THE STABILITY OF COLLAGEN CROSS-LINKS WHEN DERIVED FROM HYDROXYLYSYL RESIDUES*

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SUMMARY: The cross-linked cyanogen bromide peptide, (4x9), previously isolated after reduction of cartilage collagen, has been isolated without prior reduction of the collagen. The unreduced cross-link is cleaved by periodate allowing recovery of the component peptides. When isolated after borotritide reduction of the collagen, (4x9) contains a single residue of radioactive hydroxylysinohydroxynorleucine. Radioactivity in the cross-link remains in the component peptides when the cross-link is cleaved with periodate. Performic acid oxidation removes this radioactivity and produces an additional glutamic acid residue in each peptide. These data indicate that dehydrohydroxylysinohydroxynorleucine undergoes an Amadori rearrangement producing a more stable keto-amine form of the cross-link.

It has been concluded that the major reducible, intermolecular cross-link in the relatively insoluble collagens of bone and dentin (1,2) bovine tendon (3), cartilage (4), and embryonic dermis (5) is an aldimine-containing compound, dehydrohydroxylysinohydroxynorleucine. This conclusion is based on the identification of hydroxylysinohydroxynorleucine in hydrolysates of these collagens after mild reduction of the native collagens with borohydride.

Nevertheless, the presence of dehydrohydroxylysinohydroxynorleucine cannot account for the observed insolubility of the collagens cited above since dilute acids should cleave the aldimine bond rendering the collagens soluble as monomeric molecules. To account for this apparent stability, it has been proposed (6) that dehydrohydroxylysinohydroxynorleucine might

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undergo an intramolecular oxidation-reduction reaction (Amadori rearrangement) involving the formation of a keto group and saturation of the carbon-nitrogen double bond.

The present paper provides data confirming the proposal of a keto-amine structure for dehydrohydroxylysinohydroxynorleucine. These studies have been performed on the cross-linked cyanogen bromide (CNBr) peptide, (4x9), previously isolated after borohydride reduction of insoluble cartilage collagen (7). The cross-link joining CNBr peptides 4 and 9 in peptide (4x9) is derived from the hydroxylysyl residue of peptide 4 and one of the hydroxylysyl residues of peptide 9 in the α1(II) chain of cartilage collagen (7). It is an intermolecular cross-link which joins the NH_2-terminal region of one molecule to the COOH-terminal portion of an adjacent molecule as judged by the respective locations of peptides 4 and 9 in the α1(II) chain (8).

MATERIALS AND METHODS. Insoluble cartilage collagen was prepared from the sternal cartilages of 10-week-old chickens (9). A portion of the collagen was reduced with sodium borohydride-borotritide as previously described (7). Both unreduced and reduced cartilage collagen preparations were cleaved with CNBr and the resulting peptides chromatographed on carboxymethyl (CM-) cellulose (9). Peptide (4x9) was isolated from each preparation by rechromatographing appropriate regions of the CM-cellulose eluant on agarose beads and phosphocellulose as described previously (7).

Periodate cleavage of reduced and unreduced peptide (4x9) was performed as previously described (7). The cleavage products were separated by chromatographing the reaction mixture on a 1.5 x 90 cm column of Bio-Gel P-6 equilibrated with 0.1 M acetic acid. The P-6 eluant was collected in 2.0-ml fractions and radioactivity in each fraction was monitored by mixing a 1.0-ml aliquot with 10 ml of scintillator solution (Aquasol, New England Nuclear Corp.) and counting in a liquid scintillation counter.

Performic acid oxidation of the periodate cleavage products eluted from the P-6 column was carried out according to the procedure of Moore (10). Following the 4-hr reaction, the reaction mixtures were diluted 10-fold with distilled water and the protein was recovered by lyophilization.

