Isolation and Characterization of the Cyanogen Bromide Peptides from the α1(II) Chain of Chick Cartilage Collagen*

Edward J. Miller†

ABSTRACT: The 13 peptides obtained by cleavage of the α1(II) chain of chick sternal cartilage collagen with cyanogen bromide have been isolated by ion-exchange and molecular sieve chromatography and characterized with regard to amino acid composition and molecular weight. The isolated peptides are clearly distinguishable as unique portions of the α1(II) chain and together they account for all the amino acids and molecular weight of the chain. The peptides are recovered from digests of α1(II) in equimolar amounts. On the basis of size, compositional features, and chromatographic properties, five of the cyanogen bromide peptides derived from α1(II) may be tentatively identified as homologs of peptides derived from the α1(I) chain of chick bone and skin collagen as well as the skin collagens of several other species. The remaining eight peptides from α1(II) have no obvious counterparts in digests of α1(I) indicating a markedly different distribution of methionyl residues. The COOH-terminal end of α1(II) was found to have the sequence Met-Tyr which accounts for the presence of tyrosine in cyanogen bromide digests of the chain.

Preliminary comparisons of peptides derived from chick cartilage α1(II) with those derived from human cartilage collagen indicate a striking similarity in the number of peptides released as well as their amino acid compositions and chromatographic properties. These results indicate that cartilage collagens represent a separate and genetically distinct family of collagens that may be specifically adapted to the function of cartilaginous tissues.

Recent studies on collagen extracted from various tissues of the lathyritic chick have demonstrated that collagen derived from sternal cartilage contains a genetically distinct type of α1 chain, α1(II), which is not present in collagen extracted from chick bone or skin (Miller and Matukas, 1969). Subsequent work indicated that the α1(II) chain comprises approximately 90% of the extractable collagen in chick sternal cartilage, and that this collagen may be characterized as a mixture of molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ which are common to bone, skin, and cartilage, and $[\alpha 1(II)]_3$ which is apparently restricted to cartilaginous structures (Trelstad *et al.*, 1970; Miller, 1971). These studies further indicated that by employing repeated short extraction periods for cartilage preparations, the α1(II) chain could be isolated in pure form by CM-cellulose chromatography of the collagen solubilized in later extracts.

In order to characterize further the α1(II) chain, the technique of cyanogen bromide (CNBr) cleavage has been applied. The results of this study, the isolation and characterization of 13 unique peptides accounting for all the amino acids and molecular weight of the α1(II) chain, provide additional evidence confirming that synthesis of α1(II) proceeds from a different genetic locus than that responsible for synthesis of α1(I) (Miller and Matukas, 1969; Trelstad *et al.*, 1970; Miller, 1971). In addition, this work not only provides relatively short fragments of α1(II) which are suitable for further studies, but also affords the opportunity for a detailed comparison of chick cartilage collagen with the homologous skin and bone collagens of several species as well as with cartilage collagen from other species.

In the present paper, the previously established nomenclature for collagen CNBr peptides (Miller *et al.*, 1969b) is not used, since several of the peptides from cartilage collagen α1(II) bear no obvious homology to the peptides derived from the α1(I) chain of rat skin (Butler *et al.*, 1967), chick bone (Miller *et al.*, 1969b), chick skin (Kang *et al.*, 1969), calf skin (Rauterberg and Kühn, 1968), baboon skin (Epstein *et al.*, 1971), and human skin (Click and Bornstein, 1970; Epstein *et al.*, 1971). Therefore, the peptides have been assigned numbers which, for the most part, denote their order of elution from cation-exchange columns.

Materials and Methods

Source and Preparation of α1(II). α1(II) was prepared by CM-cellulose chromatography of the collagen obtained in third and fourth extracts of sternal cartilages from lathyritic chicks as previously described (Miller, 1971).

