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Isolation and Characterization of the Peptides Derived from the α1 Chain of Chick Bone Collagen after Cyanogen Bromide Cleavage*

Edward J. Miller, Joseph M. Lane, and Karl A. Piez

ABSTRACT: Cleavage of the α1 chain of chick bone collagen with CNBr gives rise to ten peptides which have been separated by ion-exchange and molecular sieve chromatography. The peptides constitute unique portions of the α1 chain as demonstrated by chromatographic properties, amino acid composition, and molecular weight. They account for all of the amino acids in the α1 chain. The molecular weights of the peptides vary from 242 to 26,000 and total 92,000, in good agreement with the measured molecular weight of about 95,000 for α chains. The isolation of ten unique peptides in approximately equimolar amounts from a chain containing nine methionyl residues indicates that the two α1 chains of the bone collagen molecule have identical or very similar primary structures. Variation in the degree of hydroxylation of lysine is evident from the presence of nonintegral numbers of hydroxylysyl and lysyl residues in many of the peptides. The peptide from the cross-link region, α1-CB1, contains a lysyl residue (amino acid number 7 from the NH_2 terminus) which is about 50% hydroxylated. Cleavage of this peptide with trypsin demonstrated that hydroxylysine and lysine occupy the same position. Heterogeneity of this type cannot be the result of differences in the original primary structure, but arises after synthesis of the protein. The CNBr peptides derived from the α1 chain of chick bone collagen are clearly homologous to those obtained previously from the α1 chain of rat skin collagen. An apparent exception was noted in that a dipeptide (α1-CB0) was present in digests of the α1 chain of chick bone collagen that was not among the cleavage products of rat skin collagen α1. α1-CB0 was placed at the NH_2-terminal end of the α1 chain, preceding α1-CB1. These two residues, plus two additional residues at the NH_2-terminal end of α1-CB1 not found in rat skin collagen α1-CB1, placed the lysyl residue that is a precursor of cross-links at position 9 in the α1 chain of chick bone collagen. Other than the addition of these four residues, the cross-link region of the chick bone collagen α1 chain, as represented by α1-CB1, is identical with the same region in rat skin collagen α1 except for an alanine–serine substitution.

The COOH-terminal peptide from rat skin collagen α1 (α1-CB6) is represented by two peptides (α1-CB6A and α1-CB6B) from chick bone collagen α1 indicating the presence of an extra methionyl residue in the COOH-terminal region.

Cleavage of the α1 and α2 chains of collagen with CNBr has proved to be a highly useful procedure in the study of these large polypeptide chains of about 95,000 molecular weight (Bornstein and Piez, 1965, 1966; Bornstein *et al.*, 1966; Butler *et al.*, 1967). Cleavage of the seven methionyl residues of rat skin collagen α1 led to the isolation of eight unique peptides accounting for all the amino acids and the molecular weight of the chain (Butler *et al.*, 1967). These peptides are suitable for sequence studies, some of which have been reported (Kang *et al.*, 1967; Bornstein, 1967a,b).

In order to extend our knowledge to a collagen of another species and with a different function, we have applied CNBr cleavage to the α1 chain from chick bone collagen. The solubility and chromatographic properties of this collagen have been studied (Miller

* From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014. *Received August 20, 1968.*

et al., 1967). It is a highly cross-linked and insoluble collagen. However, in animals made lathyritic, cross-linking is inhibited and soluble, largely monomeric collagen can be obtained from the bones. The α1 chain of this collagen has served as the starting material for the studies reported here.

In this paper we continue and extend the nomenclature previously used (Butler *et al.*, 1967) for CNBr peptides from collagen. The chain is indicated first, followed by CB (CNBr) and a number denoting the position of elution of the peptide (determined initially for the peptides from rat skin collagen) from a phospho- or CM-cellulose column, *e.g.*, α1-CB3 for the peptide in the third peak. Where a lysine-derived aldehyde is present in a peptide, the superscript "Ald" is used, *e.g.*, α1-CB1 becomes α1-CB1^{Ald} (previously α1-CB1a). Where the lysine form is meant, no superscript is used. For a peptide that contains an uncleaved methionyl residue and therefore represents two adjacent regions, both numbers are used separated by a dash, *e.g.*, α1-CB(3-7) for α1-CB3 and α1-CB7 joined by a methionyl residue.

Since it is now evident (Piez *et al.*, 1968) that many animal collagens have a close homology, the peptides from these collagens, including chick bone collagen discussed here, are numbered on the basis of inferred homology to the rat skin collagen peptides irrespective of the position in the effluent from a column. If an extra methionyl residue is present in a collagen (as in chick bone collagen), the two resulting peptides that correspond to a single peptide in rat skin collagen are designated "A" and "B," *e.g.*, α1-CB6A and α1-CB6B. If a methionyl residue is missing, the peptide that corresponds to two peptides from rat skin collagen is designated by both numbers separated by a comma, *e.g.*, α1-CB(0,1) where the methionyl residue between the regions represented by α1-CB0 and α1-CB1 is missing. An example of this type has been found in human skin collagen (P. Bornstein, in preparation; Piez *et al.*, 1968).

To retain the numbers given to CNBr peptides from rat skin collagen α1, the designation α1-CB0 is used for a peptide that was not found in CNBr digests of rat skin collagen α1 but is present in digests of α1 from other collagens (see Piez *et al.*, 1968) and precedes α1-CB1 in the elution pattern from a phosphocellulose column.