Samples were hydrolyzed in 6 N HCl and amino acid analyses were performed on an automatic amino acid analyzer (Model 119, Beckman Instruments, Inc.) as previously described (9). In some experiments one-half of the analyzer eluant was collected in 1.0-ml fractions by means of a stream divider pump. Radioactivity in the eluant was then determined by counting the fractions as described above.

RESULTS. Figure 1 depicts a CM-cellulose chromatogram of the CNBr peptides

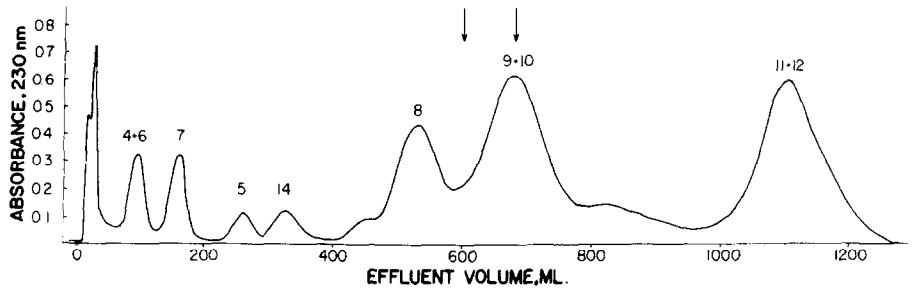

Figure 1. CM-cellulose chromatogram illustrating the elution pattern of the CNBr peptides prepared from insoluble cartilage collagen. Arrows denote the portion of the eluant chosen for isolation of peptide (4x9).

prepared from unreduced insoluble cartilage collagen. Arrows denote the portion of the eluant containing peptide (4x9). This portion of the eluant was rechromatographed on agarose to remove higher molecular weight overlapping peptides (peptides 8 and 10). Final purification of peptide (4x9) and resolution from peptide 9 was achieved by rechromatography of appropriate portions of the agarose eluant on phosphocellulose (Figure 2).

As shown in Table I, the amino acid composition of peptide (4x9) from unreduced cartilage collagen is virtually identical to that for

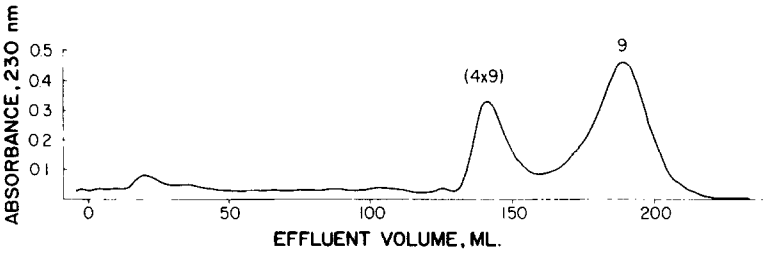

Figure 2. Phosphocellulose chromatogram illustrating the resolution of peptide (4x9) from peptide 9. Elution was achieved on a 1.0 x 10 cm column in 0.001 M (Na^+) sodium acetate buffer, pH 3.8, using a linear salt gradient from 0.1 to 0.4 M NaCl over a total volume of 400 ml.

Table I

Amino Acid Compositions[a] of Peptide (4x9) from Reduced Cartilage Collagen, Unreduced Cartilage Collagen, and the Component Peptides Released by Periodate Cleavage.

Amino Acid	Reduced Collagen (4x9)[b]	Unreduced Collagen (4x9)	4^P	9^P
3-Hydroxyproline	1	1(1.0)	0	1(1.0)
4-Hydroxyproline	4	4(4.1)	0	4(4.2)
Aspartic acid	5	5(5.2)	1(1.1)	4(3.8)
Threonine	2	2(1.9)	0	2(1.9)
Serine	4	4(3.9)	0	4(4.0)
Glutamic acid	8	8(7.8)	2(2.2)	6(6.1)
Proline	10	10	0	10
Glycine	27	27	4(4.1)	23
Alanine	7	7(6.9)	3(3.1)	4(4.0)
Valine	1	1(1.0)	0	1(0.9)
Leucine	3	3(3.0)	0	3(3.1)
Phenylalanine	2	2(1.9)	1(0.9)	1(1.0)
Hydroxylysine	1	1(1.1)	0	1(0.9)[d]
Lysine	1	1(1.0)	0	1(1.0)
Histidine	1	1(1.0)	0	1(1.0)
Arginine	4	4(3.9)	0	4(3.8)
Homoserine	2	2(1.9)	1(0.9)	1(0.9)
Hydroxylysinohydroxy-norleucine[c]	1	0	0	0
	84	83	12	71