Cleavage with CNBr. Cleavage with CNBr was performed as described previously (Miller *et al.*, 1971; Epstein *et al.*, 1971). In the present study, samples of α1(II) weighing 40–50 mg were dissolved in 10 ml of 70% formic acid. The solution was flushed with nitrogen and a weight of CNBr equal to a 150-fold molar excess relative to the methionyl residues of the dissolved collagen was added. The reaction mixture was incubated at 30° for 4 hr, diluted 10-fold with distilled water, and lyophilized. The dried peptides were redissolved in 50 ml of water and relyophilized to ensure complete removal of CNBr and formic acid.

Molecular Sieve Chromatography, P-4. Initial separations of the CNBr peptides from α1(II) were achieved on a 1.7 × 80 cm column of Bio-Gel P-4 (100–200 mesh, Bio-Rad Laboratories) equilibrated with 0.1 N acetic acid. The lyophilized peptides were dissolved at a concentration of 20 mg/ml in 0.1 N acetic acid and 1-ml aliquots were applied to the column which was eluted with the same solvent at a flow rate of 60 ml/hr. The column effluent in this procedure and all other chromatography (see below) was monitored and recorded as

* From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014. *Received April 9, 1971.*

† Present address: Department of Biochemistry and Institute for Dental Research, University of Alabama in Birmingham, Birmingham, Ala. 35233.

previously described (Miller *et al.*, 1969b). Appropriate fractions comprising the peaks in the P-4 eluent were combined, lyophilized, and rechromatographed in the same system.

Chromatography of CNBr Peptides on Phosphocellulose. The peptides eluted in various peaks from the P-4 column were further resolved by chromatography on a 1.7 × 8.0 cm column of phosphocellulose (Whatman, floc, capacity 7.4 mequiv/g) utilizing conditions similar to those previously described (Bornstein and Piez, 1966; Miller *et al.*, 1969b). In the present studies, gradient conditions were modified to allow optimal resolution of the peptides from α1(II) (see legends for Figures 2, 3, and 4).

Chromatography of CNBr Peptides on CM-cellulose. Several of the larger peptides eluted in the exclusion volume of the P-4 column were not resolved on phosphocellulose and were rechromatographed on a 1.7 × 9.0 cm column of CM-cellulose (Whatman CM 32, microgranular, capacity 1.0 mequiv/g). The column was equilibrated with starting buffer (0.02 M sodium citrate, adjusted to pH 3.6 with citric acid) and the peptides were dissolved in this buffer prior to application to the column. Elution was achieved with a linear salt gradient prepared from 500 ml of starting buffer and 500 ml of limit buffer (starting buffer containing 0.14 M NaCl) at a flow rate of 100 ml/hr and a column temperature of 42°.

Molecular Sieve Chromatography, Bio-Gel A-1.5. With the exception of peptides 1, 2, 3, and 13, all peptides eluted from ion-exchange columns were desalted on a column of Bio-Gel P-2 and further purified and resolved by chromatography on a 1.8 × 230 cm column of Bio-Gel A-1.5 (200–400 mesh, Bio-Rad Laboratories) as previously described (Miller *et al.*, 1969b). Molecular weight determinations were also made on the peptides chromatographed on the agarose column in the manner described by Piez (1968).

Amino Acid Analyses. Amino acid analyses of purified peptides were performed on an automatic amino acid analyzer (Miller and Piez, 1966) after hydrolysis and preparation of the samples as previously described (Miller *et al.*, 1969b). In calculating results, corrections were made for the destruction of threonine, serine, and tyrosine, and incomplete release of valine during hydrolysis (Piez *et al.*, 1960).

Digestion of α1(II) with Carboxypeptidase A. In order to characterize more definitively the amino acid sequence at the COOH terminus of α1(II), digestion with carboxypeptidase A was employed (Potts, 1967). A suspension of carboxypeptidase A (CPA-DFP, Worthington Biochemical Corp.) containing 5 mg of enzyme was washed three times with distilled water and dissolved in 2.5 ml of 2.0 M NH_4HCO_3 (pH 8.0). An aliquot of the enzyme solution, 0.5 ml, was added to 20 mg of α1(II) dissolved in 4.5 ml of water. Incubation of substrate and enzyme was allowed to proceed at 25° for 8 and 16 hr, one-half the sample being utilized at each time period. Samples taken at 8 and 16 hr were imeediately passed over a calibrated 3.0 × 35 cm column of Bio-Gel P-2 equilibrated with 0.2 M NH_4HCO_3. The column was eluted with the same solvent and that portion of the eluent representing the total included volume plus additional eluent (in order to collect fractions containing adsorbed material) was lyophilized. Lyophilization of the samples was repeated twice to remove essentially all NH_4HCO_3. Amino acid analyses on amino acids released by carboxypeptidase were performed as described above.

