Differences in the Degradation of Native Collagen by Two Microbial Collagenases

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The early stages of degradation of native collagen by two bacterial collagenases were studied by electron microscopy and by automatic Edman degradation. The purified collagenase from *Clostridium histolyticum* was shown to cleave native collagen at several sites, but not progressively from the *N*-terminus, as had been previously suggested. The homogeneous collagenase from *Achromobacter iophagus* cleaves native collagen preferentially at two sites corresponding to the interbands 33–34 and 41–42. The latter lies within the region cleaved by the eukaryotic collagenases.

The function of the extracellular collagenases of prokaryotes is to enable the micro-organism to invade the host by rapid degradation of native collagen of the connective tissues. The vertebrate collagenases, on the other hand, play an essential role in the regulatory mechanisms of growth, tissue remodelling and resorption.

According to previous studies, native collagen is degraded by prokaryote and eukaryote collagenolytic proteinases by two different pathways. The bacterial collagenase most thoroughly studied in this respect is the enzyme from *Clostridium histolyticum* (EC 3.4.24.3). Electron-microscopic studies were undertaken to identify the early products of digestion of native calf and rat skin collagen by this enzyme (Kühn & Eggl, 1966; Stark & Kühn, 1968). At 10°C crude collagenase was found to degrade the α -chain of native collagen inward from the ends, although the *C*-terminus resisted further degradation at a point corresponding to the interband 52–53 [numbering of Bruns & Gross (1973)]. However, if the digestion is carried out at 37°C the native collagen chains are degraded to give low-molecular-weight peptides (Bornstein, 1967; Harper & Kang, 1970; Kang & Gross, 1970; Mark *et al.*, 1970; Balian *et al.*, 1971; Wendt *et al.*, 1972). This very unspecific action of the bacterial collagenase is in contrast with an extremely strict specificity observed in the action of most of the eukaryotic collagenases: they cleave collagen α -chains, containing more than 1000 amino acid residues, exclusively in one region situated at three-quarters of the chain length from the *N*-terminus (Harris & Krane, 1974*a,b,c*).

The very low specificity of *Clostridium* collagenase compared with vertebrate collagenases could be attributed to the presence of other bacterial proteinases present in the crude collagenase used in the early studies. A homogeneous prokaryotic collagenase of very high specific activity was isolated in our labora-

tory from the aerobic micro-organism *Achromobacter iophagus* (EC 3.4.24.8) (Lecroisey *et al.*, 1975; Keil-Dlouha, 1976). In the present paper we report a study of the early stages of the degradation of native collagen by this collagenase and compare it with that brought about by a purified sample of collagenase from *Clostridium histolyticum*. The sites of degradation of collagen were identified either by the electron microscopy of segment-long-spacing crystallites (Bruns & Gross, 1973; Schmitt *et al.*, 1953; Gross *et al.*, 1954; Gross & Nagai, 1965) or by automated Edman degradation.

Materials and Methods

Collagenases and collagen

Crude collagenase from *Achromobacter iophagus* was a gift from Institut Pasteur Production. Homogeneous *Achromobacter* collagenase of specific activity 1.78 μ kat/mg was prepared in our laboratory by I. Trocheris as described by Keil-Dlouha (1976). Collagenase from *Clostridium histolyticum* (type III, fraction A, substantially free of proteinase and peptidase activity), was from Sigma Chemical Co (St. Louis, MO, U.S.A.). Native salt-soluble collagen was prepared and purified as described by Kang *et al.* (1966). It was freeze-dried and stored at –20°C. Freshly prepared 0.1% solutions in 0.6M-acetic acid were dialysed successively against phosphate buffer, pH 7.2, 1.0M, against 0.4M-NaCl and finally against the appropriate buffer. All operations were performed at 4°C.

Collagenase assay

Specific activity of the collagenases used was determined on the synthetic substrate 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-

D-arginine obtained from Fluka A.G. (Buchs, Switzerland) (Wünsch & Heidrich, 1963).

Digestion

The digestion of collagen by both collagenases was performed in 0.05M-Tris/HCl/0.4M-NaCl/0.001M-CaCl₂ buffer, pH 7.2, at 10°C, for different times and enzyme/substrate weight ratios, as indicated in Table 1. It was stopped by adding an equal volume of chilled 0.5M-acetic acid.

