing 65% of the total theoretical amount of elastase in the purified complex prior to cleavage. The dissolved protein had a single band on SDS polyacrylamide gels, corresponded to a molecular weight of 26,400 and had amino-terminal valine (3). This material is referred to herein as "elastase component" of the complex. Control samples of elastase used in the methods of analysis described below were as follows: (a) elastase dissolved at 5 mg protein/ml in 0.1 M sodium phosphate, pH 8.0, at 5°, then reacted with a 100-fold molar excess of DFP at room temperature for 3 hr (DFP-inhibited elastase) and (b) elastase dissolved in the same way in 0.1 M Tris-HCl, pH 7.6, containing 3 mM sodium azide, but not treated with DFP (native elastase).

The presence of dehydroalanine in the proteins tested was determined as described elsewhere after first converting dehydroalanine to pyruvate (5, 12). Samples for analysis were lyophilized, dissolved in 1 ml of 3 N HCl, and heated at 100°C for 1 hr. Approximately 250 µl of 10 N NaOH was added to

bring the pH to 7.6 and the total volume was brought to 2.3 ml with 0.1 M sodium phosphate, pH 7.6. Then 0.1 mg (0.1 ml) of β-NADH (Sigma Chemical Co.) in phosphate buffer was added, followed by 0.1 ml (0.02 mg) of LDH , No. L-2500 (Sigma Chemical Co.). After 5 min at room temperature the change in absorbance following addition of LDH was measured at 340 nm. A standard curve was made using sodium pyruvate (Sigma Chemical Co.) in amounts of 0.8 µg to 8 µg (calculated as pyruvic acid) per assay cuvette.

In experiments involving use of [^{32}P]-DFP the solvent-free material (The Radiochemical Centre, Amersham, England) was first dissolved in propylene glycol at a concentration of 0.01 M. A 10-µl aliquot of this solution contained 5.44 x 10^5 dpm. Combinations of 3 mg of alpha-1-antitrypsin and 0.5 mg of elastase or 0.5 mg of elastase (each test sample in a total volume of 1.8 ml of 0.1 M Tris-HCl, pH 7.6, containing 3 mM sodium azide), under specified conditions of pH, temperature and time, were treated with 0.2-ml aliquots of the [^{32}P]-DFP solution (100-fold molar excess of DFP over enzyme). Unbound radioactivity was removed from all samples by extensive dialysis at 5° in 1 mM HCl.

Polyacrylamide gel electrophoresis (7.5% acrylamide) in SDS was carried out by the method of Weber & Osborn (13). Radioactive samples were dissolved in 10 ml of Biofluor scintillation liquid (New England Nuclear, Boston, Mass.). Radioactivity was then measured in a Searle Isocap 300 liquid scintillation spectrometer by repeatedly monitoring until the counting rate was stable. Correction for quenching was made using the external standard ratio method.

RESULTS AND DISCUSSION

The quantitation of dehydroalanine residues was carried out for the elastase component derived from the alpha-1-antitrypsin-elastase complex, native elastase and DFP-inhibited elastase, and mixtures of elastase/DFP-inhibited elastase and alpha-1-antitrypsin (Table I). The same quantity of pyruvate was detected in both elastase inhibited by DFP and treated at pH 12, the native enzyme treated at pH 12, and the elastase released from alpha-1-antitrypsin (see Group 1, Table I). The data for Group 2 of Table I show that exposure of DFP-inhibited elastase to alpha-1-antitrypsin results in the occurrence of the same amount of dehydroalanine as for the enzyme fully inhibited by alpha-1-antitrypsin and then released.

Since the above data did not prove whether the active site serine residue of elastase had undergone modification in the alkaline medium, whether or not first reacted with alpha-1-antitrypsin, use was made of [^{32}P]-DFP. This inhibitor reacts exclusively and stoichiometrically with the active site serine moiety of elastase (14) and other serine proteases. Combinations of 3 mg of alpha-1-antitrypsin and 0.5 mg of elastase or 0.5

TABLE I

TEST FOR PRESENCE OF DEHYDROALANINE IN ELASTASE RELEASED FROM ALPHA-1-ANTITRYPSIN-ELASTASE COMPLEX[a]

Test Material	Treatment	Pyruvate (nanomoles)	Nanomoles Pyruvate / Nanomole Elastase
Group 1			
Elastase component	pH 12 (2 hr)	32.2	1.70
DFP-inhibited elastase	"	32.6	1.72
Native elastase	"	31.8	1.68
Group 2			
Native elastase + Alpha-1-antitrypsin	pH 12 (2 hr)	65.2	1.71[b]
DFP-inhibited elastase + Alpha-1-antitrypsin	"	65.8	1.74[b]
Alpha-1-antitrypsin	"	32.9	--

[a] All results based on analysis of 3.0 mg of inhibitor and/or 0.5 mg enzyme protein.