Results

Chromatography of CNBr Peptides on P-4. As depicted in Figure 1, the CNBr peptides from α1(II) could be resolved into four fractions by molecular sieve chromatography on P-4. The peak eluted at approximately 110 ml and designated peptide 13 has a K_{av} greater than unity. Amino acid analyses of the material in this peak, both with and without hydrolysis, demonstrated that the peak contained free tyrosine which had been separated from other components in the digestion mixture by its tendency to adsorb to the gel. No other amino acids were detected in this portion of the P-4 eluent, and the amount of tyrosine recovered in each instance represented that expected for a "peptide" equivalent based on the amount of sample applied to the column.

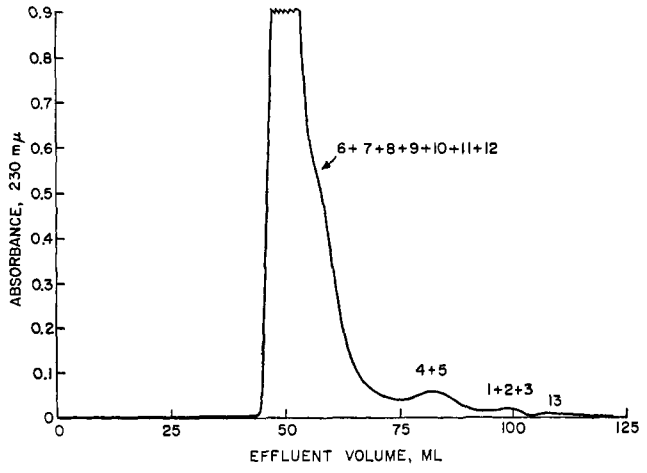

FIGURE 1: Elution pattern of the CNBr peptides derived from 30 mg of α1(II) from chick sternal cartilage collagen when chromatographed on Bio-Gel P-4. The column was equilibrated and eluted with 0.1 N acetic acid. Fractions chosen for further chromatography comprised the elution volumes between 45–70 75–90, and 95–110 ml.

These results indicating that tyrosine is the COOH-terminal amino acid in α1(II) and that methionine is the penultimate amino acid were verified by carboxypeptidase A digestion of the chain. Within 8 hr, digestion of 20 mg of α1(II) (approximately 0.2 μmole assuming a molecular weight of 100,000) with carboxypeptidase A released 0.184 μmole of tyrosine and 0.039 μmole of methionine. At 16 hr, the amount of tyrosine released remained unaltered while methionine increased to 0.061 μmole. No other amino aicds were detected as products of enzyme treatment. The slow and incomplete release of methionine observed in these studies may possibly be attributed to the presence of a glycyl or prolyl residue in the next position in the chain.

Phosphocellulose Chromatography of CNBr Peptides. The peptides eluted from the P-4 column at approximately 100 ml (the total fluid volume of the column) were further resolved by chromatography on phosphocellulose (Figure 2). The doublet peak emerging at the start of the gradient contained largely nonprotein ultraviolet-absorbing material along with a dipeptide, designated peptide 1. The presence of two small peptides, 2 and 3, in the other peak was ascertained on the basis of the amount of homoserine in this fraction when compared to that in single peptides (see Table II). No attempts were made to further resolve peptides 2 and 3.

Peptides 4 and 5 which were eluted in the second peak from the P-4 column were resolved by rechromatography on phosphocellulose as shown in Figure 3.

Two of the peptides emerging in the first peak from the P-4 column, peptides 6 and 7, were resolved by rechromatography

TABLE I: Amino Acid Compositions[a] of the CNBr Peptides of the α1(II) Chain of Chick Sternal Cartilage.