Segment-long-spacing fragments for electron microscopy

Segment-long-spacing fragments were obtained by dialysis of the digest against 0.1M-acetic acid and then against 0.4% ATP in 0.1M-acetic acid, each for 48h at 4°C [in Spectrapor tubing calibrated for low molecular weights (up to 3500) obtained from Spectrum Medical Industries (Los Angeles, CA, U.S.A.)]. After application to 75-mesh Formvar-coated grids, the segments were stained for 15 min with 0.5% phosphotungstic acid, pH 3.5, and for 10 min with 0.1%

uranyl acetate. The grids were washed with water after each stain. They were examined in an Elmiskop 101 Siemens microscope and micrographs were taken at a direct magnification of 80000. The notation of the segment-long-spacing pattern is that proposed by Bruns & Gross (1973).

Edman degradation

Calf skin collagen was digested with collagenase from *Achromobacter iophagus* for 72h in 0.4M-(NH₄)₂CO₃ at pH 7.2 and 10°C. The enzyme/substrate weight ratio was 1/5000. The reaction was stopped by freeze-drying and the digestion mixture was subjected to an automated Edman degradation in a Beckman Sequencer model 890 C by the method of Edman & Begg (1967) as modified by Hermodson *et al.* (1972). The resulting phenylthiohydantoin of amino acids were identified by g.l.c. and t.l.c.

Results and Discussion

The degradation of native collagen from calf and rat skin by two different bacterial collagenases was performed under very mild conditions to obtain

Table 1. *Segment-long-spacing crystallites identified after digestion of native collagen by bacterial collagenases*
Fragments are characterized by N- and C-terminal bands by the notation of Bruns & Gross (1973). For full details see the text. Abbreviations: E:S, enzyme/substrate ratio (w/w); n.d., not determined; X, N-terminal distorted.

Collagenase	Calf skin collagen						Rat skin collagen					
	E:S	Time of digestion (h)					E:S	Time of digestion (h)				
		18	24	42	48	66		18	24	42	48	66
<i>Clostridium histolyticum</i>	1/2500	Intact	1-33	Intact	27-58	n.d.	1/2500	Intact	23-58	Intact	27-58	1-18 23-58
	1/500	27-58	1-26 1-33 27-52 27-58 34-58 36-58	1-17 1-33 27-58 34-52 36-52	1-33 27-58 34-52 27-58	1-30 1-33 27-58	1/500	23-58	1-18 23-58	1-18 23-58	1-18 23-58	1-18 23-58
	1/100	1-33 27-58 34-58	1-33 27-58 36-58	27-58	27-58	n.d.	1/100	27-52	27-52	27-52	27-52 27-58	27-52 27-58 34-52 34-58
	1/10000	1-33 27-58	1-33 34-58 42-58	1-40 34-58	1-26 1-33 1-40 34-58 46-58	1-33 1-40 34-58	1/25000	Intact	Intact	Intact	1-33 1-40 X-58	Intact
	1/5000	1-17 1-33 1-40 34-58 46-58	1-17 1-33 1-40 34-58	1-33 1-40 34-58	34-58 46-58 34-58	1-33 1-40 34-58	1/5000	1-33 1-40	1-33 34-58 X-58	1-33 1-40 34-58	1-33 1-40 34-58	1-33 1-40 34-58
<i>Achromobacter iophagus</i>	1/2000	1-40 34-58	1-33 1-40 14-40 14-58 34-58	1-33 1-40 34-58	1-33 1-40 34-58	n.d.	1/1000	34-58	n.d.	34-58	n.d.	34-58

information on the early stages of fragmentation. Some of the collagen always remained unaltered. The fragments observed as long-spacing crystallites at different enzyme concentrations and digestion times are summarized in Table 1.

