Further Studies on Elastase-Like Esterases in Human Leukocyte Granules[1] (35424)

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In an earlier report (1), we showed that a lysosome fraction of human peripheral blood leukocytes contained neutral esterase activity against tertiary butyloxycarbonyl (*t*-BOC)-*l*-alanine *p*-nitrophenol. Because alanine esters are considered specific substrates for elastase (2, 3), the foregoing observation complemented our previous finding of neutral elastoproteolytic activity in these same granules (4). Human leukocyte elastoprotease has since been implicated in the pathogenesis of several inflammatory diseases (5, 6). It therefore seemed worthwhile to extend our initial investigation of the elastase-like esterase(s) in human leukocyte granules. Accordingly, additional granule fractions have been prepared and tested for hydrolytic activity against a second, more specific elastase substrate, *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester (3). Second, isoelectric-focusing experiments have been carried out in two different supporting media to determine the isoelectric pH of the alanine *p*-nitrophenyl esterase. Third, commercially available *t*-BOC-*l*-alanine and *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine (corresponding to the prodcuts of hydrolysis of their respective esters) have been tested as inhibitors of proteolysis by leukocyte granules. The present communication describes the results of each of these studies.

Materials and Methods. Leukocyte granule extracts and fractions. Lysosome-enriched granule fractions were prepared by differential centrifugation of human peripheral blood leukocytes, and the water-soluble proteins of the granules were extracted by freeze-thawing in sodium phosphate-buffered saline at pH 7.0. These methods have been described in detail elsewhere (4). For the isoelectric-focusing experiments, a chromatographic subfraction of the granule extract was employed in which the sp act of the alanine *p*-nitrophenyl esterase had first been enhanced about 5-fold. Preparation of this material (referred to below as the "Sephadex-fraction") has also been described in a previous report (5).

Substrates and purified enzymes. The *t*-BOC-*l*-alanine *p*-nitrophenol, *t*-BOC-*l*-alanine, *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester, and *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine were purchased from the Cyclo Chemical Corp., Los Angeles, Calif. Elastase [chromatographically purified pancreatopeptidase E, (EC 3.4.4.7)] was obtained from Worthington Biochemical Corp., Freehold, N.J.

Isoelectric-focusing procedures. Isoelectric-focusing of granule proteins was carried out in two different supporting media. In the first procedure, a modification of that described by Awdeh *et al.* (7), 5% acrylamide gels (19 parts acrylamide:1 part *N,N'*-methylenebisacrylamide:380 parts H_2O) were used to stabilize a 1% solution of ampholytes with a pH range of 3–10 (Ampholine Carrier, LKB Instrument Co., Rockville, Md.). The gels were cast in Leucite tubes 22 cm long with a 0.6-cm i.d., using tetramethylethylenediamine and ammonium persulfate to catalyze the polymerization. One percent phosphoric acid was used as anodal electrolyte, and 1% ethanolamine as the cathodal electrolyte solution. Forty μg of granule proteins (Sephadex fraction) in 10% sucrose were layered under the anodal electrolyte at the top

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of the gel. Focusing was carried out for 20 hr at 4° with a starting current of 3.5 mA/tube. At equilibrium, this value had decreased to 0.25 mA/tube. At the end of the isoelectric-focusing procedure, protein distribution in the sample-containing gel was determined by UV-scan at 280 nm. The sample containing gel was then cut into 0.2-cm thick slices, and the latter were incubated in 1.15 ml of 0.05 *M* sodium phosphate buffer (pH 7.4) containing 0.2 m*M* *t*-BOC-*l*-alanine *p*-nitrophenol. After 1 hr at 4°, absorbance of *p*-nitrophenol cleaved from the substrate by enzyme present in the gel was measured spectrophotometrically at 347.5 nm. Duplicate gels containing ampholyte mixture but not sample protein, were also exposed to isoelectric-focusing. These gels were subsequently used to determine the pH gradient at equilibrium. For this purpose, 0.5-cm thick slices were cut and eluted for 1 hr in 1.0 ml of water and the pH of the eluates then was measured.

