Degradation of Type IV (Basement Membrane) Collagen by a Proteinase Isolated from Human Polymorphonuclear Leukocyte Granules*

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A serine esterase with potent proteolytic activity against native bovine lens capsule type IV collagen was isolated and purified from extract of human polymorphonuclear leukocytes (PMN). The type IV collagenolytic activity co-purified with N-t-benzyloxy carbonyl-L-alanine nitroanilidase, and was inhibited by phenylmethanesulfonyl fluoride and N-acetyl-Ala-Ala-Ala-Ala chloromethyl ketone. In addition, the purified enzyme had elastolytic activity, reacted with a specific antibody to PMN elastase, and, therefore, appeared to be identical with this enzyme.

A simple, reproducible assay for the detection of type IV collagenase activity using insoluble bovine anterior lens capsule collagen was defined. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the enzyme released large molecular weight peptides (>30,000) from the insoluble substrate. The enzyme was also active against native, pepsin-solubilized type IV collagen; five reaction products could be identified. These data suggest that PMN elastase may be involved in the degradation of basement membrane collagen in physiologic and pathologic states.

The isolation of collagen molecules from tissues rich in basement membrane has led to the identification of two classes of collagen, type IV and type V (AB), which are distinct from each other and from the interstitial collagens (types I, II, and III). Type IV collagen has been partially characterized by pepsin extraction of tissues such as anterior lens capsule (1), renal glomeruli (2), and placenta (3, 4). This collagen has certain unusual properties, including a high content of hydroxylysine, over 80% of which is glycosylated, the presence of 3-hydroxyproline and disulfide bonds (5). Although earlier studies suggested that type IV collagen was composed of three identical chains (5), recent observations from several laboratories indicate that there are at least two genetically distinct chains, C and D, in basement membranes (3, 4). Despite these important observations, the molecular organization and configuration of the native type IV collagen molecule remain unknown.

Another unique feature of type IV collagen is its resistance to the action of vertebrate collagenases. It has been established that interstitial collagens are resistant to the action of general proteases, but are cleaved by vertebrate collagenases at a single site (6) (an exception to this is a trypsin-sensitive bond in the native type III molecule) (7). It has been reported that type IV collagen is resistant to the action of skin collagenase (8, 9) and we have found a similar resistance to rheumatoid synovial collagenase. This has led investigators to search for other proteinases which may be active against this collagen. Davies et al. (10) reported that elastase, cathespin G, and cathespin B were able to solubilize hydroxyproline from isolated renal glomeruli. Liotta et al. (8) have described a neutral proteinase extracted from an invasive murine tumor which cleaved type IV collagen. Sage et al. (4) have shown that mast cell protease, a chymotrypsin-like enzyme, had proteolytic activity against native type IV collagen extracted from human placenta.

The present report describes the degradation of type IV collagen prepared from bovine anterior lens capsule (ALC)1 by a protease extracted and purified from human polymorphonuclear leukocytes (PMN). Our data clearly indicate that this activity is due to PMN elastase, a neutral serine esterase. The enzyme is active against insoluble native substrate, solubilizing up to 79.9% of the hydroxyproline, and against native soluble collagen extracted from ALC, completely degrading both C and D components.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: Sucrose, Trizma base, agaropectin, chloramine-T, casein, ovalbumin, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanyl chloromethyl ketone (TPCK), N-t-benzyloxy carbonyl-L-alanine-p-nitroanilide (Boc-Ala-Np), N-Benzoyl-DL-phenylalanine-N-octyl ester (Bz-DL-Phe-2-ONap), Fast Garnet G, and porcine pancreatic elastase from Sigma; acrylamide, N,N,N'-methylenebisacrylamide, sodium dodecyl sulfate, glycine, Coomassie Brilliant Blue R 250, N,N,N',N'-tetramethylethlenediamine, ammonium persulfate, and Affi Gel 10 from Bio-Rad; 4-phenylbutylamine from Aldrich; soybean trypsin inhibitor and elastin from Worthington; carboxymethylcellulose (CM-52) from Whatman; Ultro-Gel AcA-44 LKB; and SeaKem agarose from Marine Biologicals. N-Acetyl-Ala-Ala-Ala-chloromethyl ketone was obtained from Dr. James Powers, and the mono-specific antibody to human PMN elastase was a gift from Dr. Zena Werb, Laboratory of Radiobiology, University of California at San Francisco. All other chemicals were analytical grade.

