ANIMAL COLLAGENASES: SPECIFICITY OF ACTION, AND STRUCTURES OF THE SUBSTRATE CLEAVAGE SITE*

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SUMMARY:

Two highly purified animal collagenases, one derived from homogenates of the rabbit V_2 ascites cell carcinoma growing in muscle and the second isolated from the media of tadpole tissue cultures cleaved isolated non helical α chains from chick and rat skin collagen, and the CNBr peptide CB7 from chick skin $\alpha 1$ chains at one, and the same peptide bond. Although two other Gly-Ile bonds exist elsewhere in the $\alpha 1$ chain they were not cleaved.

A unique feature of the mode of action of most of the known animal collagenases (1, 2) is their production of a single cleavage at a specific locus one quarter the length of the intact collagen molecule from the COOH-terminus in each of the three α chains, first demonstrated for the prototype enzyme, the tadpole collagenase (3).

Nagai and colleagues (4) reported the release by this purified enzyme of one leucine and two isoleucine NH_2-terminal residues, and three glycine COOH-termini from native collagen molecules in solution following attack by purified tadpole collagenase at neutral pH and 20°C. Using denatured collagen as a substrate however, resulted in the release of NH_2-terminal leucine, isoleucine,

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alanine, phenylalanine, and valine. Recently Harris et al. (5) observed that a purified collagenase obtained from homogenates of rabbit ascites cell carcinoma (6) cleaved both native and denatured collagen at a single locus liberating the characteristic one quarter and three quarter length fragments from α chains. Harris and Krane (7) had previously noted that gelatin, as compared with native collagen, was only weakly attacked and reduced to large peptide fragments by partially purified rheumatoid synovial and rheumatoid nodule collagenase.

McCroskery and associates (6) proposed that purified collagenase cleaves both collagen and gelatin at the same single locus and that the latter is a poor substrate; the earlier results of Nagai et al. (4) probably reflected contamination of the tadpole enzyme with other protease(s).

The high degree of purification of the rabbit tumor and tadpole collagenases has now made it possible to study the amino acid sequence around the unique cleavage site in triple helical molecules, α chains and cyanogen bromide peptides. This paper reports the amino acid sequences at the NH_2-terminal end of the COOH terminal fragment released by these enzymes.

The rabbit tumor collagenase was purified from homogenates of V_2 ascites cell carcinoma formed after injecting tumor cells into the thigh muscle of adult rabbits as described previously (6). The homogenates were freeze-thawed and the collagenolytic activity was extracted in 0.1 M Tris-HCl, buffer pH 7.6, containing 0.005 M $CaCl_2$, and purified by ammonium sulfate fractionation (20-50% saturation) followed by ion exchange chromatography on DEAE Sephadex A-50. Final fractionation by molecular sieve chromatography was accomplished on agarose (Bio Gel A-1.5). The resulting enzyme preparation had a specific activity (mg collagen degraded per hr per mg of enzyme) over 5000 times that of the original homogenate. Highly purified tadpole tail fin

collagenase was prepared from crude third day culture medium by the method of Harper et al. (8) which includes ammonium sulfate precipitation (0-30% saturation precipitate) followed by chromatography on agarose. The ascending limb of the first of the two collagenase peaks was then further purified by affinity chromatography on collagen-Sephadex 4B columns (9).

Acid-extracted collagen from skins of 3 week old white Leghorn chicks was prepared, and purified (10), and separated into $\alpha 1$ and $\alpha 2$ chains by chromatography on carboxymethylcellulose (11). Purified homogeneous cyanogen bromide peptides $\alpha 1$-CB8 and $\alpha 1$-CB7 were prepared from $\alpha 1$ chains (12).

Purified tadpole collagenase and $\alpha 1$ or $\alpha 2$ chains were mixed in an enzyme-substrate ratio of about 1:80, and the reaction mixture was incubated at 37° C in a pH stat for various time periods. After a maximum rise in alkali addition was reached, more enzyme was added without further cleavage (at about 3 hours incubation). Twenty mg samples of reaction mixture were dialyzed extensively against 0.01 M acetic acid, lyophilized and prepared for sequencing without further fractionation.

Since the T_m for $\alpha 1$-CB7 was about 22° C and cleavage by rabbit tumor collagenase was negligible at this temperature the reaction with this enzyme was run with denatured CNBr peptides. Incubation was carried out at 37° using 200 µg purified rabbit tumor collagenase and 9.0 mg of either $\alpha 1$-CB7 or $\alpha 1$-CB8 in 0.1 Tris-HCl pH 7.6, 0.005 M $CaCl_2$ for 26 hours. The reaction products were monitored by SDS polyacrylamide electrophoresis and the bulk of it was fractionated on a Bio-Gel column into a low molecular weight fragment, another larger peptide and lesser amount of undigested starting material. The lowest amount was undigested starting material. The lowest molecular weight fragment presumably from the COOH-terminal region of the cleaved $\alpha 1$-CB7 was desalted, lyophilized, analyzed for amino acid composition and sequenced.

Automated sequential degradation was performed with a Beckman model 890B sequencer according to the method of Edman and Begg (13). The samples were dissolved in glass-distilled water and sequentially degraded with the Quadrol Single Cleavage Program. Identification of residues was accomplished by gas-liquid chromatography (14) on 10% SP-400 in the Beckman GC-45 instrument and also in an automated JEOLCO model 5AH amino acid analyzer after back hydrolysis.