The second isoelectric-focusing procedure was performed on a preparative scale using the LKB 8101 column (LKB Instrument Co., Rockville, Md.) and following the instructions supplied by the manufacturer. The gradient used was from pH 7–10 and was selected on the basis of the results obtained in the smaller scale, acrylamide gel-supported, isoelectric-focusing procedure just described. A 400-μg protein sample (Sephadex fraction) containing 0.16 n*M* of total inorganic salts was placed on the column in the middle of the sucrose-gradient. Isoelectric focusing was carried on in 1% ampholyte for 40 hr at 4°, and at 600 V and 1.0 mA of current (equilibrium values). Fractions were then collected as follows: four 1.5-ml fractions to eliminate the bulk of the dense electrode solution in the extreme lower region of the column, sixty-five 0.25-ml fractions corresponding to the gradient zone extending from 11.3 to 9.0 (pH values greater than 10 were due to mixing with dense electrode solution), and fifty 1.25-ml fractions which essentially exhausted the remainder of the gradient. The pH of the fractions was measured directly. The *t*-BOC-*l*-alanine *p*-nitrophenyl esterase in the fractions was determined by mixing 50-μl aliquots with 1.5 ml of 0.2 m*M* substrate in 0.05 *M* sodium phosphate buffer (pH 7.4) and measuring absorbance of liberated *p*-nitrophenol after 10 min of incubation at room temperature.

Assays. Hydrolysis of *t*-BOC-*l*-alanine *p*-nitrophenol was measured according to Visser and Blout (2), as modified by Janoff (1). Additional changes were introduced in the assay of the isoelectric-focused granule fractions as described above.

Measurement of *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester hydrolysis was carried out essentially as described by Gertler and Hofmann (3) with the following modifications: The final substrate concentration in the reaction vessel was 0.004 *M* (about 10-fold greater than the K_m reported for the hydrolysis of this ester by pancreatic elastase). The substrate was dissolved in 0.002 *M* Tris buffer adjusted with HCl to pH 8.0 (at 25°) and containing 0.05 *M* KCl. Measurements were made with a TTT1A Titrator pH Stat and an SBR2C Titrigraph recorder (Radiometer A/S, Copenhagen, Denmark).

Digestion of denatured hemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio), was monitored by the method of Anson (8) as modified by Press *et al.* (9). Protein was determined according to Lowry *et al.* (10) using crystallized bovine serum albumin (Pentex, Inc., Kankakee, Ill.) as reference standard.

Results and Discussion. Esterolysis. Figure 1 shows that at pH 8.0 and 25°, an extract of human leukocyte granules hydrolyzed *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester, a highly specific synthetic substrate for elastase. This result is consistent with our previous observation of alanine *p*-nitrophenyl esterase activity in these granules (1). Examination of Fig. 1 reveals that rates of hydrolysis of the acetyl-trialanine methyl ester by purified pancreatic elastase and by crude leukocyte granule extract varied uniformly with the amount of enzyme employed over the range of values tested except for amounts of granule protein below 25 μg. Fifty to 100 μg of crude granule protein hydrolyzed the trialanine ester at rates which were nearly the same as those given by 5–10 μg of pure elastase, suggesting that the leukocyte en-

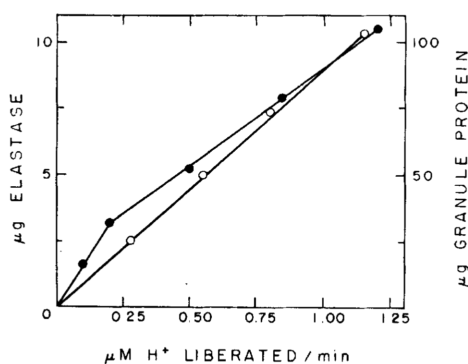

FIG. 1. A comparison of the hydrolysis of *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine methyl ester by leukocyte granule extract (●); and pancreatic elastase (O). Points represent μM hydrogen ion liberated/min by different amounts of enzymes (μg) present in the reaction mixture. Assay conditions: pH 8.0, 25°, 4 mM substrate concentration, 3.0 ml of reaction volume.

zyme had appreciable affinity for this substrate.