Preparation of Type IV Collagen Substrates—Insoluble type IV collagen was prepared by a modification of the method of Tanzer and Kefalides (11, 12). The anterior capsule was dissected from fresh

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2 The abbreviations used are: ALC, anterior lens capsule; Me2S0, dimethyl sulfoxide; PMN, polymorphonuclear leukocyte; PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyl-L-lysyl chloromethyl ketone; TPCK, tosyl-L-phenylalanyl chloromethyl ketone; Boc-Ala-ONp, N-t-benzyloxy carbonyl-L-alanine-p-nitroanilide; Bz-DL-Phe-ONap, N-Benzoyl-DL-phenylalanine-N-octyl ester; SDS, sodium dodecyl sulfate.
bovine lens followed by washing with distilled water and lyophilization. The freeze-dried material was then pulverized in a freezer mill (Spex) in liquid nitrogen and resuspended at 2 mg/ml in 0.1 M acetic acid and extracted for 24 h at 4°C. After centrifugation at 10,000 × g for 20 min, the pellet was resuspended in 0.05 M citrate, 0.5 M NaCl, pH 7.6, and solubilized. After extraction, the pellet was centrifuged at 18,000 × g for a second time with 0.1 M acetic acid and the resulting supernatant was dialyzed extensively against the same and lyophilized. The lyophilized material was pulverized again in a freezer mill in liquid nitrogen and resuspended at 0.01 M Tris-HCl, pH 7.6, centrifuged at 10,000 × g for 20 min and finally resuspended at 1 mg/ml in the same buffer with 0.02% sodium azide and stored in suspension at 4°C.

Soluble ALC collagen was prepared by suspending the pulverized ALC (1 g) in 200 ml of 0.5 M acetic acid, the pH of which was adjusted to 2.5 by the addition of formic acid. Pepsin, 100 mg, was added and the mixture shaken gently for 40 h at 4°C. At the end of the incubation, the supernatant was collected by centrifugation at 18,000 × g for 1 h, and the collagens precipitated by the addition of NaCl to a final concentration of 10% (w/v). After centrifugation, the pellet was solubilized in 0.5 M acetic acid, the salt precipitation was repeated, and the final pellet was solubilized in 0.5 M acetic acid, dialyzed extensively against the same, and stored lyophilized. The final product was solubilized in 0.1 M Tris-HCl, 0.02% sodium azide, pH 7.6, and stored at 4°C.

Preparation of PMN Granules—PMN granules were isolated and extracted according to the method of Baugh and Travis (13). PMN were isolated from fresh human blood by dextran sedimentation and hypotonic lysis of contaminating red blood cells. The PMN were lysed by stirring in 0.2 M sucrose containing heparin (100 units/ml) for 16 h at 4°C followed by forcing the suspension through a 25-gauge needle. Intact granules were then harvested by differential centrifugation in 0.34 M sucrose. The granules were suspended in 0.01 M phosphate buffer, 1.0 M NaCl, 0.02% sodium azide, pH 7.2, sonicated, and extracted for 16 h at 4°C, and centrifuged at 10,000 × g for 20 min. The extraction procedure was repeated until proteolytic activity was no longer detectable in the supernatant using azocasein as substrate.

Enzyme Assays—Azocasein was prepared according to a modification of the method of Charney and Tomarelli (14) and azocaseinase assays were done according to the method of Starkey and Barrett (15). Elastase activity was detected by two separate methods. For column screens, the synthetic substrate Boc-Ala-ONp was utilized and esterolytic activity against this substrate was determined according to the method of Visser and Blout (16). Elastinolytic activity was assessed by a modification of the method of Schumacher and Schill (17) in which SeaKem agarose was used because of its low gelling temperature. The elastin G activity was detected using the synthetic substrate Bz-Me2SO-Phe-2-ONap esterase (elastase) activity, and Bz-Me2SO-Phe-2-ONap esterase (cathepsin G) activity. For elastinolytic activity, a column was prepared by applying activity separated from elastase and that the cathepsin activity was eluted in the Me2SO gradient. It is also clear from Fig. 2B that the type IV collagen-degrading activity correlated with elastase, but the separation of elastase from cathepsin G is suboptimal using this technique and thus, a conclusion could not be drawn from this column run.

