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Identification of Hydroxypyridinium Cross-Linking Sites in Type II Collagen of Bovine Articular Cartilage†

Jiann-Jiu Wu and David R. Eyre*

ABSTRACT: In mature cartilage, collagen fibrils are strengthened by covalent intermolecular bonds provided by 3-hydroxypyridinium cross-linking residues. To determine the location of these trifunctional cross-links within the type II collagen molecule, CNBr peptides were analyzed from pepsin-soluble collagen and from guanidine hydrochloride insoluble collagen of bovine articular cartilage. The presence of hydroxypyridinium residues in collagen α chains and CNBr-derived peptides was detected by their characteristic natural fluorescence. Quantitatively, about one in three α chains from pepsin-soluble collagen was found to contain a hydroxypyridinium residue. Its distribution in the chains was limited to two CNBr peptides, which were purified by column chromatography on CM-cellulose and Bio-Gel P-30 followed by slab-gel electrophoresis in sodium dodecyl sulfate–polyacrylamide. The composition and properties of the two peptides indicated that the main component of one was α1(II)-CB9,7 and of the other α1(II)CB12. It was suspected that two amino-terminal telopeptides were cross-linked by hydroxylysylpyridinoline to α1(II)CB9,7 and two carboxy-terminal telopeptides to α1(II)CB12. The properties of fluorescent CNBr peptides isolated from digests of insoluble cartilage collagen supported this conclusion. Cleavage of the 3-hydroxypyridinium ring by UV light was exploited to confirm the identity of the cross-linked peptides. On UV irradiation, one cross-linked peptide released α1(II)CB9,7, and the other, α1(II)CB12. The findings indicate there are only two hydroxypyridinium cross-linking sites within the triple-helical region of the type II collagen molecule, probably placed symmetrically at opposite ends at residues 87 and 930, where telopeptide aldehydes are known to react to form the initial "head to tail" intermolecular bonds.

A naturally fluorescent amino acid, that embodies three hydroxylysine residues in a 3-hydroxypyridinium ring, was found in acid hydrolysates of bovine achilles tendon and proposed to be a trivalent cross-linking residue of collagen (Fujimoto et al., 1977, 1978). The cross-link, which was named pyridinoline, has since been found in the collagen of most connective tissues other than skin (Fujimoto & Moriguchi, 1978; Moriguchi & Fujimoto, 1978; Eyre & Oguchi, 1980; Kuboki et al., 1981; Eyre et al., 1984) and is especially abundant in articular cartilage (Eyre & Oguchi, 1980; Eyre et al., 1981). During cartilage growth, the divalent reducible cross-link hydroxylysino-5-ketonorleucine predominates (Robins & Bailey, 1974; Parsons & Glimcher, 1976; Shapiro et al., 1979), but in the mature tissue it is almost totally replaced by hydroxypyridinium residues (Eyre et al., 1981). The reducible cross-links are thought to be direct precursors of the hydroxypyridinium cross-links, and there is evidence that two reducibles give rise to one hydroxypyridinium (Eyre, 1980; Eyre et al., 1981).

Two forms of the cross-link have been identified, a major one which embodies three hydroxylysine residues [hydroxylysylpyridinoline (HP)[1]] and a minor one which embodies two hydroxylysines and one lysine [lysylpyridinoline (LP)] (Eyre,

† From the Laboratory for Skeletal Disorders, Children's Hospital and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received August 11, 1983.* The work was supported in part by National Institutes of Health Grants AM15671 and AM30774.

* Address correspondence to this author at the Laboratory for Skeletal Disorders, Children's Hospital, Boston, MA 02115.

[1] Abbreviations: HP, hydroxylysylpyridinoline; LP, lysylpyridinoline; CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Gdn·HCl, guanidine hydrochloride.

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1981). The structure of LP has recently been confirmed (Ogawa et al., 1982). Among normal tissues, LP is prominent only in bone and dentin (Eyre et al., 1984) but is abundant in cartilage from patients with Ehlers-Danlos Syndrome type VI, the hydroxylysine-deficient collagen disease (Eyre, 1982). Both types of hydroxypyridinium cross-linking residues are stable to acid hydrolysis, and neither is reduced by borohydride. However, the 3-hydroxypyridinium ring is unstable to UV light (Sakura & Fujimoto, 1981), which destroys the compound and can produce hydroxylysine among the destruction products (Sakura et al., 1982; Koob et al., 1983).