Materials and Methods

Source and Preparation of α1. Acid-extracted collagen from the diaphyseal region of the tibiae of 3-week-old lathyritic chicks was obtained and purified as previously described (Miller *et al.*, 1967). The collagen extracted for these studies represented approximately 20% of the total bone collagen. The α1 chain was isolated by chromatography of 200-mg samples of denatured collagen at 42° on 25 × 90 cm columns of CM-cellulose (Whatman CM 32) as described previously (Piez *et al.*, 1963). Elution was achieved by use of a linear gradient pumped from a two-chamber constant level device containing 400 ml of starting buffer (0.06 M sodium acetate, pH 4.8) in the first chamber and 400 ml of limit buffer (0.06 M sodium acetate containing 0.1 M NaCl, pH 4.8) in the second chamber. The column was operated at a flow rate of 150 ml/hr, the effluent was monitored continuously at 230 mμ in a Beckman DB-G spectrophotometer, and absorbance was recorded on a Beckman 10-in. linear–log potentiometric recorder at a chart speed of 5 in./hr. The column effluent was collected in 6-ml fractions. The fractions comprising the α1 peak (about 80 ml) were combined, lyophilized, redissolved in 20 ml of 0.1 M acetic acid, desalted at room temperature on a 3.0 × 35 cm column of Bio-Gel P-2 (Bio-Rad Laboratories), equilibrated with 0.1 M acetic acid, and relyophilized.

Cleavage with CNBr. Cleavage with CNBr was achieved essentially as described previously (Bornstein and Piez, 1966; Butler *et al.*, 1967). Samples of α1 weighing from 50 to 100 mg were dissolved in 20 ml of 0.1 N HCl. The collagen solution was flushed with nitrogen and a weight of CNBr equal to the weight of α1 (a 150-fold molar excess relative to the methionyl residues of the dissolved collagen) was added. After swirling the flask to dissolve the crystals, incubation was carried out at 30° for 4 hr. The reaction mixture was diluted with five volumes of water, lyophilized, redissolved in 20 ml of water, and relyophilized to ensure complete removal of CNBr.

Chromatography of CNBr Peptides on Phosphocellulose. The CNBr digests of α1 were first chromatographed on a 17 × 80 mm column of phosphocellulose (Whatman, floc, capacity 7.4 mequiv/g) under conditions similar to those described previously (Bornstein and Piez, 1966). A weighed sample of α1-CNBr peptides (50–100 mg) was dissolved in 4 ml of starting buffer (0.001 M sodium acetate, pH 3.8) and applied to the phosphocellulose column equilibrated with the same buffer. The column temperature was maintained at 42°, and chromatography was performed in 0.001 M sodium acetate (pH 3.8) with a linear gradient of NaCl from 0 to 0.3 M. The total volume of the gradient was 500 ml and best results were obtained at a flow rate of 100 ml/hr. The more basic peptides which were not eluted by this gradient were removed as one peak by eluting with 0.5 M NaCl. The column effluent was continuously monitored as described above for the CM-cellulose column and 3-ml fractions were collected. The combined fractions representing a given peptide peak were immediately lyophilized and subsequently desalted on Bio-Gel P-2 as described for the α1 chain. The phosphocellulose column was regenerated and repoured after each run.

Some of the peptides eluted from phosphocellulose under the conditions just described were rechromatographed on the same column employing gradients which were modified with regard to salt concentration and total volume. Where applicable, these modifications will be noted.

Chromatography of CNBr Peptides on CM-Cellulose. The peptides which were eluted with 0.5 M NaCl as a single peak at the end of the phosphocellulose run were desalted and chromatographed on a 17 × 90 mm column of CM-cellulose (Whatman CM 32) essentially

as described by Butler *et al.* (1967). The column was equilibrated with 0.02 M citrate buffer (pH 3.6) containing 0.04 M NaCl, and 35–50-mg samples of the CNBr peptides were dissolved in 3 ml of this buffer before application to the column. The column temperature was 42° and chromatography was performed at a flow rate of 145 ml/hr. Elution was achieved with a linear gradient derived from 450 ml each of 0.02 M citrate–0.04 M NaCl (pH 3.6) and 0.02 M citrate–0.14 M NaCl (pH 3.6). The column effluent was monitored as described above and 6-ml fractions were collected. Fractions representing a given peak were pooled, desalted, and lyophilized.

Molecular Sieve Chromatography, G-50. Two peptides (α1-CB4 and α1-CB5) failed to separate during chromatography on CM-cellulose. Resolution of these peptides was achieved by molecular sieve chromatography on a 1.8 × 130 cm column of Sephadex G-50, fine beads (Pharmacia). The column was equilibrated with 0.1 M acetic acid and the peptides were applied to the column in 2 ml of this solvent. The column was operated at room temperature and at a flow rate of 20 ml/hr. The effluent was monitored as described above and 4-ml fractions were collected. The fractions corresponding to each peptide were pooled, lyophilized, and rechromatographed on phosphocellulose. The conditions for phosphocellulose chromatography in this instance were the same as described above with the exception that a gradient from 0.15 to 0.5 M NaCl was employed.

Molecular Sieve Chromatography, P-150. Following ion-exchange chromatography, each of the peptides was further purified by molecular sieve chromatography at room temperature on a 1.5 × 110 cm column (180-ml bed volume) of Bio-Gel P-150 (100–200 mesh) (Bio-Rad Laboratories) essentially as described by Piez (1968). The column was equilibrated with 1 M $CaCl_2$ (0.05 M Tris, pH 7.5) and 1–4-mg samples of peptide in 2.5 ml of this solvent were applied to the column. To ensure a constant flow rate, the column effluent was regulated at 10 ml/hr with a peristaltic pump. The column effluent was monitored at 230 mμ and 5-ml fractions were collected. Fractions representing the major portion of a given peptide peak (excluding both front and tail) were pooled, lyophilized, and desalted on Bio-Gel P-2 as described above.

Molecular Weight Determinations. The molecular weights of peptides α1-CB1 through α1-CB8 were determined from their elution volumes on a Bio-Gel P-150 column as described by Piez (1968). The conditions under which chromatography was performed differed from those outlined above only with regard to sample preparation. When molecular weights were to be determined, 2 mg of α1 (rat skin collagen) was added to the peptide sample (0.5–2 mg) to serve as an indicator of column void volume, V_0. In addition, 0.5 ml of the $CaCl_2$ solution was replaced by an equal volume of tritiated water (38,000 cpm/ml). Elution of the tritiated water was detected by counting a 1-ml aliquot of the collected fractions in a liquid scintillation counter (Packard Tri-Carb, Model 3375) and the peak of the radioactivity curve was employed as a marker for the total liquid volume of the column, V_L. For all runs, the ratio V_L/V_0 was in the range 3.25–3.42. The molecular weights of the peptides were then calculated from their elution volumes, V_E, employing a standard curve of log molecular weight *vs.* V_E/V_0 determined by chromatographing CNBr peptides from rat skin collagen of known molecular weight (Butler *et al.*, 1967; Piez, 1968).