The fractions containing type IV collagen-degrading activity from this procedure were pooled, dialyzed, and applied to a column of 4-phenylbutylamine-Affi-Gel and cathepsin G activity separated from the elastase activity by the method of Feinsteen and Janoff (21). As can be seen in Fig. 2A, the cathepsin G activity did not bind to the resin, whereas the elastase activity was eluted in the Me2SO gradient. It is also clear from Fig. 2A that the type IV collagen-degrading activity correlated with elastase and that the cathepsin G peak contained no activity against type IV collagen.

The type IV collagen-degrading activity was pooled and further purified using CM-cellulose according to the method of Baugh and Travis (13). Once again, the type IV collagenase activity co-eluted with elastase (Fig. 2B). This highly purified material was used for all further experiments after dialysis against 0.05 M Tris-HCl, 0.02% sodium azide, pH 7.6.

Characterization of the Type IV Collagen-degrading Enzyme—Although the elution patterns of type IV collagen degradation and elastase correlated well, further evidence was necessary to be certain that the same enzyme was responsible for both effects. The purity of the enzyme preparation was tested in two manners. The specific activity of the purified material was determined against azocasein. One unit of azocaseinase activity was defined as the amount of enzyme nec-

RESULTS

Purification of Type IV Collagen-degrading Enzyme—Having determined that crude PMN granule extract solubilized hydroxyproline from insoluble ALC, an attempt was made to characterize this enzymatic activity further by gel filtration on Ultrogel Aca-44. The column fractions were assayed for type IV collagen-degrading activity using insoluble substrate, Boc-Ala-ONp esterase (elastase) activity, and Bz-Me2SO-Phe-2-ONap esterase (cathpsin G) activity. It can be seen in Fig. 1 that the type IV collagen-degrading activity eluted in a sharply defined peak co-eluting with the peak of elastase activity, but the separation of elastase from cathepsin G is suboptimal using this technique and thus, a conclusion could not be drawn from this column run.

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Basement Membrane Collagen Degradation by PMN Elastase

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Fig. 1. Gel filtration of crude PMN granule extract on Ultrogel AcA-44. The column was equilibrated with 0.01 M phosphate buffer, 0.5 M NaCl, 0.02% sodium azide, pH 7.5, at a flow rate of 45 ml/h. Fractions of 14 ml were collected and assayed for Boc-Ala-ONp esterolytic activity (ΔA436nm/3 min/100 μl), ○; Bz-DL-Phe-ONap esterolytic activity (ΔA436nm/30 min/100 μl), ○; and type IV collagen-degrading activity expressed as micrograms of hydroxyproline released/2 h, ○. Absorbance at 280 nm is indicated by solid line.

Absorbance (280 nm) related with Boc-Ala-ONp esterase. B, purification of type IV collagen-degrading activity on CM-cellulose. The type IV collagen-degrading fractions from the 4-phenylbutylamine Affigel column were pooled, dialyzed against 0.02 M sodium acetate, 0.15 M NaCl, pH 5.5, and applied to a column (1.5 X 30 cm) of CM-cellulose equilibrated with the same buffer. The absorbed proteins were eluted in a linear gradient of NaCl from 0.15 M to 0.5 M which was begun at Fraction 0. Fractions 8 to 15 were pooled and used for all further experiments. Absorbance at 280 nm is indicated by solid line.