Amino Acid	1	2 + 3	4	5	6	7	8	9	10	11	12	13	Total CNBr Peptide	α1[b] (II)
3-Hydroxyproline	0	0	0	0	0	1 (1.3)	0	1 (0.8)	0	0	0	0	2	2.2
4-Hydroxyproline	0	0	0	0	5 (5.2)	8 (7.8)	16	4 (3.7)	30	30	11	0	104	106
Aspartic acid	0	0	1 (1.0)	0	1 (1.0)	2 (2.1)	5 (5.2)	4 (3.9)	17	9 (9.1)	4 (4.0)	0	43	43
Threonine	0	0	0	0	1 (1.0)	1 (1.0)	5 (5.0)	2 (2.1)	7 (6.9)	8 (7.8)	2 (2.2)	0	26	27
Serine	0	0	0	1 (1.0)	0	1 (1.1)	4 (3.8)	4 (4.0)	6 (6.0)	7 (7.0)	4 (3.8)	0	27	27
Glutamic acid	1 (0.9)	0	2 (2.1)	0	4 (4.1)	2 (2.0)	15	6 (5.8)	29	22	7 (7.1)	0	88	89
Proline	0	1 (1.1)	0	2 (2.2)	7 (6.9)	8 (7.9)	14	10	36	29	6 (6.2)	0	113	118
Glycine	0	2 (2.2)	4 (4.1)	4 (4.0)	12	14	49	23	105	90	28	0	331	336
Alanine	0	0	3 (2.8)	2 (2.1)	3 (3.1)	2 (2.1)	13	4 (3.9)	40	31	5 (5.0)	0	103	106
Valine	0	1 (0.9)	0	0	0	0	3 (3.1)	1 (1.0)	6 (6.2)	4 (4.1)	1 (1.0)	0	16	16
Isoleucine	0	0	0	0	0	2 (2.2)	0	0	2 (2.1)	3 (3.0)	0	0	7	7.2
Leucine	0	0	0	0	0	0	8 (8.0)	3 (3.0)	6 (6.1)	7 (7.1)	2 (2.1)	0	26	27
Tyrosine	0	0	0	0	0	0	0	0	0	0	1 (1.0)	1 (1.0)	2	2.0
Phenylalanine	0	0	1 (1.0)	0	1 (1.0)	0	3 (2.8)	1 (.10)	3 (3.2)	4 (4.2)	1 (1.0)	0	14	15
Hydroxylysine[d]	0	0	1.0	0	0	0	3.0	2.2	8.4	7.6	2.9	0	25.1	24
Lysine[d]	0	0	0	0	0	0	3.0	0.9	3.6	2.3	3.1	0	12.9	13
Histidine	0	0	0	0	0	0	0	1 (1.0)	0	0	1 (1.0)	0	2	2.0
Arginine	0	0	0	1 (1.0)	1 (1.0)	2 (2.0)	7 (6.9)	4 (4.1)	16	17	4 (3.9)	0	52	51
Homoserine	1 (1.0)	2 (1.8)	1 (1.0)	1 (0.9)	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.1)	1 (1.0)	1 (1.1)	1 (1.0)	0	12	12[c]
Total	2	6	13	11	36	44	149	72	316	272	84	1	1006	1023

[a] Residues per peptide rounded off to the nearest whole number. Actual values are listed in those instances where less than ten residues are present. A value of zero indicates less than 0.1 residue. [b] Values in this column have been calculated on the basis of an average residue molecular weight of 92.7 and a molecular weight of 95,000 for the α1(II) chain. [c] Represents methionine in α1(II). [d] Values for lysine and hydroxylysine have not been rounded off since there is evidence for partial hydroxylation giving rise to noninteger values (Butler, 1968; Miller *et al.*, 1969b).