Molecular Sieve Chromatography, Bio-Gel A-1.5. In order to chromatograph all of the CNBr peptides in a single chromatographic procedure, the complete CNBr digest of 30 mg of α1 was applied to a 1.8 × 230 cm column (585-ml bed volume) of Bio-Gel A-1.5 (200–400 mesh) (Bio-Rad Laboratories). The column was equilibrated with 1 M $CaCl_2$ (0.05 M Tris, pH 7.5) and the sample was applied along with 2 mg of untreated α1 as a marker in 3 ml of this solvent. The column was eluted at a flow rate of 15 ml/hr, the effluent was monitored at 230 mμ, and 5-ml fractions were collected. Fractions corresponding to the major peaks were pooled, lyophilized, and desalted on Bio-Gel P-2. The peptides in the peaks eluted from the agarose column were subsequently identified from their chromatographic properties on phosphocellulose and CM-cellulose as well as amino acid composition.

Tryptic Digestion of α1-CB1. α1-CB1 was cleaved by trypsin (TRTPCK, Worthington) which had been treated by the supplier with L-[tosylamido-2-phenyl]-ethyl chloromethyl ketone (Kostka and Carpenter, 1964) to inhibit contaminating chymotryptic activity. Enzyme and substrate were incubated at 37° for 4 hr at a molar ratio of 1:10 in 0.1 M NH_4HCO_3 (pH 7.8) containing 2.5 × 10^{-3} M $CaCl_2$. The digestion was terminated by lowering the pH to 4 by the addition of 0.5 M acetic acid. The digest was lyophilized and redissolved in 2 ml of 0.001 M sodium acetate (pH 3.8) and the tryptic peptides were chromatographed on phosphocellulose employing the conditions described above. Fractions of the column effluent representing each of the tryptic peptides were lyophilized, desalted on Bio-Gel P-2, and relyophilized. However, α1-CB1-T1, which contains two tyrosyl residues, was apparently adsorbed to the gel and was not completely freed of salt.

Spectroscopy. The tyrosine content of the tryptic peptide, α1-CB1-T1, was determined from its absorption spectrum in 5 M guanidine hydrochloride at pH 6.5 (Edelhoch, 1967) since hydrolysis of the peptide in the presence of salt resulted in almost complete loss of the tyrosyl residues. The peptide was dissolved in 3 ml of 6 N HCl in preparation for hydrolysis and amino acid analysis, and a 1-ml aliquot was taken for spectroscopy. HCl was removed by lyophilization, and the sample was redissolved in 1 ml of 6 M guanidine hydrochloride (pH 6.5). The absorption spectrum of the sample was determined in a Cary recording spectrophotometer (Model 14) and the tyrosine concentration was calculated from the absorbance at 275.5 mμ using a value of 1500 for the molar extinction coefficient of tyrosine (Edelhoch, 1967). The peptide contains no tryptophan or cystine which would contribute to the absorbance at 275.5 mμ. The concentration of the

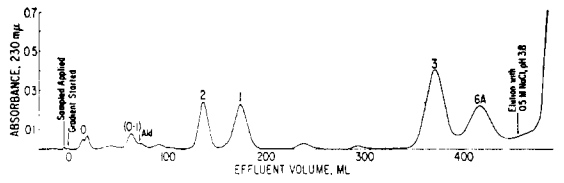

FIGURE 1: Phosphocellulose elution pattern of the CNBr peptides derived from the α1 chain of chick bone collagen. Elution was achieved in 0.001 M sodium acetate (pH 3.8) using a linear gradient of NaCl from 0 to 0.3 M. The total volume of the gradient was 500 ml. In this and other figures the peptides are indicated by a number only, omitting "α1-CB," which applies to all the peptides.

peptide was determined after hydrolysis by amino acid analysis of the remaining 2-ml aliquot.

Tryptic Digestion of α1-CB(0-1). In order to determine the position of α1-CB0 relative to α1-CB1 in the α1 chain, the uncleaved peptide containing the sequences of α1-CB0 and α1-CB1, designated α1-CB(0-1), was treated with trypsin in the same manner as described above for α1-CB1. After lyophilization, the digestion mixture was dissolved in 2 ml of 0.5 M acetic acid and chromatographed on a column of Bio-Gel P2. The peaks eluted from P2 were then rechromatographed separately on phosphocellulose.

Amino Acid Analysis. Samples of peptide (1–5 mg) were hydrolyzed in 3 ml of constant-boiling 6 N HCl at 108° for 24 hr under nitrogen in sealed tubes. After removal of acid on a rotary evaporator at about 50° under vacuum, the sample was redissolved in water and amino acid analyses of 0.5–1-mg portions were performed on a single-column automatic amino acid analyzer equipped for high-speed analysis (Miller and Piez, 1966). To resolve homoserine, which chromatographs with glutamic acid when the column temperature is maintained at 60° throughout the run, the column was kept at 50° for the first 45 min and at 60° thereafter. In the final calculations, losses of threonine, serine, and tyrosine and incomplete release of valine were corrected for by the application of factors determined for collagen (Piez *et al.*, 1960). As in the case of α1 from rat skin collagen (Butler *et al.*, 1967), it was found that methionine was completely recovered if hydrolysis was not accompanied by oxidation.