Characterization of Type IV Collagenase Assay using Insoluble ALC as Substrate—To determine whether there was a significant quantity of noncollagenous protein in the substrate, a precipitin line formed with the purified enzyme solution (data not shown). Despite the evidence cited above, it was still possible that another protease was present that was responsible for the type IV collagen-degrading activity. The effect of various inhibitors was therefore tested on the collagenolytic activity. Because the organic solvents necessary to solubilize some of these inhibitors interfered with the colorimetric determination of hydroxyproline, another system had to be utilized. Thus, ALC-agarose plates were utilized as a semiquantitative assay for inhibitor studies. A plate of agarose-elastin was run simultaneously with the identical enzyme/inhibitor solution. PMSF, TPCK, TLCK, and N-acetyl-Ala-Ala-Ala-Ala-chloromethyl ketone were dissolved in Me2S0 and added to the enzyme solution at a final concentration of 1 mM. Fig. 4B demonstrates that PMSF and N-acetyl-Ala-Ala-Ala-Ala-chloromethyl ketone totally inhibited the type IV collagen degradation, the former indicating that the enzyme is a serine esterase and the latter being a specific inhibitor of elastase (25, 26). An identical inhibitory pattern was seen when elastin was used as a substrate (Fig. 4A).

Characterization of Type IV Collagenase Assay using Insoluble ALC as Substrate—To determine whether there was a significant quantity of noncollagenous protein in the substrate, amino acid composition was determined. The analysis confirmed that the substrate was essentially all collagen as evidenced by the fact that the glycine content was 325 residues/1000, the 4-hydroxyproline content was 325 residues/1000, the hydroxylysine/lysine ratio was 39:12 residues/1000.
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**Fig. 3.** SDS-electrophoresis of crude PMN extract (Tract 1) and purified enzyme after gel filtration on Ultrogel AcA-44, affinity chromatography on 4-phenylbutylanine Affigel, and cation exchange on CM-cellulose (Tract 2). Acrylamide concentration is 10%. Tract 3 indicates molecular weight standards, OA, ovalbumin (45,000); CA, carbonic anhydrase (31,700). BF indicates buffer front. Ribonuclease A (14,000) ran with the buffer front in this gel.

and the remainder of the composition was consistent with that of type IV collagen (2).

The initial step in the characterization of the assay was the definition of a time course. Two hundred microliters of collagen suspension was incubated at 37°C with 100 µl of enzyme solution in a final volume of 1 ml. The reaction was stopped by centrifugation at various time points and hydroxyproline content determined in hydrolysates of both the pellet and supernatant. The final concentration of enzyme in each assay was 3.2 µg/ml or 0.22 azocaseinase unit. Each point represents mean of triplicate determinations. The amount of substrate varied slightly from sample to sample because of the difficulties in pipetting a suspension of insoluble material. The total hydroxyproline content of the experiment shown was 23.07 ± 3.68 µg. Fig. 5 shows a typical time course demonstrating linearity of the reaction up to 4 h.

Fig. 6 demonstrates a dose-response curve obtained with a substrate concentration of 16.96 ± 2.2 µg of hydroxyproline/determination and an incubation time of 1 h with increasing enzyme concentration. The highest point in this experiment represents only 18.90 ± 2.05% hydroxyproline released. However, when the substrate concentration was decreased to 8.45 ± 1.45 µg of hydroxyproline/ml, the incubation time doubled, and maximum enzyme concentration increased to 0.97 unit/ml, 79.30 ± 2.11% of the total hydroxyproline was released.

In order to be certain that the assay was truly solubilizing collagen peptides and not simply dispersing fibrillar material, the supernatant from an enzyme-substrate mixture preincubated at 37°C for 1 h was examined by electron microscopy. No fibrillar material could be detected in the supernatant using this technique. To confirm this further, duplicate samples of enzyme-substrate were incubated for 1 h at 37°C and the mixture subjected to either routine centrifugation in the Microfuge or centrifuged at 78,000 × g for 2 h in a Beckman model L5-65 ultracentrifuge. There was no difference in the hydroxyproline content of the supernatants of these samples.