The action of the collagenase from *Achromobacter iophagus* on native collagen is shown in Fig. 1. This enzyme cleaves both calf and rat skin collagens preferentially at two sites, corresponding to the interbands 33–34 and 40–42 (Fig. 2). It was impossible to determine the points of cleavage with a higher precision by using electron microscopy. This is because a certain distortion is often found at the extremity of the segment-long-spacing filaments, which makes the exact identification of the site of cleavage difficult. The fact that we could identify a 42–58 fragment might suggest that cleavage occurs at the interband 41–42 rather than at 40–41. Furthermore, the fragment X–58 that we sometimes found could be the fragment 42–58, but it was too distorted to be identified with certainty. The additional cleavages that we could observe in calf skin collagen may be due to a partial denaturation of that substrate. Their random occurrence may correspond to the fact that insoluble long-spacing crystallites are formed only above a critical concentration of the corresponding fragments. In any case they did not appear progressively from the *N*-terminus with time or concentration of the enzyme.

The first step of the Edman degradation of the newly formed *N*-terminal sequences after the digestion of rat skin collagen gave exclusively glycine. At

Fig. 2. Most frequently observed segment-long-spacing fragments from the mild digestion of calf skin collagen by *Achromobacter collagenase*. The figure shows segment-long-spacing fragments 1–33 (a), 1–40 (b) and 34–58 (d), and intact collagen (c). For experimental details see the text.

Fig. 1. Sites of cleavage of native calf skin collagen (a) and rat skin collagen (b) by the collagenase from *Achromobacter iophagus*. For experimental details see the text. Key: h, time of digestion (h); E:S, enzyme/substrate ratio. Numbers at top of the Figure are of the segment-long-spacing bands.

the second step both proline and alanine were found, as has been reported from this laboratory (Keil *et al.*, 1975). At the third step hydroxyproline predominated and at the fourth step glycine was again found. As expected, native collagen treated in the same way in the absence of collagenase gave no detectable *N*-terminal amino acid. Although the use of silylation procedures for the g.l.c. made quantitative evaluations difficult, the results indicate that the *Achromobacter* collagenase cleaves collagen preferentially at the X-glycine bond of the sequences X-Gly-Ala-Hyp-Gly and X-Gly-Pro-Hyp-Gly at sites encompassed by the bands 33-34 and 40-42. The present results are only qualitative, so it is possible that *N*-terminal sequences other than Gly-Ala-Hyp-Gly and Gly-Pro-Hyp-Gly are present. A more exact location of the observed cleavages may have to be reconsidered when the total primary sequence of the α_2 -chain of the rat skin collagen is known.

The nature of the bonds that are cleaved in collagen corresponds to the specificity observed with other peptide and protein substrates: the peptides 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine and 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-alanyl-D-arginine yielded the tripeptides glycyl-L-prolyl-D-arginine (Keil *et al.*, 1975) and glycyl-L-alanyl-D-arginine (O. Siffert, unpublished work) respectively. The autolysis of *Achromobacter* collagenase liberates the new *N*-

terminal Gly-Ala (Keil-Dlouha, 1976); four bonds are cleaved in β -casein, yielding three new Gly-Pro *N*-terminals and one Ala-Pro (Gilles & Keil, 1976). A similar specificity was observed after the digestion of Bence-Jones κ -chains, myosin and fibrinogen (cf. Keil, 1977).

The action of the collagenase from *Clostridium histolyticum* recorded in detail in Table 1 is shown in Fig. 3. The enzyme cleaves calf skin collagen preferentially at the level of bands 26-27 and 33-34 (Fig. 4a). Even when the minor fragments are taken into consideration, the results indicate that the preferential sites of cleavage are situated close to the central part of the α -chains, with the exception of a cleavage on the C-terminal side, at the interband 52-53. This observation is not in accordance with previously published results on the same subject, which suggested that collagen is degraded by *Clostridium* collagenase progressively inward from the *N*-terminus and, to a lesser extent, from the *C*-terminus (Kühn & Eggl, 1966). Such a degradation from the *N*-terminus might imply that each cleavage by the enzyme creates a new 'weak' region by loosening the compact helical structure of the substrate, which in turn facilitates further cleavage. Our present results, on the contrary, seem to indicate that such regions already pre-exist in the central parts of native collagen.

In rat skin collagen, the most frequently observed fragments were those containing bands 1-18, 23-58

Fig. 3. Sites of cleavage of native calf skin collagen (a) and rat skin collagen (b) by collagenase from *Clostridium histolyticum*. For experimental details see the text. Key: h, time of digestion (h); E:S, enzyme/substrate ratio. For comparison are shown results of: (i) Kühn & Eggl (1966); (ii) Stark & Kühn (1968). Numbers at top of the Figure are of the segment-long-spacing bands.