Results

Phosphocellulose Chromatography of CNBr Peptides. A representative chromatogram obtained by phosphocellulose chromatography of the CNBr peptides derived from the α1 chain of chick bone collagen is presented in Figure 1. The peak containing α1-CB6A was eluted near the end of the gradient, and the peptides remaining on the column were eluted by increasing the NaCl concentration of the eluent to 0.5 M. (The identification and justification for the designation of this and the other peptides are presented later.) The doublet peak eluting at the start of the salt gradient was comprised largely of nonprotein ultraviolet-absorbing material together with a dipeptide, α1-CB0. When rechromatographed on Bio-Gel P-2, the dipeptide was

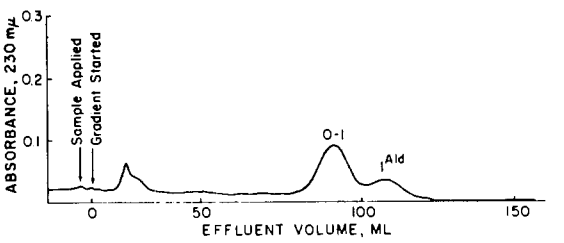

FIGURE 2: Phosphocellulose rechromatography of α1-CB-(0-1) and α1-CB1^{Ald}. The conditions for chromatography were the same as those listed in Figure 1 with the exception that the NaCl concentration in the limit buffer was reduced to 0.1 M.

eluted in an effluent volume corresponding to the total fluid volume of the column and was essentially free of contaminating peptide material. The peaks containing α1-CB(0-1) and α1-CB1^{Ald} were more completely resolved by rechromatography on phosphocellulose employing slightly different conditions as shown in Figure 2. In this case, the concentration of NaCl in the limit buffer was reduced to 0.1 M. The peptides in all other peaks were further purified by chromatography on Bio-Gel P-150.

CM-cellulose Chromatography of Remaining CNBr Peptides. A CM-cellulose chromatogram of the peptides not eluted by the gradient from phosphocellulose is illustrated in Figure 3. The first peak appearing near the start of the gradient consisted of nonpeptide material. The two following contained the same peptides (α1-CB3 and α1-CB6A) as the two last peaks in the phosphocellulose chromatogram (Figure 1) and represent material which tailed into the fraction eluted from the phosphocellulose with 0.5 M NaCl. The amount present varied with the point at which elution of the phosphocellulose column with 0.5 M NaCl was begun. Four additional peaks were routinely observed in the CM-cellulose chromatogram. Chromatography of the second of these on Bio-Gel P-150 indicated that it was heterogeneous and two peptides (α1-CB4 and α1-CB5) were partially resolved by chromatography on Sephadex G-50 as illustrated in Figure 4. Fractions corresponding to each peptide were purified by rechromatography on phosphocellulose as described above. The other peaks (α1-CB6B, α1-CB7, and α1-CB8) contained single components.

Amino Acid Composition of CNBr Peptides. Analyses of the ten CNBr peptides are presented in Table I.

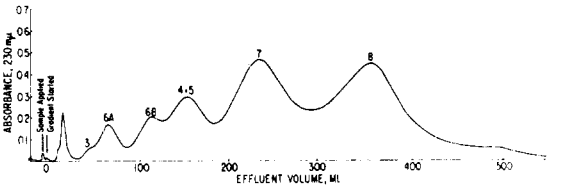

FIGURE 3: CM-cellulose elution pattern of the CNBr peptides derived from the α1 chain of chick bone collagen illustrating resolution of the peptides which were not eluted by the gradient from phosphocellulose. Elution was achieved in 0.02 M citrate (pH 3.6) using a linear gradient of NaCl from 0.04 to 0.14 M. The total volume of the gradient was 900 ml.

TABLE I: Amino Acid Compositions[a] of CNBr Peptides of the α1 Chain of Chick Bone Collagen.

Amino Acid	α1-CB0	α1-CB1	α1-CB2	α1-CB3	α1-CB4	α1-CB5	α1-CB6A	α1-CB6B	α1-CB7	α1-CB8	Total CNBr Peptides	α1[b]
3-Hydroxyproline	0	0	0	0	0	0	0	1 (1.0)	0	0	1	0.9
4-Hydroxyproline	0	0	6 (5.8)	16	6 (5.9)	4 (4.4)	7 (7.1)	9 (8.8)	31	31	110	110
Aspartic acid	0	1 (1.0)	0	7 (7.0)	3 (3.0)	2 (1.9)	5 (5.1)	3 (2.9)	11	10	42	43
Threonine	0	0	0	1 (1.1)	1 (1.0)	0	3 (2.7)	1 (1.2)	7 (6.8)	6 (6.0)	19	19
Serine	0	2 (1.8)	1 (1.0)	0	0	2 (1.8)	0	5 (4.9)	8 (7.8)	10	28	28
Glutamic acid	1 (1.1)	1 (1.1)	4 (3.9)	17	3 (3.1)	4 (3.9)	8 (8.0)	5 (4.7)	18	20	81	81
Proline	0	2 (1.8)	6 (6.3)	16	5 (4.9)	2 (2.3)	12	13	32	28	116	128
Glycine	0	3 (3.2)	12	49	16	12	31	28	92	90	333	342
Alanine	0	2 (2.0)	3 (3.0)	23	4 (4.2)	3 (3.2)	15	6 (6.3)	36	39	131	132
Valine	0	2 (1.8)	0	3 (2.9)	0	0	1 (1.0)	2 (2.0)	3 (3.0)	3 (3.1)	14	14
Isoleucine	0	0	0	0	0	0	0	1 (0.9)	4 (4.1)	2 (2.1)	7	7.0
Leucine	0	0	1 (1.0)	2 (2.0)	2 (2.0)	1 (1.1)	1 (1.0)	3 (2.9)	6 (6.1)	4 (4.1)	20	21
Tyrosine	0	2 (1.9)	0	0	0	0	0	0	0	0	2	2.2
Phenylalanine	0	0	1 (1.0)	3 (2.9)	0	1 (0.8)	0	1 (1.1)	3 (3.1)	3 (3.1)	12	13
Hydroxylysine[d]	0	0.5	0	0.4	0	1.5	0.6	0.6	2.2	1.1	6.9	6.0
Lysine[d]	0	0.5	0	4.5	2 (2.0)	1.4	2.3	1.4	8.8	8.7	29.6	30
Histidine	0	0	0	0	0	1 (0.8)	0	1 (1.0)	0	0	2	2.1
Arginine	0	0	1 (1.0)	6 (6.0)	4 (4.1)	1 (1.2)	6 (6.2)	4 (3.9)	13	15	50	53
Homoserine	1 (0.9)	1 (0.9)	1 (0.9)	1 (1.0)	1 (1.0)	1 (1.0)	1 (0.9)	0	1 (1.0)	1 (0.9)	9	8.9[c]
Total	2	17	36	149	47	37	93	85	276	272	1014	1041