When 100 μg of granule protein or 10 μg of elastase were used, substrate exhaustion occurred after 22 min. Calculated amounts of base used in the completed reactions corresponded to 12.8 and 13.0 μmoles of hydrogen ion liberated by granule extract and elastase, respectively. Since 12.0 μmoles of substrate were present initially in the reaction vessel, it was concluded that both enzymes preferentially attacked only one carboxylic acid bond per molecule of substrate. Gertler and Hofmann (3) have reported similar findings for the hydrolysis of acetyltrialanine methyl ester by pancreatic elastase. They observed alkali uptake, after completed reactions, corresponding to 1 mole/mole of substrate and concluded that only the ester bond is hydrolyzed under the assay conditions.

Isoelectric focusing. Figure 2 shows the results of the isoelectric-focusing procedures performed with leukocyte granule proteins (Sephadex fraction). As shown in Fig. 2A, the isoelectric point of the granule alanine *p*-nitrophenyl esterase was at pH 9.4 when focusing was carried out in an acrylamide-supported gradient. Figure 2B demonstrates that this same enzyme was focused at pH 10.8 when the procedure was performed in a sucrose-supported gradient system. Consideration of the methods used to place proteins on the isoelectric-focusing columns favors acceptance of the higher pH value. Lewin (11) pointed out that "placing of a protein with an alkaline isoelectric point at the acid pH end of the gel . . . could lead to denaturation" and suggested "it would be advantageous to place a protein sample in a pH region as near as possible to its isoelectric point." Of the two methods used in the present experiments, the sucrose-supported system utilized sample-loading procedures which most closely approximated these idealized conditions (see Materials and Methods). In any event, both isoelectric-focusing procedures gave results consistent with the interpretation that the alanine *p*-nitrophenyl esterase of human leukocyte granules is a strongly basic protein. The isoelectric pH reported for porcine pancreatic elastase is also in the alkaline range (pH 9.5) (12).

The yields recovered from preparative isoelectric focusing have so far proven too low

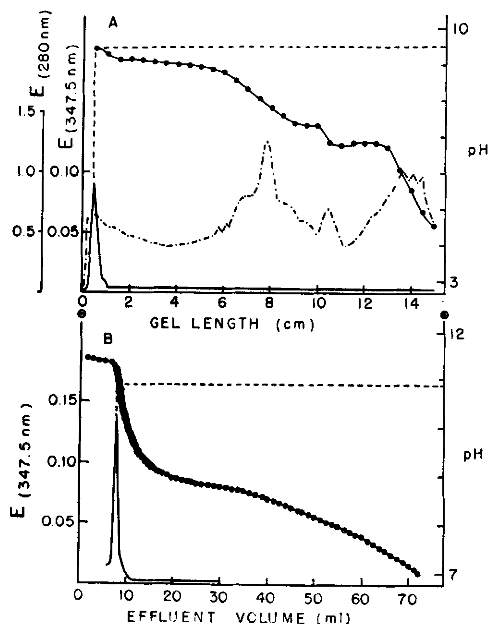

FIG. 2. Isoelectric focusing of leukocyte granule protein (Sephadex fraction) in acrylamide gel (A) and sucrose (B): (—), alanine *p*-nitrophenyl esterase activity measured as absorbance of free *p*-nitrophenol at 347.5 nm; (- . -), protein measured as absorbance at 280 nm; (●), pH values in the gel or effluent fractions; (—), cathode; (+), anode.