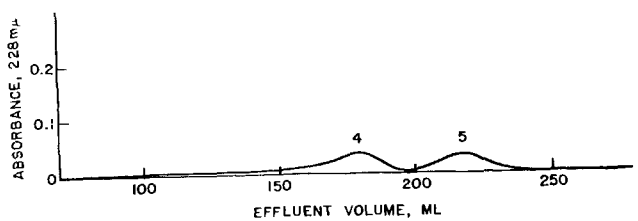

FIGURE 3: Phosphocellulose elution pattern of the peptides eluted at approximately 75 ml in the P-4 eluent (Figure 1). Elution was achieved in 0.001 M (Na^+) sodium acetate (pH 3.8) using a linear salt gradient of NaCl from 0 to 0.2 M in a total volume of 600 ml.

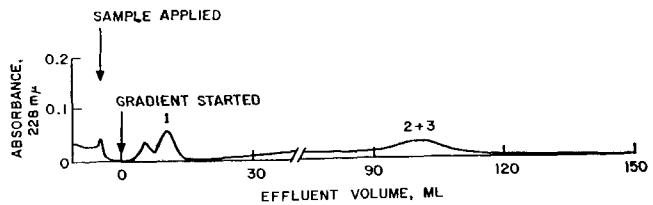

FIGURE 2: Phosphocellulose elution pattern of the peptides eluted at approximately 100 ml (the total fluid volume of the column) in the P-4 eluent (Figure 1). Elution was achieved in 0.001 M (Na^+) sodium acetate (pH 3.8) using a linear salt gradient of NaCl from 0 to 0.1 M. The total volume of the gradient was 500 ml.

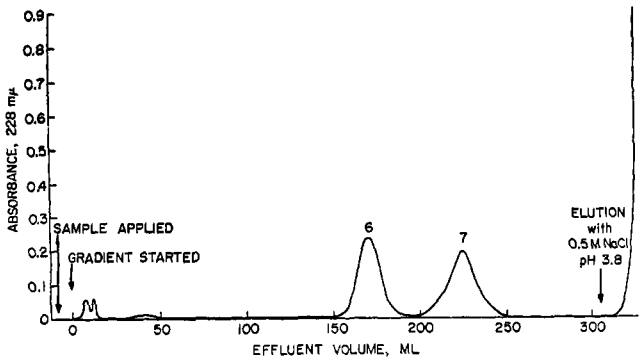

FIGURE 4: Phosphocellulose elution pattern of some of the peptides eluted in the exclusion volume in the P-4 eluent (Figure 1). Elution was achieved by employing conditions identical with those described in Figure 3. Peptides not resolved by this procedure were eluted at the designated volume by increasing the NaCl concentration to 0.5 M.

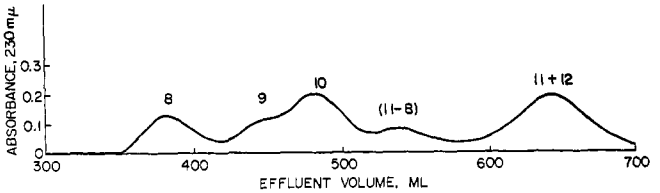

FIGURE 5: CM-cellulose elution pattern of the larger CNBr peptides from α1(II) which are eluted in the exclusion volume of the P-4 column (Figure 1). Elution was achieved in 0.02 M (Na^+) sodium citrate (pH 3.6) using a linear salt gradient of NaCl from 0 to 0.14 M in a total volume of 1000 ml.

on phosphocellulose as shown in Figure 4. The remaining peptides were eluted at the designated time as one peak by washing the column with starting buffer containing 0.5 M NaCl.

CM-cellulose Chromatography of Remaining CNBr Peptides. The peptides eluted as one peak from phosphocellulose (see above) were further resolved by rechromatography on CM-cellulose as illustrated in Figure 5. Although peptides 9 and 10 were incompletely resolved by this procedure and peptides 11 and 12 were not resolved at all, the molecular weights (see Table III) of these peptides are sufficiently different to allow resolution by rechromatography of the respective regions on Bio-Gel A-1.5. The peak labeled 11-8 contains material with a molecular weight and animo acid composition which indicates that it results from incomplete cleavage of the methionyl residue joining these two peptides.

Amino Acid Composition of CNBr Peptides from α1(II). Amino acid analyses of the 13 CMBr peptides of α1(II) are presented in Table I. The data listed in the last two columns of Table I clearly indicate that the isolated peptides account for all the amino acids in α1(II). Since the α1(II) chain contains 12 methionyl residues the 13 peptides represent the expected number after cyanogen bromide cleavage.

