COLLAGEN CROSS-LINKING: IDENTIFICATION OF TWO CYANOGEN BROMIDE PEPTIDES CONTAINING SITES OF INTERMOLECULAR CROSS-LINK FORMATION IN CARTILAGE COLLAGEN

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SUMMARY: A double-chain radioactive peptide has been isolated from cyanogen bromide digests of borotritide reduced cartilage collagen. Characterization of the peptide indicates its derivation as the result of cross-linking mediated through the hydroxylysyl residue of peptide 4 and one of the hydroxylysyl residues of peptide 9. Radioactivity in the peptide is associated with the cross-link, providing direct evidence that reducible compounds in native fibrillar collagen preparations have a cross-linking function. The reduced cross-link is susceptible to periodate cleavage leading to the dissociation and recovery of the component peptides suggesting that cross-linking in this instance occurs through the formation of a Schiff base or aldol condensation product.

Studies on the cyanogen bromide (CNBr) peptides from soluble collagens have demonstrated that intramolecular cross-links are formed by an aldol condensation of lysine-derived aldehydes located in the non-helical amino-terminal region of the molecule (1-3). In contrast, information concerning the nature, location, and biosynthesis of intermolecular cross-links is less complete although several advances have been made using the technique of borohydride reduction of reconstituted collagen fibers and native collagen. These studies have shown that reduction modified the physical and chemical properties of the preparations in a manner suggesting the stabilization of intermolecular cross-links and have led to the identification in reduced form of several possible cross-links. These include: dehydrolysinonorleucine, formed by a Schiff base condensation between a residue of lysine and a residue

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of the δ-semialdehyde of α-aminoadipic acid (allysine) (4,5); dehydrohydroxylysinonorleucine, which could arise either through Schiff base condensation between a residue of allysine and a residue of hydroxylysine or a residue of lysine and a residue of hydroxyallysine (6,7); dehydrohydroxylysinohydroxynorleucine, a Schiff base compound originating through condensation of a residue of hydroxylysine and a residue of hydroxyallysine (8); and syndesine, an aldol condensation product derived from a residue of allysine and a residue of hydroxyallysine (9).

Although these compounds exhibit structural features compatible with cross-linking, direct evidence for a role in cross-linking and specifically, intermolecular cross-linking, is currently unavailable. Recent studies indicating that hydroxylysinonorleucine is present in peptides isolated from reduced calf skin collagen strongly suggest that dehydrohydroxylysinonorleucine is a cross-link (10), however, it was not established that the peptides were double-chain peptides.

The present report describes studies demonstrating a cross-linking role for reducible components in collagen. Cartilage collagen has been studied since this collagen is comprised largely of molecules of the chain composition $\{\alpha 1(II)\}_3$ (11-15) greatly facilitating interpretation of the CNBr peptide pattern derived from the total collagen pool. In addition, the CNBr peptides accounting for all the amino acids and molecular weight of α1(II) from soluble chick cartilage collagen have been isolated and characterized (16), providing data useful in the detection of cross-linked peptides in insoluble cartilage collagen.

MATERIALS AND METHODS. Sternal cartilages were dissected from three-week-old normal chicks, and prepared for extraction of collagen (11,14). Particles remaining after extraction were washed with water and freeze dried. For reduction, 500-mg quantities of dried insoluble cartilage were suspended in 30 ml of 0.1 M Tris, pH 7.8, to which was added 17 mg of sodium borohydride and 3 mg of sodium borohydride-H^3 (New England Nuclear Corp., specific

activity >100 mc/mM). Reduction was allowed to proceed for 1 hr and the reaction was stopped by the addition of 50% acetic acid to pH 3.0. The reduced particles were washed with water and lyophilized.

CNBr peptides were prepared from the reduced insoluble cartilage collagen after cleavage in 70% formic acid and chromatographed initially on CM-cellulose as previously described (15). Radioactivity in the CM-cellulose effluent (and during subsequent chromatographic procedures) was monitored by mixing a 1.0-ml aliquot from alternate fractions with 10 ml of scintillator solution (Aquasol, New England Nuclear Corp.) and counting in a Packard Tri-Carb liquid scintillation spectrometer model 3375. The major radioactive component in the CM-cellulose effluent was further purified by rechromatography on a 1.8 x 230 cm column of agarose beads (17) and subsequently on phosphocellulose (for conditions, see legend for Fig. 3).

For periodate cleavage, a sample of the radioactive component (5 mg) was dissolved in 2 ml of 0.025 M periodic acid, pH 3.8. The solution was allowed to stand at 22° C for 4 hr in the dark and the cleavage products were separated by immediately passing the solution over a 1.8 x 70 cm column of Bio-Gel P-4 equilibrated with 0.1 M acetic acid.

Samples were hydrolysed by 6 N hydrochloric acid and amino acid analyses were performed on an automatic amino acid analyzer (18). Radioactivity in the analyzer column effluent was monitored continuously (19).