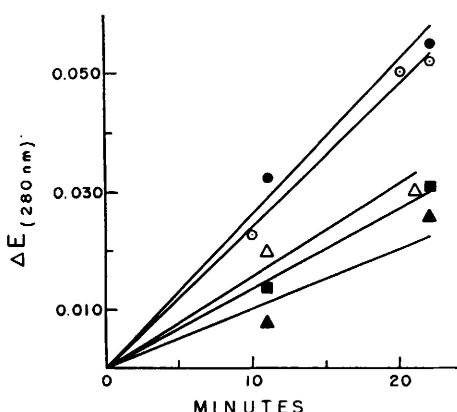

FIG. 3. Digestion of denatured hemoglobin substrate by human granule extracts at neutral pH in the presence of substituted alanine compounds: (ordinate), increment in absorbance of trichloroacetic acid soluble peptides at 280 nm; (abscissa), length of incubation; (○), granule extract alone; (△), granule extract with 0.01 *m* *t*-BOC-*l*-alanine; (▲), granule extract with 0.02 *M* of above; (●), granule extract with 0.01 *M* *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine; (■), granule extract with 0.02 *M* of above. Each point represents the average of duplicate assays. Incubations carried out at pH 7.0 in 0.075 *M* sodium phosphate buffer at 37°. Twenty μg of granule protein used in all reactions.

to permit any analyses other than those required to identify enzyme-containing fractions. Isoelectric focusing on a larger scale with greater amounts of starting protein will be required before it can be determined whether enzyme fractions isolated in this manner will also hydrolyze acetyl trialanine methyl ester. Similarly, preparations of purified enzyme will be required to determine the extent to which the elastase-like esterase(s) contribute to the extensive neutral proteolytic activity of human leukocyte granules (4, 5, 13). Some preliminary support for a "unified" enzyme hypothesis is available from studies with partly-purified granule fractions. The latter show parallel enhancement of elastolytic and hemoglobin-digesting activities along with esterolytic activity against *t*-BOC-*l*-alanine *p*-nitrophenol (1, 5). In addition, the results of inhibition experiments described next lend further support to this assumption.

Inhibition of proteolysis. Figure 3 shows the effect of two different concentrations of *t*-BOC-*l*-alanine and *N*-acetyl-*l*-alanyl-*l*-alanyl-*l*-alanine on the release of trichloroacetic acid-soluble peptides from denatured hemoglobin by human granule preparations at neutral pH. A 0.01 *M* concentration of *t*-BOC-*l*-alanine inhibited hemoglobin digestion by 34% while 56% inhibition was achieved with 0.02 *M* reagent. *N*-acetyltrialanine showed similar inhibition at the highest concentration tested (44% at 0.02 *M*). Conversely, 0.025 *M* tosyl arginine methyl ester, a substrate and competitive inhibitor of trypsin-like esterases, does not inhibit hemoglobin digestion by human leukocyte granules (13). It should be noted that the inhibition of hemoglobin digestion by granule extract in the presence of 0.02 *M* *t*-BOC-*l*-alanine proved identical to the inhibition of hemoglobin digestion by trypsin (also 56%) in the presence of 0.025 *M* tosyl arginine methyl ester (13).

In conclusion, our results show that an esterase (esterases) present in human leukocyte granules hydrolyzes specific synthetic substrates of elastase and suggest that this elastase-like enzyme may be responsible for the extensive digestion of hemoglobin by human leukocyte granules at neutral pH. The high isoelectric pH should facilitate purification of the enzyme and further exploration of its biochemical and biological properties.

Summary. 1. Human peripheral blood leukocyte granules contain a neutral esterase which hydrolyzes *t*-BOC-l-alanine *p*-nitrophenol, a synthetic substrate of elastase. The alanine *p*-nitrophenyl esterase was identified following isoelectric focusing of granule proteins in acrylamide gel and sucrose density-gradient systems. The enzyme proved highly basic, focusing at pH 9.4 in acrylamide gel and at pH 10.8 in the sucrose gradient.

2. At pH 8.0, preparations of human leukocyte granules also actively hydrolyzed *N*-acetyl-l-alanyl-l-alanyl-l-alanine methyl ester, a second highly specific elastase substrate, releasing 1 mole of acid/mole of substrate. In this reaction, the activity of 10 μg of crude granule extract protein corresponded to that of 1 μg of crystalline pancreatic elastase.

3. Products of hydrolysis of both esters; namely, *t*-BOC-l-alanine and *N*-acetyl-l-

alanyl-l-alanyl-l-alanine, were shown to inhibit the digestion of denatured hemoglobin at neutral pH by granule extract suggesting that part of the neutral protease activity of human leukocyte granules may be attributed to the elastase-like esterase(s).

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