Stoichiometry of Peptides Derived from α1(II). Assuming that each peptide (with the exception of the COOH-terminal peptide) would contain one residue of homoserine, the relative yields of the peptides isolated in this study were determined on the basis of the amount of homoserine in each peptide. For this purpose a single preparation of 35 mg of α1(II) was treated with CNBr and the peptides were isolated as indicated above. A known aliquot of each peptide was hydrolyzed and analyzed in order to confirm the purity of the preparation and determine homoserine content. In Table II the results of these determinations are presented in terms of the quantity of homoserine in each peptide and relative homoserine content. It is evident that the peptides are recovered in approximately equivalent amounts.

Molecular Weights of CNBr Peptides from α1(II). The molecular weights of the CNBr peptides from the α1(II) chain of chick sternal cartilage collagen are listed in Table III. With the exception of peptides 1, 2 and 3, and 13, the molecular weights were calculated both by amino acid analyses and from elution volumes of the peptides on a calibrated agarose molecular sieve column. The values obtained by both methods

TABLE II: Stoichiometry of the CNBr Peptides of the α1(II) Chain from Chick Sternal Cartilage Collagen.

Peptide	Homoserine Content (μmole)	Rel Homoserine Content[a]
1	0.317	1.0
2 + 3	0.588	1.8
4	0.336	1.0
5	0.309	0.9
6	0.326	1.0
7	0.297	0.9
8	0.307	0.9
9	0.273	0.8
10	0.281	0.9
11	0.300	0.9
12	0.269	0.8
13[b]	0.271	0.8

[a] The data in this column are expressed in terms of the quantity of homoserine recovered in peptide 6 which was assigned a value of 1.0. [b] For peptide 13, the data indicate tyrosine content.

TABLE III: Molecular Weights of the CNBr Peptides from the α1(II) Chain of Chick Sternal Cartilage Collagen as Determined by Amino Acid Analysis and Molecular Sieve Chromatography.

Peptide	Amino Acid Anal.	Mol Sieve Chromatography
1	278	278[a]
2 + 3	573	573[a]
4	1,156	1,200
5	957	1,000
6	3,291	3,200
7	4,101	4,000
8	13,929	13,200
9	6,775	6,400
10	28,914	30,000
11	25,162	25,000
12	8,114	7,600
13	181	181[a]
Total	93,431	92,632

[a] The molecular weights of peptides 1, 2 + 3, and 13 were calculated only from amino acid analyses.

of calculation agree quite well. The total molecular weight of the isolated peptides is approximately 93,000 by both methods. This value is well within the range of the value of about 95,000 for whole α chains as determined by sedimentation equilibrium (Lewis and Piez, 1964; Kang *et al.*, 1966) and is also quite close to the total molecular weight (92,000) of the ten CNBr peptides derived from the α1(I) chain of chick bone (Miller *et al.*, 1969b) and skin (Kang *et al.*, 1969) collagens.

Discussion

The present work has shown that cleavage of the α1(II) chain of chick sternal cartilage collagen with CNBr gives rise to thirteen unique peptides which have been characterized with regard to amino acid composition and molecular weight. It has been further demonstrated that the isolated peptides which correspond to the number expected in CNBr digests of α1(II) are present in equimolar amounts and together the peptides account for all the amino acids and molecular weight of the chain. These results extend and confirm the previous conclusion based on chromatographic properties of collagen extracted from chick sterna, that the majority of the collagen in this tissue is comprised of molecules containing three identical α1(II) chains (Trelstad *et al.*, 1970; Miller, 1971). In addition, these studies demonstrate that α1(II) and α1(I) have identical or very similar lengths and molecular weights as previously indicated by molecular weight determinations of these chains by molecular sieve chromatography (Miller, 1971).

