Specific Cleavage of Human Type III Collagen by Human Polymorphonuclear Leukocyte Elastase*

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Purified polymorphonuclear leukocyte elastase degraded native human liver type III collagen at 27°C by making a cleavage through the triple helix. The enzyme had no effect on human type I collagen. The reaction was inhibited by phenylmethanesulfonyl fluoride (PhCH₂SOF) but not by EDTA. The collagen reaction products were identical with those generated by human rheumatoid synovial collagenase when analyzed by polyacrylamide gel electrophoresis and gel filtration. NH₂-terminal sequence analysis indicated that the enzyme cleaved at an isoleucyl-threonyl bond located 4 residues on the carboxyl side of the established cleavage site for animal collagenases. Therefore, it is likely that in pathologic states, type III collagen can be selectively depleted from the matrix by this enzyme.

The degradation of the collagenous component of the extracellular matrix is an important function in both physiologic remodelling and pathologic tissue destruction. The investigative interest in collagenolysis is in part due to the fact that the triple helical configuration of the interstitial collagens renders them resistant to the action of general proteases. The degradation of these collagens is felt to be initiated by the specific action of a group of metalloproteases, the vertebrate collagenases, which make a single cleavage through all three chains of type I, II, and III collagen by purified polymorphonuclear leukocyte elastase (PMN) enzymatic activity to be important in tissue destruction in certain pathologic states.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: Trizma base, phenylmethanesulfonyl fluoride (PhCH₂SOF), L-arginine, and adenosine triphosphoric acid from Sigma; acrylamide, N,N',N'N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, sodium dodecyl sulfate (NadDSO₄), glycine, dithiothreitol, ammonium persulfate, Coomassie Brilliant Blue R-250, agarose (A-1.5m), and Bio-Gel P-2 from Bio-Rad; ammonium sulfate from Schwarz/Mann. All other chemicals were analytical grade.

Preparation of Type III Collagen—Human circulatory blood vessels were obtained after autopsy and type III collagen extracted and purified as previously described (8) except that an additional step of purification involving trichloroacetic acid-ethanol precipitation according to the method of Gross (9) was performed.

Preparation of Human PMN Elastase—PMN granules were isolated from fresh human blood and extracted according to the method of Baugh and Travis (10). Elastase was purified using affinity chromatography on 4-phenylbutylamine Affigel (11), ion-exchange chromatography on CM-cellulose (10), and gel filtration. Its purity and specificity were determined by NaDodSO₄ electrophoresis, by determination of specific activity, and by reaction with a specific antibody as previously documented (12). Protease-free collagenase was prepared from cultures of adherent rheumatoid synovial cells as previously described (4).

Determination of Type III Collagenolytic Activity—Purified type III collagen was lyophilized, dissolved in 0.1 M acetic acid, and dialyzed against 0.05 M Tris-HCl, 0.005 M CaCl₂, 0.2 M NaCl, 0.02% sodium azide, pH 7.6. All reactions were carried out at 27°C with a final collagen concentration of 0.8 mg/ml in the same buffer. For viscometric analysis, L-arginine was added to the reaction mixture at a final concentration of 0.05 M to prevent fibril formation. The viscosity of a 1-ml sample was monitored in a semi-mini viscometer (Cannon) in a water bath maintained at exactly 27°C. Reactions were terminated by the addition of PhCH₂SOF to a final concentration of 1 mM.

Isolation of Reaction Products—Eighty milligrams of collagen was incubated with 0.622 mg elastase at 27°C. The Slow Peptide—DMAA (071472) was performed. The Slow Peptide—DMAA (071472) was performed.

Electron Microscopy—Drops of each SLS preparation were placed on 4-phenylbutylamine Affigel (11), ion-exchange chromatography on CM-cellulose (10), and gel filtration. Its purity and specificity were determined by NaDodSO₄ electrophoresis, by determination of specific activity, and by reaction with a specific antibody as previously documented (12). Protease-free collagenase was prepared from cultures of adherent rheumatoid synovial cells as previously described (4).

Preparation of Segment-Long-Spacing (SLS) Crystallites—SLS crystallites were prepared according to the method of Gross and Nagai (16). One-milliliter aliquots (containing 0.8 mg of collagen) of control collagen solution, and elastase and collagenase-treated collagen solutions were dialyzed extensively against 0.05 M acetic acid (eight changes over 2 days). A 1% solution of ATP was added to the sample to attain a final concentration of 0.4%. The samples were then allowed to stand on ice for 2 h.