The low molecular weight fragment isolated from the reaction mixture of the chick α1-CB7 and purified tumor collagenase gave the unique sequence shown in line 3, Table 1. This is identical to the sequence of 12 amino acids in α1-CB7 from both calf (15) and chick (16) skin beginning at the previously established cleavage site, Gly-Ile. Since there is only one Gly-Ile sequence

TABLE I

NH_2-TERMINAL SEQUENCES AT ANIMAL COLLAGENASE CLEAVAGE SITE

	1	2	3	4	5	6	7	8	9	10	11	12
1) Calf Skin α1-CB7*	Ile	Ala	Gly	Gln	Arg	Gly	Val	Val	Gly	Leu	Hyp	Gly
2) Chick Skin α1-CB7*	Ile	Ala	Gly	Gln	Arg	Gly	Val	Val	Gly	Leu	Hyp	Gly
3) (RT)** Chick α1-CB7	Ile	Ala	Gly	Gln	Arg	Gly	Val	Val	Gly	Leu	Hyp	Gly
4) (Tp)** Chick α1-CB7'	Ile	Ala	Gly									
5) (Tp) Chick α1	Ile	Ala	Gly	Gln	Arg	Gly	Val	-	Gly	Leu	-	Gly
6) (Tp) Chick α2	Leu	Ala	Gly									

*Sequence taken from that published by Fietzek et al. (16) for entire α1-CB7 of calf skin, and the sequence for chick skin was obtained from unpublished data of Highberger, Corbett, Kang and Gross.

**RT = Rabbit Tumor Collagenase; Tp = Tadpole Collagenase

in α1-CB7 the order of amino acids on either side of cleavage site for calf and chick skin α1 type chains is now known. Purified chick skin α1-CB7', probably an artifactual variant of α1-CB7 (either a partially deamidated product or one containing a free carboxyl homoserine instead of a lactone (17)) was reacted with tadpole collagenase and the sequence obtained from the resulting product was identical with the first three residues of the NH_2-terminal side of the cleavage site of α1-CB7 (Table 1).

Since Gly-Ile is also present in α1-CB8, located near the NH_2-terminus of the α1 chain, an effort was made to cleave this peptide with the rabbit tumor collagenase; no degradation was noted.

Analysis of the unfractionated reaction products of chick skin α1 chain yielded a single sequence from the amino terminal end of the 1/4 length fragment (line 5; Table 1); pyrrolidone-carboxylic acid blocks the amino terminus of the intact α1 chain (18). Although two residues, at positions 8 and 11 in the chick skin sequence, were unresolved, it is apparent that these are identical with those obtained from chick skin α1-CB7 (Table 1, line 2). Cleavage of purified chick α2 chains with tadpole collagenase was also accomplished, yielding a predominant sequence of three residues beginning with leucine as predicted from the earlier studies (4).

We have shown that highly purified animal collagenases from two widely divergent species, one mammalian and one amphibian, cleaved the collagen molecule in both the native form or as isolated α chains at one specific peptide bond; the Gly-Ile sequence in α1-CB7. In addition, the tadpole enzyme cleaved the homologous and adjacent peptide Gly-Leu in the same region of the isolated α2 chain. Thus the substrate specificity for these two different animal collagenases is identical. The tadpole collagenase, like that from the mammalian tumor enzyme, cleaves the denatured α chain at the same single peptide bond

Figure I

	Sequence Around Animal Collagenase Cleavage Site (α1-CB7)
Chick* Skin	Gly·Ala·Hyp·Gly·Thr·Pro·Gly·Pro·Gln·Gly·Ile·Ala·Gly·Gln·Arg·Gly·Val·Val·Gly·Leu·Hyp
Calf** Skin	Gly·Ala·Hyp·Gly·Thr·Pro·Gly·Pro·Gln·Gly·Ile·Ala·Gly·Gln·Arg·Gly·Val·Val·Gly·Leu·Hyp
	Sequence Around Gly · Ile in other Parts of α1 - Uncleavable
CB6** (Calf)	Gly·Glx·Thr·Gly·Glx·Glx·Gly·Asx·Arg·Gly·Ile·Hyl·Gly·His·Arg·Gly·Phe·Ser·Gly·Leu·Gln
CB8 + (Rat)	Gly·Ala·Lys·Gly·Ala·Asn·Gly·Ala·Hyp·Gly·Ile·Ala·Gly·Ala·Hyp·Gly·Phe·Hyp·Gly·Ala·Arg

* Highberger, Corbett, Kang, Gross (in preparation)
** Reference 20
\+ Reference 19

and does not make multiple scissions as previously reported.

The isolated 268 residue CNBr peptide, α1-CB7, containing the cleavage site is a substrate for both enzymes, whereas α1-CB8 (279 residues), also containing a Gly-Ile peptide bond, is not cleaved. Apparently, the entire α chain is not required for the action of either enzyme.

Since we know that the 10 residues on either side of the cleavage site in the α1 chain are unique in that they are not repeated anywhere in the 1,052 residues, it is worth comparing this region with those surrounding Gly-Ile in other known regions of the α1 chain, namely, calf skin α1-CB6 and rat skin α1-CB8 (Figure 1). These two peptides from the α1 chain of a single species have not yet been sequenced, however we expect close homologies between species. The 20-residue sequence in the cleavage site region of α1-CB7 of both chick (16) and calf skin (15) are identical. Those surrounding Gly-Ile in calf α1-CB6 and rat α1-CB8 differ from those in α1-CB7 in essentially every position except that for glycine. There is no reason to suspect from simple examination of sequences that the physical characteristics of the helix in the cleavage site region of α1-CB7 should be qualitatively different from those in other regions of the molecule. It seems likely that the animal collagenases have a specific affinity for the particular amino acid sequence found in α1-CB7 and in the homologous region of the α2 chain for reasons yet to be determined.

We would predict that the sequences in the cleavage site region in α2-CB5 are very closely homologous to that in α1-CB7. A study of this region in type II and III collagens and of the relative susceptibility of synthetic substrates homologous to this region but varying in length and specific amino acid residues will be profitable.

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