[a] Residues per peptide rounded off to the nearest whole number. Actual values are listed in parentheses in those cases where less than ten residues were found. A value of zero indicates less than 0.2 residue. [b] Values in this column have been calculated on the basis of an average residue molecular weight of 91.2 and a molecular weight of 95,000 for the α1 chain. [c] Represents methionine in the case of α1. [d] The values for lysine and hydroxylysine are not rounded off where both are present since there is evidence for partial hydroxylation giving rise to noninteger values (see text).

TABLE II: The Amino Acid Compositions of Peptides α1-CB(0-1) and α1-CB1^{Ald}.

	Residues/Peptide	
	α1-CB(0-1)	α1-CB1^{Ald}
Aspartic acid	1 (0.1)	1 (1.1)
Serine	2 (1.8)	2 (1.7)
Glutamic acid	2 (2.0)	1 (1.2)
Proline	2 (1.9)	2 (1.6)
Glycine	3 (3.1)	3(3.4)
Alanine	2 (1.9)	2 (2.0)
Valine	2 (1.9)	2 (2.0)
Methionine*	1 (0.7)	0
Tyrosine	2 (1.9)	2 (1.8)
Hydroxylysine	0.5	0
Lysine	0.5	0
Homoserine	1 (0.9)	1 (1.0)
Total	19	16

* Analyzed as the methionine sulfoxides.

The values for amino acids present in low concentration (less than five residues per peptide) were used to calculate a factor to convert micromoles into residues per peptide. It was assumed that each peptide (with the exception of the COOH-terminal peptide) would contain one residue of homoserine.

The smallest peptide, α1-CB0, was found to contain glutamic acid and homoserine after hydrolysis. The amino acid composition of α1-CB1 resembles that of α1-CB1 from rat skin collagen (Bornstein *et al.*, 1966; Bornstein and Piez, 1966; Kang *et al.*, 1967), but it differs in that it has one more residue of both alanine and tyrosine and the single lysyl residue is approximately 50% hydroxylated.

All of the remaining peptides are comprised of one-third glycyl residues, yet each has a characteristic amino acid composition differentiating it from the other peptides as well as from the α1 chain. For example, α1-CB2 has a high content of imino acids and contains neither lysine nor hydroxylysine. α1-CB3 is characterized by relatively large amounts of aspartic and glutamic acids. α1-CB4 is the only lysine-containing peptide which does not also have hydroxylysine. α1-CB5 contains one of the two histidyl residues of the α1 chain. α1-CB6A has a relatively high content of threonine and no serine. α1-CB6B contains single 3-hydroxyprolyl, isoleucyl, and histidyl residues, and represents the COOH-terminal peptide since it has no homoserine. α1-CB7 and α1-CB8 are relatively large peptides of similar molecular weight, but are clearly distinguishable in that α1-CB7 contains less serine, glutamic acid, alanine, and arginine, and more isoleucine and leucine than does α1-CB8.

The total number of residues of each amino acid found in the ten CNBr peptides is listed in Table I along with values obtained from amino acid analyses

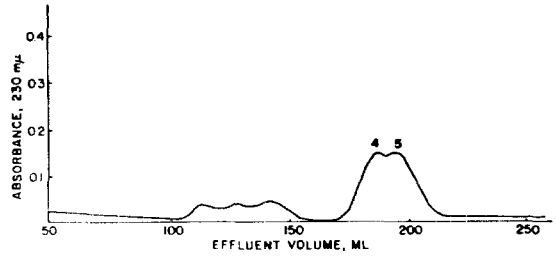

FIGURE 4: Molecular sieve chromatography (Sephadex G-50, 330-ml column) of α1-CB4 and α1-CB5 which were not resolved during CM-cellulose chromatography. The eluent was 0.5 M acetic acid.

of the whole α1 chain. It is clear that the isolated peptides account for all of the amino acids in α1, the observed differences being well within experimental error. Since the α1 chain contains nine methionyl residues, the ten peptides correspond to the number expected after CNBr cleavage.

Amino Acid Compositions of α1-CB(0-1) and α1-CB1^{Ald} in Phosphocellulose Chromatograms. The amino acid composition of the peptide designated α1-CB(0-1) (Figure 2) is given in Table II. The analysis indicates that this peptide resulted from incomplete cleavage at the methionyl residue joining peptides α1-CB0 and α1-CB1. The amino acid composition of α1-CB1^{Ald} (Figure 2) is also given in Table II. The peptide contains 16 amino acids and differs from α1-CB1 only in the absence of the partial residues of lysine and hydroxylysine. Therefore, this peptide must represent the same region as α1-CB1 in which the lysyl residue is oxidatively deaminated preparatory to cross-link formation as shown for α1-CB1 from rat skin collagen (Bornstein *et al.*, 1966; Bornstein and Piez, 1966). The collagen employed in these studies represents an easily solubilized fraction from the bones of animals given a lathyrogen, which is known to inhibit aldehyde formation. Thus, the relatively small amount of α1-CB1^{Ald} may be ascribed to the nature of the collagen.