Despite growing information on the chemical nature of these cross-links, on their distribution in different collagenous tissues, and on changes in their concentration with age, little is known about their location within the collagen molecule or of their fibril-stabilizing properties.

As an essential step in understanding their function, we set out to determine their intermolecular loci in collagen fibrils. Articular cartilage seemed a good tissue to study because it is especially rich in hydroxypyridinium residues, virtually all of which have the HP structure. Hyaline cartilage also contains almost exclusively the homotrimeric type II collagen molecule $[\alpha 1(II)]_3$ which gives a relatively simple peptide profile after CNBr cleavage. The chosen approach was to purify $\alpha 1$(II) chains from pepsin-soluble collagen and then fractionate and identify the HP-containing chains and cyanogen bromide derived peptides by monitoring for the natural fluorescence of the cross-link during column chromatography and on gel electrophoresis.

Materials and Methods

Preparation of Cartilage Collagen. Bovine articular cartilage was sliced from the femoral surfaces of the knee (stifle) joints of 2-year-old steers. Proteoglycans were extracted from the finely diced tissues with 4 M guanidine hydrochloride and 0.05 M Tris-HCl, pH 7.5, for 24 h at 4 °C, and the collagenous residue was washed thoroughly with water and freeze-dried. Collagen was solubilized by stirring the residue with pepsin (10 to 1 by weight) in 3% (v/v) acetic acid for 24 h at 4 °C (Miller, 1972; Eyre & Muir, 1975). The solubilized type II collagen was clarified by centrifugation and precipitated by addition of NaCl to 0.7 M. the centrifuged pellet was suspended in and dialyzed against 0.1 M acetic acid and freeze-dried.

CM-cellulose Chromatography. Pepsin-soluble type II collagen was denatured at 50 °C and chromatographed on a column (1.8 cm × 10 cm) of CM-cellulose (Whatman CM52) at 42 °C (Eyre & Muir, 1975), eluting with a linear gradient of 0–0.1 M NaCl in 500 mL of 0.04 M sodium acetate, pH 4.8, containing 2% (v/v) 2-propanol [modified from Fukae & Mechanic (1980)]. The 2-propanol improved the recovery of protein applied to the column to at least 90%. The column effluent was monitored for protein absorbance at 230 nm. Collected fractions (4 mL) were assayed for 3-hydroxypyridinium fluorescence. At pH 4.8, the hydroxyl group of the hydroxypyridinium ring was partially ionized and showed a single emission maximum at 395 nm and a double excitation peak with maxima at 297 and 330 nm. Therefore, to measure fluorescence, fractions were acidified to below pH 3 by addition of 0.4 mL of glacial acetic acid which shifted the spectrum to a single excitation peak at 297 nm.

High-Performance Liquid Chromatography. The α chains of pepsin-soluble type II collagen were chromatographed by reverse-phase HPLC on an Aquapore RP-300 column (4.6 mm × 25 cm, 10-μm beads, 300-nm pores; Brownlee Labs) using an Altex instrument. The samples for injection were dissolved in 1% trifluoroacetic acid (TFA) at 5 mg/mL and heat denatured briefly at 40 °C. Solvent A contained 0.1% TFA (v/v), and solvent B contained 0.085% TFA (v/v) in acetonitrile–1-propanol (3:1 v/v). Solvents and samples were filtered through nylon membranes (0.45-μm pores). Collagen samples were eluted at a flow rate of 1 mL/min by using a composite linear gradient of solvent B, 20–23% in 4 min and then 23–32% in 50 min at room temperature. The effluent was monitored for UV absorbance at 220 nm. Fractions of 2 mL were collected and assayed for fluorescence by exciting at 297 nm and measuring the emission at 395 nm using 5-nm slits on a Perkin-Elmer Model 650-10S spectrofluorometer. Fractions of interest were pooled, freeze-dried, and characterized by slab-gel electrophoresis in SDS–5% polyacrylamide.

Cleavage with CNBr. Samples of guanidine hydrochloride insoluble cartilage collagen, of pepsin-soluble type II collagen (0.7 M NaCl precipitate), and of isolated α chains were treated with CNBr in 70% formic acid under N_2 at room temperature for 24 h on a shaker. Digests were filtered through glass wool, diluted 15-fold with water, and freeze-dried.