RESULTS. Figure 1 depicts a CM-cellulose chromatogram of the CNBr peptides prepared from reduced insoluble chick sternal cartilage collagen. The

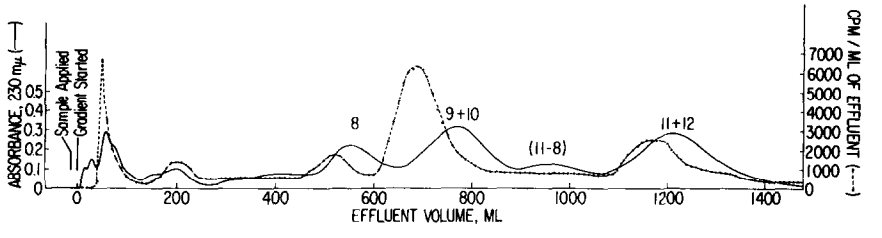

Figure 1. Carboxymethyl cellulose chromatogram depicting the elution pattern of the CNBr peptides prepared from insoluble cartilage collagen after reduction with sodium borohydride-borotritide. Also shown is the distribution of radioactivity in the peptides.

elution profile of the peptides (designated by numbers corresponding to their order of elution) resembles that observed for CNBr peptides derived from soluble α1(II) when chromatographed under similar conditions (16). Five of the thirteen CNBr peptides of chick α1(II) are significantly retarded on the column and chromatographed in this procedure, the remainder eluting in a single complex peak in the first 100 ml of eluent. A plot of the radioactivity associated with the peptides indicates a major peak, accounting for approximately 60% of the total sample radioactivity, appearing in the effluent between 600 and 750 ml. Rechromatography of this portion of the CM-cellulose effluent on an agarose column separated the radioactive component from overlapping peptides 10 and 8 (Figure 2) and indicated that it had a molecular weight

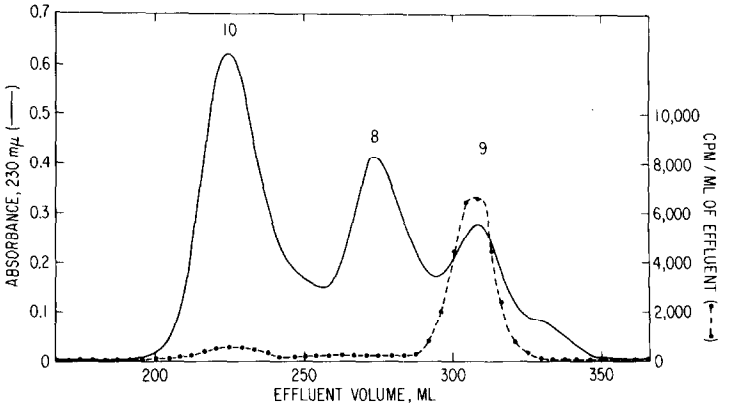

Figure 2. Chromatogram illustrating the elution pattern obtained during rechromatography of the major radioactive peak in Figure 1 on a 585-ml column of agarose beads (Bio-Rad A-1.5 m, 200-400 mesh).

closely corresponding to that of peptide 9. Final purification of the radioactive component and resolution from peptide 9 was achieved by rechromatography of appropriate portions of the agarose effluent on phosphocellulose (Figure 3).

The amino acid composition of the radioactive component (Table I) is compared with the individual compositions of peptides 4 and 9 isolated from soluble α1(II). The composition of the radioactive component {designated (4x9) to denote cross-linking between peptides 4 and 9} corresponds well with

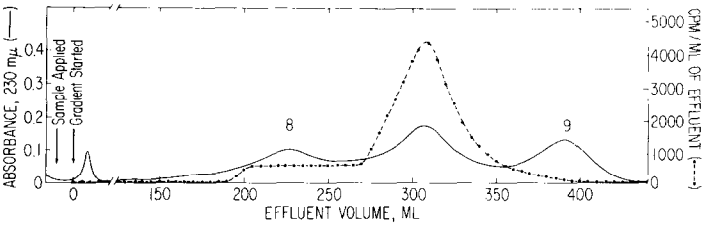

Figure 3. Phosphocellulose rechromatography of the radioactive component eluted from agarose (Figure 2). Elution was achieved in 0.001 M (Na^+) sodium acetate buffer, pH 3.8, using a linear salt gradient from 0.1 to 0.4 NaCl over a total volume of 600 ml.

that expected for the sum of peptides 4 plus 9 except for the absence of two hydroxylysyl residues and the appearance of an additional amino acid listed as the cross-link. The latter was the only radioactive amino acid in hydrolysates

Table I

Amino Acid Compositions of Peptides 4 and 9 from Soluble α1(II), the Cross-Linked Peptide (4x9) from Reduced Insoluble Cartilage Collagen, and the Component Peptides Released by Periodate Cleavage