As may be seen in Figure 1, α1-CB(0-1) chromatographs on phosphocellulose in a manner indicating that it has fewer basic groups than α1-CB1. Under the chromatographic conditions used, carboxyl groups are largely protonated and do not contribute significantly to the chromatographic behavior. Indeed, the chromatographic properties of α1-CB(0-1) are very similar to α1-CB1^{Ald} which is identical with α1-CB1 except that it has one less positive charge owing to the loss of the ε-amino group of the lysyl residue. Although the lysyl residue of α1-CB(0-1) is unaltered, the loss of a positive charge relative to α1-CB1 could arise if (as shown later) α1-CB0 were on the amino side of α1-CB1 and if the glutamic acid derived from α1-CB0 were in the form of pyrrolidone-5-carboxylic acid. It has been shown that this residue is NH_2 terminal in the α2 chain from rat skin collagen (Kang *et al.*, 1967).

Molecular Weights of CNBr Peptides. The molecular weights of the CNBr peptides from the α1 chain of chick bone collagen are given in Table III. With the exception of α1-CB0, the molecular weights were determined by molecular sieve chromatography on

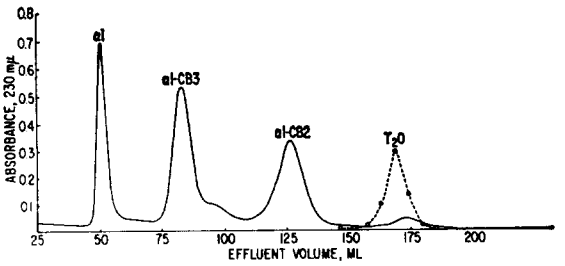

FIGURE 5: Molecular sieve chromatography (Bio-Gel P-150, 180-ml column) of α1-CB2 and α1-CB3 illustrating the method whereby the molecular weights of the CNBr peptides were determined. Relative to the elution volume of α1, the elution volumes of α1-CB2 and α1-CB3 are 2.52 and 1.65, respectively. These elution volumes correspond to a molecular weight of 3200 for α1-CB2 and 12,800 for α1-CB3 (Piez, 1968).

Bio-Gel P-150. A chromatogram showing the elution pattern of α1-CB2 and α1-CB3 on Bio-Gel P-150 is given in Figure 5. In all cases, the molecular weights calculated on the basis of amino acid composition are in good agreement with the values obtained by molecular sieve chromatography. The sum of the molecular weights determined by molecular sieve chromatography is 92,200, and 92,400 by amino acid analysis. These values compare favorably with the value of about 95,000 for the whole α1 chain as determined by sedimentation equilibrium (Lewis and Piez, 1964; Kang *et al.*, 1966).

Tryptic Digestion of α1-CB1. Following digestion of the α1-CB1 with trypsin, phosphocellulose chromatography revealed that the peptide had been cleaved into two chromatographically distinct fragments (Figure 6). The amino acid compositions of the fragments are given in Table IV. It may be seen that the two fragments contain all of the amino acids in α1-CB1 and that hydroxylysine and lysine total a single residue in α1-CB1-T1. The presence of homoserine, which must be COOH terminal, in one fragment and lysine and hydroxylysine, which the specificity of trypsin indicates must also be COOH terminal, in the other fragment demonstrate that the partial residues of hydroxylysine and lysine both occupy position 7 in α1-CB1.

Tryptic Digestion of α1-CB(0-1). Phosphocellulose chromatography of the tryptic digest of α1-CB(0-1) indicated that the resulting peptides chromatographed together in a position corresponding to that of α1-CB1-T2 (Figure 6). Therefore, the digest was chromatographed on Bio-Gel P2 taking advantage of the tendency of tyrosyl-containing peptides to be adsorbed to the gel. In this instance, the peptide containing the tyrosyl residues was adsorbed less strongly than α1-CB1-T1 and was eluted midway between the excluded volume and the volume at which salt appeared in the effluent. The peptide was subsequently chromatographed on phosphocellulose and desalted on Bio-Gel P-2. The amino acid composition of the peptide is given in Table V. It is apparent that this peptide is similar to α1-CB1-T1 (Table IV) but contains an additional glutamic acid residue and one methionyl residue. These results demonstrate that the sequence represented by α1-CB0 is on the amino side of the sequence represented by α1-CB1.

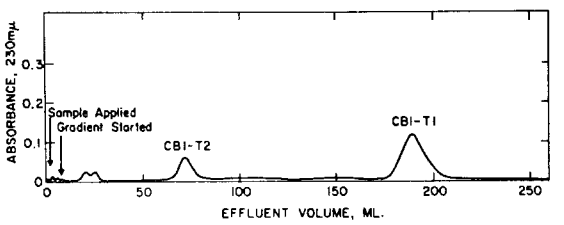

FIGURE 6: Phosphocellulose chromatography of the peptides obtained after cleavage of α1-CB1 with trypsin. The conditions for chromatography were identical with those given in Figure 1.

TABLE III: Molecular Weights of the CNBr Peptides of the α1 Chain of Chick Bone Collagen as Determined by Amino Acid Analysis and by Molecular Sieve Chromatography.

Peptide	Amino Acid Anal.	Molecular Sieve Chromatography
α1-CB0	242	242[a]
α1-CB1	1,690	1,575
α1-CB2	3,283	3,200
α1-CB3	13,527	12,800
α1-CB4	4,405	4,250
α1-CB5	3,585	3,500
α1-CB6A	8,432	8,900
α1-CB6B	7,836	7,200
α1-CB7	24,867	24,500
α1-CB8	24,525	26,000
Total	92,392	92,168

[a] The molecular weight of α1-CB0 was calculated from its amino acid composition only, assuming the composition pyrrolidone-5-carboxylmethionine.

Stoichiometry of Isolated α1 Peptides. Although the peptides isolated in this study account for all the amino acids and all the molecular weight of the α1 chain, an estimate of the relative yields of the peptides was made in order to rule out the possibility that there were repeating sequences within α1 which would yield 2 or more equiv of some peptides or that a component resulting from nonspecific cleavage had been included among the α1 peptides. It was assumed that the peptides (except α1-CB0 and α1-CB1; see below) would have similar extinction coefficients at 230 mμ. Therefore, the area under each peptide peak would have a constant relation to molecular weight if the peptides were present in equivalent amounts.