**Fig. 5.** Time course of solubilization of hydroxyproline from insoluble anterior lens capsule collagen by purified enzyme. Two hundred microliters of the collagen suspension (23.07 ± 3.68 µg of hydroxyproline) was incubated with 3.2 µg of the enzyme at 37°C. Reaction was stopped periodically by centrifugation and hydroxyproline content of pellet and supernatant were determined separately.

The pH optimum of elastase has been determined using various substrates (26) and found to be between 7.5 to 9.0 depending upon the substrate used. In order to determine the
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**Fig. 6.** Dose response of purified enzyme against insoluble anterior lens capsule collagen. Two hundred microliters of the collagen suspension (16.96 ± 2.2 μg of hydroxyproline) was incubated for 1 h at 37°C with increasing doses of enzyme. Reaction was stopped as described and hydroxyproline determined in both supernatant and pellet.

**Fig. 7.** SDS-electrophoresis of supernatants of purified enzyme and insoluble ALC collagen. Track 1 is the buffer control which had been incubated for 24 h at 37°C. Tracks 2 to 5 are supernatants from 2-h, 4-h, 8-h, and 24-h incubation, respectively. Track 6 is the pepsin-solubilized mixture of collagen chain extracted from ALC, identifying the C-1 component (140,000), C component (95,000), and D component (70,000). Acrylamide concentration was a linear gradient from 5 to 20%. BF indicates buffer front.

pH optimum of the type IV collagen-degrading activity, the enzyme was incubated with insoluble substrate for 2 h at 37°C in 0.1 M phosphate buffer, the pH ranging from 6.0 to 9.0 in steps of 0.5 pH unit. The reaction had a broad pH optimum from 7.0 to 8.5. At pH 9.0, a significant amount of hydroxyproline was found in the supernatant of the buffer control indicating that there was an adverse effect on the integrity of the substrate at this pH.

**Identification of the Reaction Products of Insoluble Type IV Collagen and PMN Elastase**—Two milligrams of the insoluble substrate was incubated with 23 μg of enzyme in a final volume of 5 ml in the buffer described. The reaction was stopped at intervals by the addition of PMSF to a final concentration of 1 mM followed by centrifugation at 10,000 × g for 10 min. The supernatants were harvested, lyophilized, and the residues were resuspended in 1 ml of distilled water. SDS-electrophoresis was performed on the reaction mixture on a linear gradient of 5 to 20% polyacrylamide in the presence of 2-mercaptoethanol. The identical peptide pattern was obtained if the samples were run in the absence of 2-mercaptoethanol. Fig. 7 shows a typical gradient gel of the reaction products which range in molecular weight from 20,000 to 140,000. The time course shows that most of the reaction products are stable at 37°C and are not further degraded by the purified enzyme.

**Type IV Collagen-degrading Activity against Native, Soluble Collagen Chains**—Soluble, pepsin-extracted type IV collagen (400 μg) was incubated with 3.2 μg of enzyme in a volume of 1 ml and the viscosity of the solution monitored at 33°C.

The specific viscosity of a 400 μg/ml solution of type IV collagen was found to be approximately 1.5, somewhat lower than that observed for other types of collagen. Fig. 8 shows that the viscosity of the control collagen solution remained unaltered while that of the mixture of collagen and enzyme rapidly decreased.

In order to identify the reaction products generated by this enzyme, the reaction was stopped at intervals with PMSF and SDS-electrophoresis performed on gradient slab gels as described above. Only five reaction products could be identified (Fig. 9A). The molecular weight of these products was estimated using a mixture of type I collagen (guinea pig skin) which had been partially cleaved by purified rheumatoid synovial collagenase. The molecular weight standards were: β₁, 100,000; α₁, 150,000; α₁, 100,000; α₁, 75,000; and α₁, 25,000. By plotting the migration distance against the molecular weight on a semilogarithmic scale, the molecular weights were estimated to be 90,000, 68,000, 48,000, 37,000, and 31,000. The concentration of enzyme was apparently insufficient to detect a band on the gels. These same reaction products were identified if the reaction was carried out at 37°C or at 33°C. If the reaction was carried out for 18 h at either 33 or 37°C the major components of the native collagen solution, C-1, C, D, and the 50,000 component were no longer detected indicating that the reaction had gone to completion and all chains were susceptible to degradation.