a. Residues per peptide rounded off to the nearest whole number. Parentheses are used to designate actual values where less than 10 residues are found.
b. Data from reference 7.
c. Calculated by assuming a color yield twice that of leucine.
d. Resistance to periodate indicates that this hydroxylysyl residue is glycosylated (7).

peptide (4x9) previously isolated from reduced cartilage collagen (7). The only difference is the absence of the cross-link in the unreduced peptide, indicating that the unreduced cross-link is not stable to acid hydrolysis. It is of interest to note that hydrolysates of the unreduced peptide contain the same amount of hydroxylysine as hydrolysates of the reduced peptide. Were the cross-link to contain an aldimine function derived from the condensation of a residue of hydroxylysine and a residue of α-amino-δ-hydroxy adipic acid δ-semialdehyde (hydroxyallysine), one would expect to find an additional hydroxylysine residue in hydrolysates of the unreduced peptide.

To substantiate that unreduced peptide (4x9) is a double-chain peptide, it was treated with periodate and the component peptides (4^P and 9^P) liberated by periodate cleavage were recovered after chromatography on P-6 (Figure 3A). Amino acid analyses of peptides 4^P and 9^P (Table I)

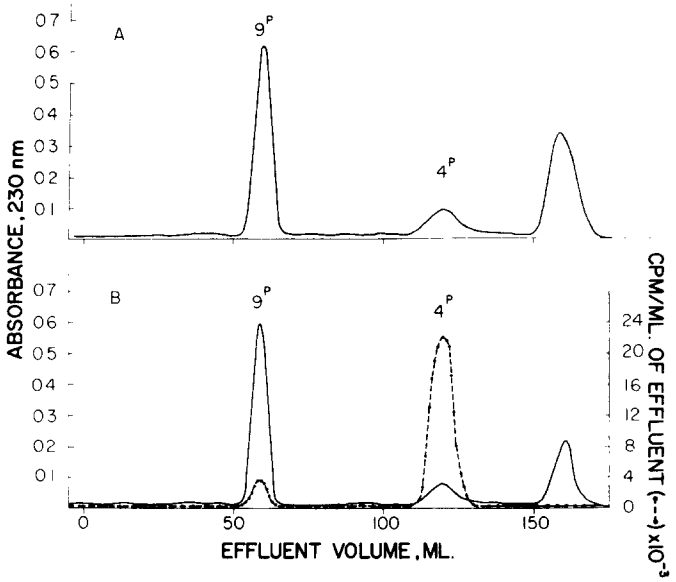

Figure 3. P-6 molecular sieve chromatography depicting the resolution of peptides 4^P and 9^P following treatment of unreduced (A) and borotritide reduced (B) peptide (4x9) with periodate.

demonstrated that they are identical to the component peptides liberated by periodate cleavage of reduced (4x9) and that each peptide contains one less hydroxylysyl residue than the parent peptide from α1(II) chains (7,11). These data establish that peptide (4x9) had been isolated without prior reduction of the cross-link and that the unreduced cross-link is susceptible to periodate cleavage.

Amino acid analyses of peptide (4x9) isolated from reduced cartilage collagen indicated the presence of one residue of an additional amino acid which was the only radioactive amino acid in the hydrolysate (7). In the system used for analysis (9), the latter amino acid chromatographs just prior to tyrosine as does authentic hydroxylysinohydroxynorleucine (kindly provided by Dr. Gerald Mechanic, Univ. of North Carolina). Periodate cleavage and destruction of the cross-link liberates the component peptides, 4^P and 9^P, while the radioactivity originally associated with the cross-link remains in the component peptides (Figure 3B).