A chromatogram illustrating the elution pattern of the α1 peptides on a 585-ml column of agarose beads is presented in Figure 7. The first eluted peak represents untreated α1 which was mixed with the CNBr digest at the time the sample was applied to the column. Following the α1 peak is an area in which the base line remains elevated indicating that a small amount of

TABLE IV: The Amino Acid Compositions of the Peptides Obtained after Tryptic Digestion of α1-CB1.

Amino Acid	Residues/Peptide		
	α1-CB1	α1-CB1-T1	α1-CB1-T2
Aspartic acid	1	1 (0.9)	0
Serine	2	1 (0.9)	1 (1.1)
Glutamic acid	1	1 (1.0)	0
Proline	2	0	2 (1.8)
Glycine	3	1 (1.1)	2 (2.1)
Alanine	2	0	2 (2.1)
Valine	2	0	2 (2.0)
Tyrosine[a]	2	2 (2.2)	0
Hydroxylysine	0.5	0.5	0
Lysine	0.5	0.5	0
Homoserine	1	0	1 (1.0)
Total	17	7	10

[a] The tyrosine content of α1-CB1-T1 was determined spectrophotometrically (Edelhoch, 1967).

high molecular weight components representing partially cleaved components still remain in the sample. The second peak is eluted in a position corresponding to a molecular weight of 37,000. CM-cellulose chromatography and amino acid analyses of the material contained in this peak indicated that it represented a mixture of two peptides both of which contained methionine and therefore resulted from incomplete cleavage during CNBr digestion. All of the other peaks in Figure 7 contain the designated peptides which were resolved into molecular weight classes. Assuming that the true base line of the chromatogram was the dash line, the area under each of the peaks was calculated by means of a planimeter. Comparisons of the area under each of the peaks and the molecular weight of the peptides in each peak are presented in Table VI. Similar comparisons of peak area and peptide molecular weight were made on individual peptides eluted from phosphocellulose and CM-cellulose (Figures 1 and 3). These data are also summarized in Table VI. These calculations cannot be expected to give more than approximate numbers of equivalents but the results indicate the presence of 1 equiv of each peptide within experimental error. None of the peptides is present in trace amount and there are not two or more equivalents of any peptide. A similar conclusion was reached (Butler *et al.*, 1967) for the CNBr peptides from the α1 chain of rat skin collagen.

Similar calculations could not be made for α1-CB0 and when made for α1-CB1, they indicated the presence of much greater than 1 equiv. However, the assumption that these peptides have an extinction coefficient similar to the larger peptides would not be expected to be valid for the dipeptide α1-CB0, owing to its small size, or for α1-CB1, owing to the presence of two tyrosyl

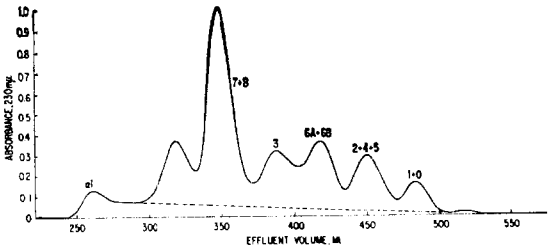

FIGURE 7: Molecular sieve chromatography (Bio-Gel A-1.5, 585-ml column) of all of the products obtained after cleavage of 30 mg of α1 (chick bone collagen) with CNBr. The eluent was 1 M $CaCl_2$ (pH 7.5) (0.05 M Tris).

residues. Although numbers cannot be assigned to the number of equivalents of these peptides found, they were certainly not present in trace amount. The amounts isolated as indicated by the number of micromoles in hydrolysates were at least consistent with the presence of 1 equiv of each.

Discussion

We have shown that cleavage of the α1 chain of chick bone collagen with CNBr gives rise to ten unique peptides which have been separated by ion-exchange and molecular sieve chromatography. Characterization of the peptides with regard to amino acid composition and molecular weight has demonstrated that the isolated peptides account for all of the amino acids and molecular weight of the α1 chain.

The CNBr peptides from the α1 chain of chick bone collagen are, except for α1-CB0, clearly homologous to the CNBr peptides previously isolated from the α1 chain of rat skin collagen. α1-CB1 and α1-CB2 of chick bone are very similar in size and amino acid composition to α1-CB1 and α1-CB2 of rat skin collagen (Bornstein *et al.*, 1966). α1-CB3, α1-CB4, α1-CB5, α1-CB7, and α1-CB8 of chick bone collagen are likewise each related to the peptide with the same designation from the α1 chain of rat skin collagen (Butler *et al.*, 1967).

TABLE V: The Amino Acid Composition of the Tyrosyl-Containing Peptide Obtained after Tryptic Digestion of α1-CB(0-1).

Amino Acid	Residues/Peptide
Aspartic acid	1 (1.0)
Serine	1 (0.9)
Glutamic acid	2 (1.9)
Glycine	1 (1.2)
Methionine[a]	1 (0.7)
Tyrosine	2 (1.8)
Hydroxylysine	0.5
Lysine	0.5
Total	9

[a] Analyzed as the methionine sulfoxides.

TABLE VI: Stoichiometry of CNBr Peptides from α1 of Chick Bone Collagen.

Column	Peptide(s)	Area[a]	Area/Mol Wt[b]
Agarose	α1-CB7 + 8	1732	0.0351 (0.73)
	α1-CB3	652	0.0482 (1.00)
	α1-CB6A + 6B	570	0.0351 (0.73)
	α1-CB2 + 4 + 5	437	0.0387 (0.81)
Phosphocellulose	α1-CB2	135	0.0411 (1.40)
	α1-CB3	395	0.0292 (1.00)
	α1-CB6A	205	0.0244 (0.84)
CM-cellulose	α1-CB6B	196	0.0250 (0.86)
	α1-CB4 + 5	287	0.0359 (1.23)
	α1-CB7	1007	0.0403 (1.38)
	α1-CB8	978	0.0399 (1.36)

[a] Planimeter units. [b] Molecular weights are those determined by amino acid analysis, Table III. Values normalized to 1.00 for α1-CB3 are listed in parentheses.