Fig. 4. (a) Segment-long-spacing fragment 27–58 from the digestion of calf skin collagen by *Clostridium histolyticum* collagenase, and (b) segment-long-spacing fragment 23–58 from the digestion of rat skin collagen by *Clostridium histolyticum* collagenase. For experimental details see the text.

and 27–52 (Fig. 4b), which reflects a quite important difference from the calf skin collagen. In this case, our results are closer to those of Stark & Kühn (1968), but the fact that we frequently observed a large *N*-terminal fragment still seems to eliminate the possibility of a progressive cleavage from the *N*-terminus.

The discrepancy between our results and those found previously may originate in the different degrees of purity of the enzymes used. The previous studies on the specificity of *Clostridium* collagenase were performed with a very crude enzyme preparation, which contained an ill-defined but considerable amount of other proteolytic enzymes of different specificity (Kühn & Eggli, 1966; Stark & Kühn, 1968). The purified commercial *Clostridium* collagenase that we used had a specific activity of 33 nkat/mg. This represents about 8% of the specific activity of the pure enzyme. The major contamination is the inactive enzyme and its degradation products (I. Emöd & B. Keil, unpublished work). On the other hand, it was substantially free of proteinase and peptidase activity. This presumably contributed to the relative stability of the large *N*-terminal fragments in the incubation mixture. It is not excluded that if the digestions were run with a fully homogeneous *Clostridium* collagenase, the number of cleavage sites would be lower and closer to those observed with *Achromobacter* collagenase.

This study permits a new comparison of the specificity of prokaryotic and eukaryotic collagenases. One of the two regions cleaved by the collagenase from *Achromobacter iophagus*, the region of bands 40–42,

is the same as that cleaved by most of the animal collagenases. Animal collagenases generally cleave collagen at a site three-quarters of the length of the molecule from the *N*-terminus, yielding two fragments (Harris & Krane, 1974a,b,c). Electron-microscopy studies have localized the point of cleavage by vertebrate collagenases of different origins exactly between the bands 41 and 42 (Harper *et al.*, 1971; Leibovich & Weiss, 1973), except for the collagenase from gastric mucosa, which cleaves between bands 43 and 44 (Woolley *et al.*, 1976). Cleavage at the interband 41–42 was shown to occur at the Gly⁷⁷²–Ile⁷⁷³ bond of the α_1 -chain and at the homologous Gly–Leu bond of the α_2 -chain (Gross *et al.*, 1974). For the gastric-mucosa enzyme, the bond cleaved was not determined, but it cannot be a Gly–Ile bond, since in that region of the chain there is no other Gly–Ile bond than that corresponding to the residues 772–773. We found recently (A. Lecroisey & B. Keil, unpublished work) that an insect collagenase from the larvae of *Hypoderma lineatum* also cleaves collagen at the interband 40–42, but at an X–Ala bond. Trypsin, which does not cleave types I and II collagen, cleaves type-III collagen between arginine-780 and glycine-781, a bond that does not exist in other types of collagen at this position (Miller *et al.*, 1976). All these facts are in agreement with the hypothesis of Leibovich & Weiss (1973) for a 'weak' helical portion in the collagen molecule between the bands 41 and 44. The collagenase from *Achromobacter iophagus* apparently has a wider specificity than the vertebrate collagenases in cleaving the collagen

molecule in two loci instead of a single one. It is, however, noteworthy that some animal collagenases, which cleave native collagen initially at the interband 41–42, can continue to degrade the large three-quarter *N*-terminal fragment up to band 33 (Eisen & Jeffrey, 1969; Dresden & Gross, 1970; Jeffrey & Gross, 1970; Tokoro *et al.*, 1972). One may therefore assume that all prokaryotic and eukaryotic proteinases that cleave native collagen are strongly influenced by the spatial organization of a relatively narrow region of the substrate. However, the fact that the bonds cleaved within this region vary from one enzyme to another reflects differences in their fixation sites and, consequently, in their sequential specificity.

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