Electron Microscopy—Drops of each SLS preparation were placed...
on Formvar/carbon-coated 300-mesh grids and the crystallite suspension was allowed to settle for 5 min. Excess buffer was removed by blotting the edge of the grids with filter paper and the grids were allowed to dry. Grids were negatively stained with 1% phosphotungstic acid pH 7.3 or positively stained with 0.2% phosphotungstic acid followed by 0.2% uranyl acetate. After a brief wash and drying, the grids were examined with a Phillips EM 201 electron microscope. Micrograph magnifications were calibrated using a standard grating replica (E. F. Fullam Co., Schenectady, N.Y.).

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—**NaDodSO,

Specific Cleavage of Type III Collagen by Elastase—Type III collagen (0.8 mg) was incubated with purified elastase (0.9 unit) or protease-free rheumatoid synovial collagenase (0.1 unit) for 18 h at 27°C. NaDodSO,

**RESULTS**

**Inhibition of the action of elastase on type III collagen.** Samples were isolated as described and electrophoresis performed under reducing conditions with dithiothreitol. Track 1, collagen + buffer; Track 2, collagen + buffer + EDTA. Track 3, collagen + buffer + PhCHSO3F. Track 4, collagen + collagenase. Track 5, collagen + elastase; Track 6, collagen + collagenase + EDTA. Track 7, collagen + elastase + EDTA. Track 8, collagen + collagenase + PhCHSO3F. Track 9, collagen + elastase + PhCHSO3F.

**Inhibition Studies of Elastase Cleavage of Type III Collagen—**In order to be certain that this observation was not due to a small amount of contaminating PMN collagenase, inhibition studies were carried out. Incubation was carried out as described and reaction products examined by electrophoresis in the presence of dithiothreitol. In Fig. 2, Tracks 1 to 3 are collagen controls incubated without inhibitor (1), with EDTA (0.005 m) (2), or PhCHSO3F, 0.001 m (3). Track 4 is collagen incubated with collagenase and Track 5 is collagen incubated with elastase. Tracks 6 and 7 are collagen incubated with collagenase and elastase, respectively, to which EDTA (0.005 m) was added. As can be seen, EDTA totally inhibited the collagenase but did not inhibit the latter. Tracks 8 and 9 are collagen-incubated with collagenase and elastase, respectively, to which PhCHSO3F (0.001 m) was added. In this case, the collagenase was unaffected but the elastase effect was totally inhibited.

**Viscometric Analysis of Enzymatic Reaction—**The viscosity of type III collagen solution (0.8 mg in 1 ml) was monitored at 27°C after the addition of control buffer or elastase in varying doses. The results are shown in Fig. 3. I is the buffer control and II to IV are increasing concentrations of elastase (0.45, 0.9, and 1.35 units, respectively). As can be seen, a good correlation was observed between the dose of elastase and the rate of reaction indicated by the initial fall in viscosity.

**ISOlation and Characterization of a1(III)10 Generated by Elastase Cleavage—**Type III collagen (80 mg) was incubated with 44.5 units of elastase in a final volume of 100 ml for 18 h at 27°C. The reaction was stopped and reaction products precipitated, dissolved and chromatographed on Agarose A-15m as described above. Fig. 4 shows a typical chromatogram obtained. The peak which eluted in the M, 25,000 region a1(III)10 was collected, desalted on a column of Sephadex G-25, lyophilized, and the NH2-terminal sequence determined as described. Two sets of sequence were obtained; the major sequence would indicate that the molecule was cleaved at the Ile-Thr bond indicated in Fig. 5; the second, minor sequence observed would indicate a second cleavage site at the Ala-Arg

**FIG. 1. Degradation of type III collagens by collagenase and PMN elastase.** Samples of type III collagen were incubated with buffer, collagenase, or PMN elastase at 27°C for 18 h. The reactions were stopped by the addition of EDTA (0.005 m) and PhCHSO3F (0.001 m) and electrophoresis performed on the reaction mixture. Tracks 1 to 3 were run in the absence of dithiothreitol (DTT) and Tracks 4 to 6 in its presence. Tracks 1 and 4 are collagen + buffer, 2 and 5 are collagen + collagenase, Tracks 3 and 6 were collagen + elastase.

**FIG. 2. Inhibition of the action of elastase on type III collagen.**
Cleavage of Type III Collagen by Granulocyte Elastase

Fig. 3. Viscometric analysis of reaction between type III collagen and elastase. A 1-ml sample of collagen + buffer or elastase was added to a semi-micro viscometer (Cannon) and the viscosity monitored at 27°C. I, collagen + buffer; II, III, and IV, collagen + elastase, 0.45, 0.9, and 1.35 units, respectively.