Chromatography of CNBr Peptides. The CNBr peptides were chromatographed on a CM-cellulose column (1.5 cm × 9 cm) at 42 °C as described previously (Eyre & Muir, 1975). A linear gradient of NaCl (0–0.16 M) in a total volume of 400 mL of 0.02 M sodium citrate, pH 3.6, containing 2% (v/v) 2-propanol [modified from Fukae & Mechanic (1980)] was applied. The effluent was monitored for UV absorbance at 230 nm, and collected fractions were assayed for fluorescence as before. Pooled fractions were desalted and further purified on a column (1.8 cm × 14 cm) of Bio-Gel P-30 (100–200 mesh) equilibrated at room temperature with 0.1 M acetic acid in 10% (v/v) 2-propanol.

The fluorescence spectra of fractions under fluorescent peaks were routinely scanned to confirm the presence of hydroxypyridinium residues.

SDS–Polyacrylamide Electrophoresis. Slab gels were run according to the method of Neville & Glossman (1974). Before being stained, gels were scanned for HP fluorescence in peptide bands. The gels were routinely fixed overnight in methanol–acetic acid–water (45:10:45 v/v) and then soaked in 10 (v/v) acetic acid for 1 h before being scanned at λ_{ex} = 297 nm and λ_{em} = 395 nm on a Perkin-Elmer fluorescence spectrophotometer (Model 650-10S) equipped with a thin-layer/slab-gel scanning accessory. The fluorescent peptides could also be detected immediately after electrophoresis by scanning the gel at λ_{ex} = 330 nm and λ_{em} = 395 nm. After the fluorescence scanning procedure, gels were stained with Coomassie brilliant blue R250 and were scanned for absorbance at 600 nm by using an E-C Apparatus densitometer.

The molecular weight of fluorescent peptides was assessed by comparing their mobilities with those of known CNBr peptides derived from type II collagen. The molecular weights of the $\alpha 1$(II) peptides, calculated from the data of Eyre & Muir (1975), were the following: CB8–10, 47 200; CB10, 33 300; CB11, 25 200; CB8, 13 800; CB9,7, 10 000; CB12, 8000. The molecular weights of the nonhelical telopeptides (CB4, M_r 1250; CB14, M_r 2000) were calculated from the data of Miller & Lunde (1973) and Miller (1972).

Preparative electrophoresis was carried out on a Bio-Rad Model 220 slab apparatus equipped with the accessory for elution of bands during the run. The preparations of cross-linked CNBr peptides partially isolated by CM-cellulose and subsequent Bio-Gel P-30 column chromatography were run. Lower chamber buffer was pumped at 30 mL/h through the collecting channel in the gel. Fractions (0.5 mL) were collected

and assayed for 3-hydroxypyridinium fluorescence. Aliquots of each fraction were examined by analytical SDS–polyacrylamide electrophoresis, staining with Coomassie blue to check for peptide purity. Fractions which showed a single band for the cross-linked peptide were pooled, dialyzed against water, freeze-dried, and hydrolyzed in 6 M HCl for amino acid analysis.

In some experiments, strips containing the cross-linked peptide bands were cut out from analytical gels after locating them by briefly staining the gel with Coomassie blue. The gel strips were destained in 50% (v/v) methanol in water until clear. The peptide was then eluted with 0.05 M NH_4HCO_3 containing 0.05% SDS as previously described by Weber & Osborn (1975).

Photolysis of Cross-Linked Peptides. A Mineralight UV SL-25 (Ultra-Violet Product Inc., San Gabriel, CA) UV lamp was used for UV irradiation. A solution of each cross-linked CNBr-peptide was irradiated, immediately as isolated by preparative electrophoresis, at the long-wavelength setting (366 nm) or desalted, resuspended in 10% acetic acid solution, and irradiated at the short-wavelength setting (254 nm). Samples were placed 5 cm from the light source and irradiated for 16 h at room temperature, and the effects were assessed by SDS gel electrophoresis.

Amino Acid Analysis. Peptide samples were hydrolyzed in 6 M HCl containing 10 mM phenol to prevent destruction of tyrosine and analyzed on a Beckman 121MB amino acid analyzer. The content of the HP amino acid was estimated on a portion of the hydrolysate and in hydrolysates of cartilage, of type II collagen fractions, and of isolated α chains by a new method using reverse-phase chromatography on an Altex ODS Ultrasphere column (4.6 mm × 25 cm; 5-μm beads) (Eyre et al., 1984).