[b] Values were calculated for the enzyme, assuming amount of pyruvate in alpha-1-antitrypsin of mixture equalled that of alpha-1-antitrypsin alone.

mg of elastase in 1.8 ml of 0.1 M TrisHCl, pH 7.6, containing 3 mM sodium azide, were treated with 0.2-ml aliquots of the [^{32}P]-DFP solution in propylene glycol (see "METHODS"). DFP was added in 100-fold molar excess over the enzyme in each sample. Five variations were studied (see Table II). In the first sample, alphal-antitrypsin and elastase were combined in a molar ratio of 3:1. After 30 min at room temperature the [^{32}P]-DFP was added and the pH was raised to 12 for 2 hr and returned to 7.6 as described above. In the second sample [^{32}P]-DFP-inhibited elastase was incubated with alpha-1-antitrypsin for 30 min at room temperature, followed by the same steps as for the first sample. In the third sample, native elastase was exposed at pH 12 for two hr, following which [^{32}P]-DFP was added and incubation was continued for two hr. The pH was then returned to 7.6. In

TABLE II

REACTIVITY OF THE ACTIVE SITE SERINE MOIETY OF ELASTASE UNDER DIFFERENT CONDITIONS[a]

Sample No.	Test Material	Treatment	Mole [^{32}P]-DFP/Mole Elastase
1	Native elastase + Alpha-1-antitrypsin	[^{32}P]-DFP, pH 12, 2 hr.	0.56
2	[^{32}P]-DFP-inhibited + Alpha-1-antitrypsin	pH 12, 2 hr	0.96
3	Native elastase	pH 12, 2hr→[^{32}P]-DFP, pH 12, 2 hr	0.42
4	Native elastase	[^{32}P]-DFP, pH 12, 2 hr	0.39
5	[^{32}P]-DFP-inhibited elastase	----------	1.0

[a] All results based on analysis of 3.0 mg of inhibitor and/or 0.5 mg of enzyme protein.

the fourth sample native elastase was treated with [^{32}P]-DFP at pH 12 for two hr and the pH was returned to 7.6. The fifth sample was native elastase treated with [^{32}P]-DFP only. Unbound radioactivity was removed by extensive dialysis in 1 mM HCl at 5°C.

The results of this experiment are presented in Table II. Elastase split from alpha-1-antitrypsin during the exposure to [^{32}P]-DFP at high pH (sample 1) contained about one-half mole of DFP per mole of enzyme. Native elastase alone (samples 3 and 4) was similarly labeled. However, elastase first reacted with [^{32}]P-DFP (samples 2 and 5) contained one mole of DFP per mole of enzyme. These data indicate that elastase removed from alpha-1-antitrypsin reacts in the same way as native elastase under the same conditions and that about one-half of the enzyme molecules still contain a reactive active site serine moiety. The half of the elastase molecules which failed to bind to DFP have therefore probably been inactivated by the changes induced by high pH. The elastase component represented in

Table I would be expected to have the same DFP content as that in the first test sample of Table II, and it is thus evidence that chemical changes in the molecule occurred at loci other than the active site serine moiety.

Strumeyer et al. (15) and Weiner et al. (5) obtained clear chemical evidence for the specific conversion of the active site serine moiety of serine proteases to dehydroalanine by alkaline cleavage of the enzymes first inactivated with organic chemical agents. Although Weiner et al. (5) showed a 1:1 molar occurrence of dehydroalanine in anhydrochymotrypsin produced by base treatment of the tosylated enzyme, a significant amount of dehydroalanine (0.6 mole/mole enzyme) was found in the uninhibited, base-treated enzyme. Similar results were obtained by Ako et al. (6) for base-treated trypsin. Recent observations on the effect of alkaline treatment of proteins in general provide the basis for the most likely explanation of our observed denaturation of elastase. Thus, Nashef and collaborators (16) showed that alkaline treatment of proteins can lead to the formation of dehydroalanine from cystine residues. Porcine elastase is known to contain four disulfide bonds (9) and it is thus quite possible that alteration of a part of these residues gave rise to the dehydroalanine levels observed. Furthermore, since cysteine (16) can also give rise to dehydroalanine under alkaline conditions, the single cysteine residue known to be present in alpha-1-antitrypsin (17) could have been partially modified and given rise to the result for the alpha-1-antitrypsin control in Table I.

About one-half of the elastase which separates from the alpha-1-antitrypsin-elastase complex at high pH is capable of reacting with DFP. Therefore, at least one-half of the enzyme so derived has an intact active site. Another change which was found in the elastase, the formation of dehydroalanine residues, also occurred in the DFP-inhibited and native elastase when exposed to high pH. Therefore, the same quantity

of dehydroalanine is formed at high pH, even in the absence of an intact enzyme active site. Finally, native elastase treated with DFP at high pH undergoes the same changes in DFP incorporation, dehydroalanine formation, and catalytic inactivation undergone by the elastase which is split from the complex. These observations suggest that elastase is split from the complex in an active form. It then undergoes at least two reactions. Some of it is modified via conversion of cystine residue to dehydroalanine and some of it is still capable of reacting with DFP.

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