Among the 13 CNBr peptides derived from chick cartilage α1(II), there are several whose size and amino acid composition suggest a probable homology with the CNBr peptides from the α1(I) chain of chick bone (Miller *et al.*, 1969b) and skin (Kang *et al.*, 1969) collagens as well as the collagens of several other vertebrate species (Butler *et al.*, 1967; Rauterberg and Kühn, 1968; Bornstein, 1969; Click and Bornstein, 1970; Epstein *et al.*, 1971). Peptide 1 from α1(II) is identical with α1(I)-CB0, the NH_2-terminal peptide of α1(I). Peptide 6 closely resembles α1(I)-CB2 and a detailed comparison of these peptides from chick collagens has previously appeared (Miller and Matukas, 1969). Peptides 8 and 11 are quite similar in size, compositional features, and chromatographic properties to α1(I)-CB3 and α1(I)-CB8, respectively. Moreover, the isolation of 11-8, a peptide comprised of peptides 8 and 11 joined by a methionyl residue further indicates homology for these peptides since it is known that α1(I)-CB8 lies adjacent to α1(I)-CB3 in α1(I) (Piez *et al.*, 1969). Peptide 12 has the same number of amino acids as α1(I)-CB4 plus α1(I)-CB5 and exhibits many of the compositional characteristics of the region represented by the latter peptides. These include the presence of more hydroxyproline than proline and a histidyl residue.

The remainder of the peptides from α1(II) (2, 3, 4, 5, 7, 9, 10, and 13) have no obvious homologs among the CNBr peptides from α1(I) and, with the exception of peptide 13, the COOH-terminal amino acid, cannot be assigned a probable position along the α1(II) chain at this time.

Of further interest in this regard is the observation that a peptide obviously homologous to α1(I)-CB1 and representing a nonhelical, NH_2-terminal cross-link region (Bornstein and Piez, 1966) is lacking in the peptides derived from α1(II). If, as pointed out above, peptides 1 and 6 are homologous to α1(I)-CB0 and α1(I)-CB2, respectively, the intervening sequence of seventeen amino acids homologous to α1(I)-CB1 would most likely be comprised of peptide 4 plus 2 or 3 in α1(II). Although the analysis of peptide 4 suggests it could be derived from a helical portion of the molecule since it contains one-third glycine, the distribution of glycyl residues within the peptide is not known. In addition, peptide 4 contains a single hydroxylysyl residue which could participate in cross-link formation giving rise to cross-links the structures of which require the utilization of a hydroxylysyl (Bailey and Peach, 1968; Tanzer and Mechanic, 1970; Mechanic and Tanzer, 1970) residue or a hydroxyallysyl (Bailey *et al.*, 1969) residue. Although a hydroxylysine-derived aldehyde form of peptide 4 was not detected in these studies, this may be attributed to the nature of the collagen used, *i.e.*, an easily solubilized fraction from the cartilages of lathyritic animals in which aldehyde formation had been inhibited.

The present studies also provide basic data allowing a detailed comparison of chick cartilage collagen with cartilage collagen of other species. This is particularly important in the case of human cartilage collagen which, similar to chick cartilage collagen, is extremely insoluble (Miller *et al.*, 1969a) and where the option of enhancing cartilage collagen solubility by the administration of lathyrogens is not readily available. It has recently been demonstrated (Miller *et al.*, 1971), however, that insoluble human collagens may be studied in considerable detail by examining the peptides released after CNBr digestion of the collagen which resists extraction. These studies showed that the CNBr peptides derived from human epiphyseal cartilage collagen closely resembles those reported here for chick cartilage α1(II) and the amino acid compositions and molecular weight of the human peptides homologous to peptides 9 and 12 from chick α1(II) were reported. These data indicate a greater degree of structural homology between chick and human cartilage collagens than is found when comparing cartilage with bone and skin collagens within each species. These considerations indicate that cartilage collagens represent a separate and genetically distinct series of collagens that may be specifically synthesized for and adapted to the functional requirements of cartilaginous tissues.