Amino Acid	4[a]	9[a]	(4x9)[b]	4p[b]	9p[b]
3-Hydroxyproline	0	1	1(0.9)	0	1(0.8)
4-Hydroxyproline	0	4	4(4.0)	0	4(4.1)
Aspartic acid	1	4	5(5.1)	1(1.0)	4(3.9)
Threonine	0	2	2(2.0)	0	2(1.8)
Serine	0	4	4(4.0)	0	4(4.0)
Glutamic acid	2	6	8(7.9)	2(2.1)	6(6.2)
Proline	0	10	10	0	10
Glycine	4	23	27	4(4.0)	23
Alanine	3	4	7(7.0)	3(3.2)	4(4.1)
Valine	0	1	1(0.9)	0	1(1.1)
Leucine	0	3	3(3.1)	0	3(2.9)
Phenylalanine	1	1	2(2.0)	1(1.0)	1(1.0)
Hydroxylysine	1	2	1(1.2)	0	1(0.8)
Lysine	0	1	1(1.0)	0	1(1.0)
Histidine	0	1	1(0.9)	0	1(1.1)
Arginine	0	4	4(4.2)	0	4(4.2)
Homoserine	1	1	2(1.8)	1	1(1.0)
Cross-link[c]			1(0.8)	0	0
Total	13	72	84	12	71

a. Data from reference 16.
b. Values rounded off to the nearest whole number. Numbers in parentheses designate actual values where less than 10 residues are found.
c. Calculations were made assuming a color yield twice that of leucine.

of the labelled peptide and chromatographed between phenylalanine and hydroxylysine in the system used for analysis.

To confirm that (4x9) is a double-chain peptide, periodate cleavage was employed since in reduced form either a Schiff base or aldol condensation type cross-link would be susceptible to periodate cleavage provided at least one hydroxylysyl side chain is utilized in its formation. Figure 4 illustrates the chromatographic properties of (4x9) on Bio-Gel P-4 before (A) and after (B) treatment with periodate. Amino acid analyses of the peptides (designated 4^P and 9^P) recovered after periodate cleavage (Table I) clearly show that destruction of the cross-link is associated with liberation of the component peptides. The absence of one hydroxylysyl residue in the component peptides after periodate cleavage provides additional evidence indicating that the cross-link is mediated through a hydroxylysyl side chain in both peptides. Resistance to periodate on the part of the alternate hydroxylysyl residue in

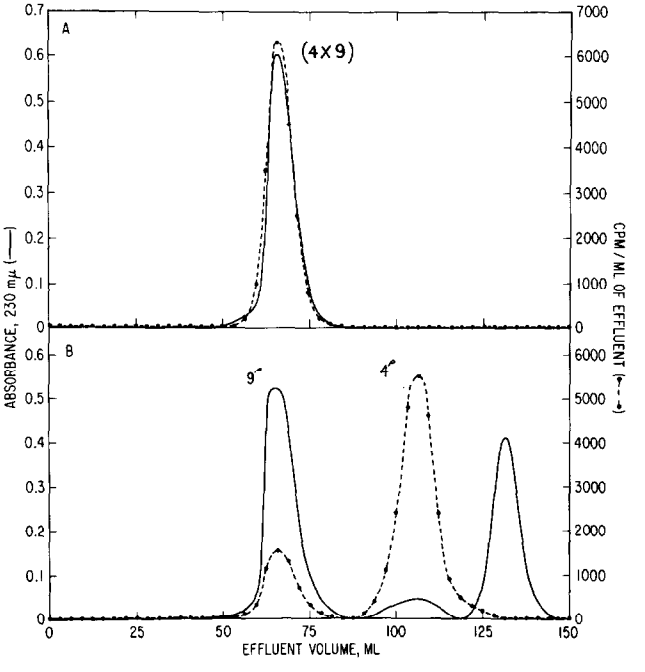

Figure 4. P-4 molecular sieve chromatography of peptide (4x9) before (A) and after (B) treatment with periodate. The peak appearing at an effluent volume of 135 ml is the salt peak.

peptide 9 indicates it is one of the several glycosylated hydroxylysyl residues of α1(II) (14). Radioactivity associated with cleavage products of the cross-link in both peptides (Figure 4) is eluted at the very front of the chromatogram during amino acid analyses indicating that the cleavage products are quite labile under the conditions required for hydrolysis.

DISCUSSION. The results described above provide direct evidence that reduction of insoluble collagen preparations leads to the stabilization and detection of cross-links. Examination of appropriate regions in chromatograms similar to those presented here indicates that quantitatively, the amounts of peptides 4 and 9 recovered in cross-linked form account for approximately one-third the total pool of each peptide. From these data, it may be estimated that on the average one cross-link per molecule is derived from the relevant sites in these peptides. Although the present results do not in themselves provide sufficient data to establish the chemical configuration of the cross-link, they are consistent with and corroborate previous studies demonstrating the presence of Schiff base and aldol condensation products in fibrillar collagens. At present, the linear order of the CNBr peptides in α1(II) is not known and the location of the cross-linking sites observed in this study cannot be assigned to specific regions of the molecule. However, in consideration of the chain composition of cartilage collagen, $\{\alpha 1(II)\}_3$, it seems certain that cross-links joining peptides 4 and 9 are intermolecular cross-links.

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