When the collagen was heat-denatured (60°C for 15 min)
prior to incubation with the purified enzyme, a different peptide pattern was observed on electrophoresis (Fig. 9B). Within 30 min, the enzyme completely degraded the large molecular weight components of the collagen (>75,000). At 90 min only small molecular weight peptides (<40,000) could be identified. These peptides were stable and not further degraded at 4 h.

**DISCUSSION**

The results presented clearly demonstrate that a serine esterase present in PMN granules possesses proteolytic activity against native type IV or basement membrane collagen. The data also show that this activity is ascribable to the well characterized elastase present in these granules. This is at slight variance with another report (10) which found that both elastase and cathepsin G, the two major neutral serine esterases of the PMN granule, had type IV collagenase activity. The explanation of the differences is presently unclear. A simple, highly reproducible assay system for the detection of type IV collagenase was defined and the reaction was found to be linear up to 4 h. Despite the ease of preparation of the substrate, the system proved to be pure. There was no appreciable contamination by noncollagenous protein and the amino acid composition was consistent with that previously described for type IV collagen (2). A second assay system was described which utilized insoluble type IV collagen incorporated into an agarose matrix. A similar system has been described for elastin (17) and type I collagen substrates (19). Its value as a quantitative assay remains to be determined but it has proven useful to screen for type IV collagen-degrading activity and to identify its inhibition.

There have been other previous reports of type IV collagen-degrading activity. Liotta et al. (8) have described an enzyme extracted from a metastatic murine tumor which is capable of degrading type IV collagen extracted from another murine tumor. The enzyme was a metalloprotease and was at least partially activated by trypsin. These two properties are common to some of the vertebrate collagenases active against interstitial collagens (6). The substrate used was somewhat unique in that it could be extracted from the tumor without the use of pepsin. Thus, the native collagen solution contained two chains with molecular weights of 140,000 and 160,000, larger than those isolated from tissues by pepsin extraction. The enzyme was active against the soluble native collagen as well as insoluble aggregates of the extracted collagen. The enzyme appeared to make a single cleavage through the 160,000 component.

Sage et al. (4) have found that mast cell protease had type IV collagen-degrading activity. It was apparently more active against the 140,000 (C-1) component and five reaction products were identified. Interestingly, mast cell protease generated an M, 65,000 fragment even after denaturation, but the peptide maps were different if collagen was denatured prior to digestion.

In view of the recent studies which have suggested that there are two major components of this molecule, it is of interest that only five reaction products were identified. This implies a single cleavage site in one chain and two cleavage sites in the other component. Further studies are necessary to identify the origin of the reaction products and the cleavage sites of the respective molecules.

The degradation of basement membrane collagen by elastase may help in the understanding of certain pathologic states. The PMN is frequently found at sites of basement membrane pathology in some diseases such as glomerulonephritis. It could be proposed that the release of elastase from PMNs stimulated by immune complex deposition might play a role in the destruction of the basement membrane. Another disorder to which this observation may be pertinent is pulmonary emphysema. Elastase-induced emphysema is a well established model in laboratory animals (27). It has been assumed that this was related to the degradation of lung
elastin. However, this report raises the possibility that the action of elastase on basement membrane collagen may be involved at least partially in the pathogenesis of this experimental disorder.

Finally, it is perhaps unfortunate that this enzyme has been called elastase as this implies substrate specificity. PMN elastase had proteolytic activity against proteoglycan, fibrinogen, and several other substrates (29). In addition, Starkey et al. (29) have shown that PMN elastase solubilized a small amount of hydroxyproline from intact articular cartilage and tendon. The solubilized collagen was in the form of intact collagen chain indicating that the site of action of elastase was in the nonhelical or telopeptide region of the molecule. Subsequent studies using soluble collagen as substrate confirmed this telopeptidase activity against types I and II and also suggested it cleaved within the helical region of type I collagen (29). We have been able to confirm the telopeptidase activity against type I collagen, but found no evidence of cleavage within the helix. We would like to add that type V collagen was unaffected by this protease.

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