α1-CB6A and α1-CB6B of chick bone collagen have no counterparts in rat skin collagen, but correspond in sum to α1-CB6 in the latter collagen. α1-CB6 is the COOH-terminal peptide in rat skin collagen (Butler *et al.*, 1967) as is α1-CB6B in chick bone collagen and both peptides contain a single residue of 3-hydroxyproline and histidine. In addition, the combined molecular weight and amino acid compositions of α1-CB6A and α1-CB6B of chick bone collagen are very similar to the molecular weight and amino acid composition of α1-CB6 of rat skin collagen. These data indicate that one of the amino acids approximately midway in the sequence of α1-CB6 of rat skin collagen is substituted by a methionyl residue in chick bone collagen. A more detailed comparison of some of these peptides with homologous peptides from other collagens has been made (Piez *et al.*, 1968).

The complete order of the ten CNBr peptides in the α1 chain of chick bone collagen cannot be determined from the present data. However, a tentative order has been established for the CNBr peptides from rat skin collagen (Piez *et al.*, 1968). This is α1-CB(1-2-4-8-5-3-7-6). On the basis of homology and since α1-CB0 from chick bone collagen α1 is on the amino side of α1-CB1, the probable order of the CNBr peptides of chick bone collagen α1 is α1-CB(0-1-2-4-8-5-3-7-6A-6B).

Since the α1 chain of chick bone collagen contains nine methionyl residues, the recovery of ten unique peptides in approximately equivalent amounts after CNBr digestion strongly suggests that the two α1 chains of the chick bone collagen molecule are identical. Differences in primary structure sufficient to cause chromatographic heterogeneity of the whole α1 chains as reported by Francois and Glimcher (1966) would have been apparent in the CNBr peptides since they are smaller than α1. Therefore, as previously suggested (Miller *et al.*, 1967), chromatographic heterogeneity of the α1 chains of chick bone collagen probably results from an artifactual alteration of the chains when extracted under conditions in which the protein is denatured.

These considerations do not rule out heterogeneity of the α1 chains resulting from *in vivo* modifications of the primary structure after synthesis of the protein. Previous studies have shown that there is incomplete hydroxylation of certain prolyl residues in rat skin collagen (Bornstein, 1967a,b). The present studies have shown that heterogeneity is introduced through incomplete hydroxylation of the lysyl residues of chick bone collagen. The most noteworthy example of this phenomenon is apparent in α1-CB1 where the lysyl residue (amino acid 7 from the NH_2 terminus) is hydroxylated to the extent of 50%. Since it has been shown that the lysyl residue in α1-CB1 of rat skin collagen participates in the formation of intramolecular cross-links, and in this case is apparently not hydroxylated (Bornstein *et al.*, 1966; Bornstein and Piez, 1966), the role which hydroxylation of the lysyl residue plays in the formation of cross-links in bone collagen warrants further investigation. The presence of hydroxylysine in this region of the α1 chain also indicates that hydroxylation of lysyl residues does not require the native collagen helicity in the substrate. A similar conclusion for proline hydroxylation has been reached after *in vitro* studies of proline hydroxylation in denatured collagen (Nordwig and Pfab, 1968). Evidence for variable hydroxylation of lysyl residues in most of the peptides of chick bone collagen may be seen in the amino acid analyses presented in Table I. α1-CB4 was the only lysine-containing peptide which did not also contain hydroxylysine. α1-CB3, α1-CB6A, and α1-CB6B each contained a partial residue of hydroxylysine. The lysine content of these peptides was such that the sum of hydroxylysyl and lysyl residues was consistent with a whole number of amino acid residues. At present, it is not known whether these data indicate that the

hydroxylation of several lysyl residues is incomplete or whether the incomplete hydroxylation is confined to a single lysyl residue.

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Comparative Studies on Myosins from Breast and Leg Muscles of Chicken*

Chuen-Shang Chung Wu

ABSTRACT: Comparative studies on chicken myosins from breast and leg muscles revealed several differences in physicochemical characteristics. Myosin from leg muscles (myosin-R) had adenosine triphosphatase activity about 82–86% of that from breast muscle (myosin-W). The former was also less extractable than the latter with the short-time extraction methods employed in this study. There was no difference in substrate specificity; both myosins hydrolyzed adenosine triphosphate and uridine triphosphate the fastest, inosine triphosphate the next, cytidine triphosphate and guanosine triphosphate the slowest. Chromatographic patterns on a DEAE-Sephadex column and sedimentation velocity in an ultracentrifuge did not reveal any differences between these two proteins; the enzyme activities of both increased somewhat after chromatography. Spectrophotometric studies showed a slightly higher extinction coefficient and tyrosine content for myosin-R; however, the absorption spectra at neutral and alkaline pH were very similar. The solubilities, when studied in 5 mM Tris-maleate buffer with various KCl concentrations and pH values, also revealed dissimilarities. The most striking differences were observed in the rate and pattern of tryptic digestion. Myosin-R was digested at a much slower rate; the course of hydrolysis was also different from that of myosin-W. The study of optical rotatory parameters, however, did not reveal any differences in conformation between these myosins.

Since the observation by Cooper and Eccles (1930) that the speed of contraction of red muscle was slower than that of white muscle, numerous physiological and biochemical studies have been conducted on these two types of muscles. However, investigations on the contractile proteins from these muscles were performed only recently. Barany *et al.* (1965) reported that ATPase activity of myosin from red muscle of rabbit was considerably lower than that from white muscle, and that the pH–ATPase profiles of the two myosins were different in the alkaline pH range. Gergely *et al.* (1965) and Maddox and Perry (1966) also found lower enzyme activity in red muscle myosin from rabbit and pigeon, respectively, but Sreter *et al.* (1966) observed no significant differences in the pH–ATPase profile or in the effect of ionic strength on the ATPase activity of red and white

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