Results

Similar concentrations of hydroxypyridinium cross-links were found in the cartilage collagen whether whole tissue, guanidine hydrochloride insoluble collagen, or pepsin-soluble type II collagen was analyzed. Whole cartilage, and the insoluble collagen after guanidine hydrochloride extraction, contained 0.5 mol of HP/100 mol of hydroxyproline, equivalent to about 1.5 mol of HP per mol of collagen. Pepsin-soluble type II collagen, which accounted for at least 50% of the total cartilage collagen, had a slightly lower HP content, at 0.42 mol of HP per 100 mol of hydroxyproline. After digestion of the collagen with CNBr in 70% formic acid, over 80% of the original HP residues were recovered in the freeze-dried peptides. Any chemical destruction of the HP residues by the CNBr treatment was minimal, therefore. Further tests indicated that 70% formic acid, and not the CNBr, was responsible for the apparent slight loss of HP residues on CNBr digestion.

Analysis of Pepsin-Soluble Type II Collagen. On CM-cellulose chromatography of the intact α1(II) chains, and of the derived CNBr peptides, consistently over 90% of the applied fluorescent material was recovered from the column. The eluted peak of α1(II) chains was heterogeneous by UV absorbance and fluorescence (Figure 1). Two main fluorescent peaks eluted ahead of the main absorbance peak and in the position of leading shoulders on it. Electrophoresis of material in the three pooled fractions (A, B, and C) spanning the composite α1(II) peak showed that all consisted primarily of α1(II) chains (Figure 1). The α component that was most fluorescent eluted earliest on CM-cellulose and hence was most acidic. It also ran slower on SDS–polyacrylamide electrophoresis, and hence appeared slightly larger, than the non-

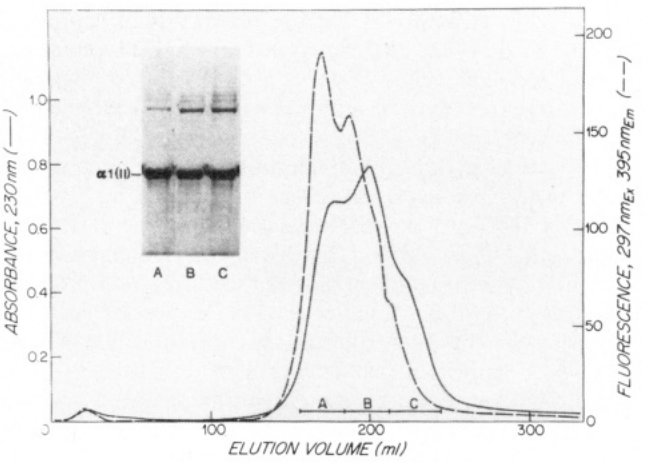

FIGURE 1: CM-cellulose chromatography of pepsin-soluble type II collagen from bovine articular cartilage. The column (Whatman CM52, 18 cm × 10 cm, at 42 °C) was equilibrated with 0.04 M sodium acetate, pH 4.8, containing 2% (v/v) 2-propanol. The sample (50 mg) was eluted with a linear gradient of 0–0.1 M NaCl in the same buffer. The effluent was continuously monitored for UV absorbance, and the collected fractions were assayed for fluorescence. The inset shows analyses by SDS–5% polyacrylamide electrophoresis of material in the pooled fractions marked by the bars.

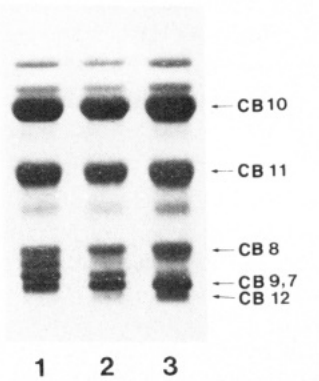

FIGURE 2: Electrophoresis of CNBr-derived peptides from α1(II) chains. Material in the pooled fractions from CM-cellulose chromatography (Figure 1) was digested with CNBr and run on SDS–10% polyacrylamide slab gels. Lane 1, fraction A; lane 2, fraction B; lane 3, fraction C.

fluorescent α1(II) chains. Quantitation of HP residues in hydrolysates of these α-chain fractions showed that the partially resolved chains in fractions A, B, and C contained 0.6, 0.3, and 0.1 mol of HP/100 mol of hydroxyproline, respectively. We suspected, therefore, that the chromatographic heterogeneity was based on the presence of a mixture of α1(II) chains, some that had telopeptides attached to their interiors via hydroxypyridinium residues and some that did not. The chromatographic results indicated that about one out of three α1(II) chains contained an HP residue with attached telopeptides. Further chromatographic heterogeneity was probably due to differences in charge between HP-bearing α1(II) chains having either amino-terminal telopeptides or carboxy-terminal telopeptides, or both, attached.