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The Zymogen of Tadpole Collagenase*

Elvin Harper,†,‡ Kurt J. Bloch, and Jerome Gross

ABSTRACT: A precursor of tadpole collagenase, enzymatically inactive and incapable of binding to collagen, has been isolated from extracts of tadpole tail-fin tissue by fractionation techniques used to isolate the active enzyme from the media of tail-fin cultures. Both enzyme and zymogen have been purified by ammonium sulfate precipitation and agarose chromatography and appear as single bands of protein on polyacrylamide disc gel electrophoresis. The molecular weight of enzyme and zymogen, as estimated by sodium dodecyl sulfate–polyacrylamide electrophoresis in 5 and 10% gels, was 104,000 and 115,000, respectively; that of zymogen in 5 and 10% gels was 106,000 and 120,000, respectively. Activation of the zymogen was accomplished by incubation with collagenase-free tail-fin culture medium, but not with trypsin or chymotrypsin. The unidentified activator in the medium is heat labile and nondialyzable. The degradation products of soluble native guinea pig skin collagen produced by activated zymogen are similar to those produced by the purified tadpole collagenase obtained from tissue culture medium. The γG fraction of rabbit anti-collagenase antiserum blocked the activity of the activated zymogen as well as that of the purified tadpole collagenase.

The animal collagenases have generally required tissue culture methods for both detection and isolation. Collagenolytic activity was first detected in the culture media of living tadpole tissue incubated on a collagen substrate in physiologic salt solutions (Gross and Lapiere, 1962). Freezing and thawing of the explant or the application of low concentrations of puromycin (Eisen and Gross, 1965), blocked the appearance of the enzyme. In addition, collagenolytic activity could not be demonstrated in tissue extracts. These observations led to the tentative conclusion that the enzyme was synthesized *de novo* in the cultured tissues, or that a zymogen requiring protein synthesis for activation (Lapiere and Gross, 1963) was the source.

By employing the methods devised for detection, isolation, and characterization of the tadpole collagenase, it has been possible to obtain collagenolytic enzymes with very similar properties from a variety of animal tissues, including inflamed gingiva (Fullmer and Gibson, 1966), human rheumatoid synovia (Evanson *et al.*, 1968), resorbing rat uterus (Jeffrey and Gross, 1967, 1970), human skin (Eisen *et al.*, 1968), skin wounds (Grillo and Gross, 1967; Donoff *et al.*, 1971), regenerating newt limb (Grillo *et al.*, 1968; Dresden and Gross, 1970), middle-ear cholesteatoma (Abramson, 1969), bone (Shimizu *et al.*, 1969; Fullmer and Lazarus, 1969), and corneal ulcerations (Brown *et al.*, 1969; Slansky *et al.*, 1969). These enzymes all have neutral pH optima, are inactive at low pH, and cleave native collagen molecules below the denaturation temperature in a limited and characteristic manner (Gross and Nagai, 1965; Sakai and Gross, 1967; Eisen *et al.*, 1968; Jeffery and Gross, 1970). The ongoing studies of tadpole collagenases continue to serve as a prototype for other collagenases in normal and diseased tissues.

Continuing investigations on the control mechanisms for the elaboration of the tadpole collagenase led to a search for an inactive enzyme precursor using an antibody prepared against highly purified tadpole collagenase (Harper *et al.*, 1970). The present study reports the detection, partial characterization, and activation of the zymogen found in extracts of tadpole tail fin.

Materials and Methods

Preparation and Purification of Collagenase from Tail-Fin Culture Medium. Crude tadpole collagenase in lyophilized form was prepared as described previously (Nagai *et al.*, 1966). Briefly, bullfrog tadpoles in late legless stages were sterilized for 24 hr with antibiotics added to the aquarium water. Strips of tail fin were placed on Whatman No. 1

* From the Developmental Biology Laboratory and the Arthritis and Clinical Immunology Units of the Medical Services, Massachusetts General Hospital and the Departments of Medicine and Biological Chemistry, Harvard Medical School, Boston, Massachusetts. *Received April 7, 1971.* This is publication 538 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Massachusetts General Hospital, Boston, Mass. These investigations were supported by Public Health Service Grants AM 3564 and 12785, AI 10129, and Training Grant AM 5067, as well as grants from the Arthritis Foundation, National and Massachusetts Chapter.

† To whom to address correspondence.
‡ Research Fellow of the Arthritis Foundation.