Peptides 4^P and 9^P from reduced (4x9) were each oxidized with performic acid and rechromatographed on P-6. Radioactivity determinations on the column effluents indicated that the oxidation procedure had been effective in removing the radioactivity from each peptide. Moreover, amino acid analyses of the oxidized peptides revealed a composition identical to that listed in Table I, with the exception that each contained an additional glutamic acid residue: 2.9 residues for peptide 4^P; 7.2 residues for peptide 9^P.

DISCUSSION. Our interpretation of the preceding results is summarized in Figure 4. The aldimine-containing cross-link (A), formed initially through the condensation of a residue of hydroxylysine and a residue of hydroxyallysine, is converted through Amadori rearrangement to a keto-amine cross-link (B). The latter cross-link would be sufficiently stable to allow the isolation of peptide (4x9) without prior reduction of the

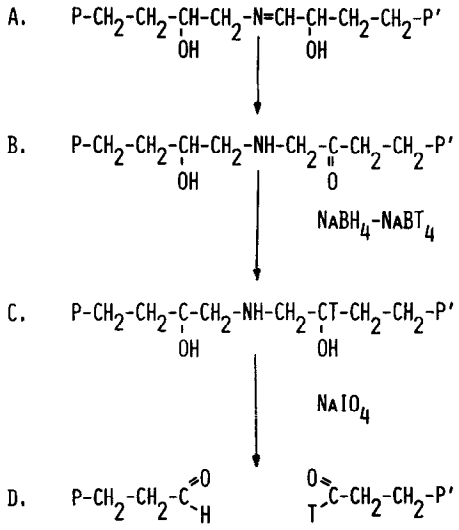

Figure 4. Summary of cross-link chemistry indicated in the present study. P and P' denote the polypeptide portions of collagen chains.

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cross-link. In addition, the unreduced keto-amine cross-link would be cleaved by periodate as demonstrated here for the cross-link joining peptides 4 and 9. Furthermore, borotritide reduction of the keto-amine compound would introduce tritium at the keto carbon atom (C). Periodate oxidation of the reduced cross-link would then produce a residue of α-amino glutaric acid γ-semialdehyde in each of the component peptides and radioactivity would remain with the peptides in the aldehyde function (D). Subsequent performic acid oxidation of the component peptides would yield an additional glutamic acid residue in each peptide and result in the loss of radioactivity through oxidation of the aldehyde function.

REFERENCES

1. Mechanic, G., Gallop, P. M., and Tanzer, M. L., Biochem. Biophys. Res. Commun., 45, 644 (1971).
2. Davis, N. R., and Bailey, A. J., Biochem. Biophys. Res. Commun., 45, 1416 (1971).
3. Mechanic, G., and Tanzer, M. L., Biochem. Biophys. Res. Commun., 41, 1597 (1970).

4. Bailey, A. J., and Shimokomaki, M. S., Fed. Eur. Biol. Soc. (FEBS) Letters, 16, 86 (1971).
5. Bailey, A. J., and Robins, S. P., Fed. Eur. Biol. Soc. (FEBS) Letters, 21, 330 (1972).
6. Robins, S. P., Shimokomaki, M., and Bailey, A. J., Biochem. J., 131, 771 (1973).
7. Miller, E. J., Biochem. Biophys. Res. Commun., 45, 444 (1971).
8. Miller, E. J., Woodall, D. L., and Vail, M. S., J. Biol. Chem., 248, 1666 (1973).
9. Miller, E. J., Biochemistry, 11, 4903 (1972).
10. Moore, S., J. Biol. Chem., 238, 235 (1963).
11. Miller, E. J., Biochemistry, 10, 3030 (1971).