Fig. 4. Agarose A-1.5M Gel Filtration of a PMN Elastase Digest of Native Type III Collagen of Human Liver. After elastase digestion at 27°C the collagen fragments were reduced in 0.1 M dithiothreitol in 5 M guanidine HCl, 0.05 M Tris at pH 7.4 for 4 h at 40°C. The reduced products were applied directly to a column (2.0 × 110-cm) of agarose A-1.5M equilibrated with 0.05 M Tris, 2.5 M guanidine HCl, pH 7.4, at 22°C. The materials eluting in the region corresponding to M, ~ 75,000 and 24,000 were desalted on columns of Sephadex G-25 in 0.1 M acetic acid and lyophilized. The identity of α(III)β and α(III)γ was confirmed by NaDodSO4 polyacrylamide gel electrophoresis and α(III)β was used for amino acid sequence analysis.

The bond indicated by the symbol * in Fig. 5. Both sequences were followed through the methionyl residue, indicating the COOH terminus of α(III)-CB5 and both sequences correlated exactly with the established sequence of that peptide (4). The interpretation of this data is that elastase cleaved the triple helix at a primary cleavage site (Ile-Thr) and a secondary cleavage occurred on the isolated one-fourth fragment. However, the possibility that elastase cleaved the triple helix at two separate sites cannot be excluded. Quantitative yields of the PTH-derivatives are presented in Table I.

Examination of SLS Crystallites Formed by the Reaction of Type III Collagen and Elastase—SLS crystallites of type III collagen incubated at 27°C with either buffer, collagenase, or elastase were prepared as described above. Fig. 6 shows electron micrographs of these crystallites. The three-fourths fragments were readily observed in the cleaved samples and the elastase-generated fragment appears identical to the collagenase-generated fragment. This is compatible with the observed cleavage site being only one triplet removed from the collagenase cleavage site.

Table I

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<th>Residue no.</th>
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<tr>
<td>2</td>
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<td>3</td>
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<td>Alanine</td>
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</table>

Fig. 5. The amino acid sequence of the specific cleavage site of human liver type III collagen with elastase. The previously described cleavage sites are also identified: C-collagenase cleavage site (4); T-trypsin cleavage site (6); Th-thermolysin cleavage site (7). The elastase cleavage site was established by Edman degradation (short arrows —) of the isolated α(III)β fragment. This cleavage site occurs between residues 792 to 793 (based on homologies with type I collagen) and is located 12 residues from the COOH terminus of α(III)-CB5. E indicates the primary elastase cleaving site and * indicates the secondary cleavage site observed.

DISCUSSION

The results presented demonstrate that human PMN elastase cleaves native human type III collagen at a specific site in the molecule. This enzyme did not cleave the helical region of human type I collagen and the same has been shown for type II collagen (18). We have previously shown that this enzyme is active against type IV collagen (12), and thus, it...
“loose helix” theory is supported by the relative paucity of imino acid residues in this region. This region of the type III molecule is particularly unique in that it is susceptible to the action of several “noncollagenolytic proteases,” and thus the phenomenon cannot be explained on the basis of a specific susceptible bond in that region. Thus, it can be postulated that if there is something peculiar about this region of the α1(I) chain, this peculiarity is amplified in α1(III).

With the present level of knowledge of the primary structure of the collagen chains, its is logical to speculate that imino acids (hydroxyproline and proline) function to “tighten” the helix, while long chain hydrophobic amino acids (leucine and isoleucine) are “bulky” and tend to loosen the helix. Fig. 7 compares a 15-residue sequence of the interstitial collagen chains containing the region discussed. If we calculate the ratio of Ile + Leu/Pro + Hyp, α1(I) has a value of 2.2/0.9, α2 is 5.2 or 2.9 whereas α1(III) is 4.1 or 4.0. This would support the hypothesis that α1(III) might be in less a degree of helicity in this region than even α1(I). The data reported here may be of biologic significance in that they are the first to describe the selective degradation of one of the interstitial collagens by a naturally occurring human white cell enzyme. This may play a role in some human pathologic disorders, especially in those where there is an increase in type I collagen relative to type III. For example, this relative increase observed in cirrhotic livers (22) may, in fact, partly be due to selective degradation of type III collagen. Also, PMN elastase is used to induce emphysema experimentally in laboratory animals (23). It is possible that the selective removal of type III and IV collagen from the matrix contributes to the pathology in this model.

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REFERENCES


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