The CNBr peptides of the three α components from CM-cellulose are compared electrophoretically in Figure 2. All three fractions showed the typical profiles of the major CNBr peptides of type II collagen. However, consistent differences were evident in the region betwen CB8 and CB12. In fraction A (lane 1), which contained the most fluorescent α1(II) chain, peptides CB12 and CB9,7 were virtually absent, suggesting their participation in cross-linking. Indeed, unknown peptide bands of higher molecular weight were evident, running be-

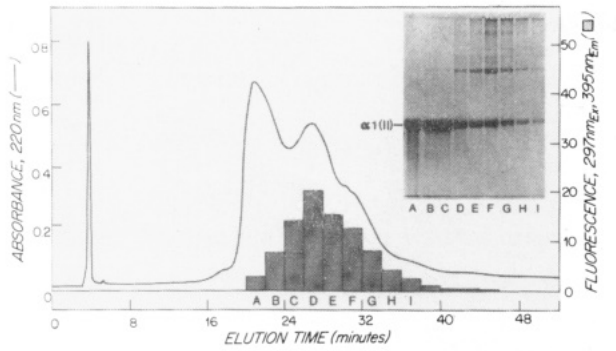

FIGURE 3: Fractionation of the α1(II) chains of pepsin-soluble type II collagen by reverse-phase HPLC. The elution profile was on a column (Brownlee Aquapore RP 300; 4.6 mm × 25 cm) using 1 mg of collagen. See Materials and Methods for details. The inset shows the results of slab gel electrophoresis in SDS–5% polyacrylamide of the fractions identified as A-I.

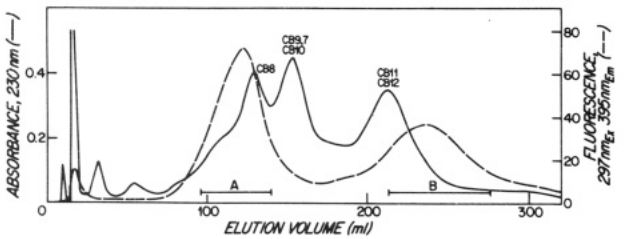

FIGURE 4: CM-cellulose chromatography of CNBr peptides derived from pepsin-soluble type II collagen. The column (Whatman CM52, 1.5 cm × 9 cm, 42 °C) was equilibrated with 0.02 M sodium citrate, pH 3.6, containing 2% (v/v) 2-propanol. The sample (40 mg) was eluted with a linear gradient of 0–0.16 M NaCl in the same buffer. The effluent was continuously monitored for UV absorbance, and collected fractions (4 mL) were assayed for fluorescence. Fractions A and B indicated by the bars were pooled for further purification.

tween CB9,7 and CB8 (Figure 2). In fractions B through C (lanes 2 and 3), peptides CB9,7 and CB12 become stronger and the unknown bands fainter, consistent with these fractions being rich in α chains that lacked telopeptides linked by an HP residue.

The α1(II) chains also appeared heterogeneous by reverse-phase HPLC chromatography, with the fluorescent α1(II) chains eluting later than the nonfluorescent α1(II) chains (Figure 3).

The CNBr digest of pepsin-soluble type II collagen was initially fractionated by CM-cellulose chromatography (Figure 4). The UV absorbance profile at 230 nm was similar to that previously reported for type II collagen from mammalian articular cartilage (Miller & Lunde, 1973; Eyre & Muir, 1975). Two peaks of fluorescence were resolved. The one eluting first contained 55% and the second one 45% of the total recovered fluorescence. The fluorescence spectra of material in the two peaks were identical, with emission maxima at 395 nm and excitation maxima at 297 nm at acid pH and 330 nm at neutral pH. Pooled fractions (Figure 4, fractions A and B) were desalted and further resolved by column chromatography on a Bio-Gel P-30 (Figure 5). The fluorescent fractions marked by bars were pooled, freeze-dried, and analyzed by electrophoresis on SDS–polyacrylamide gels.

The characteristic fluorescence of the HP residue proved to be an extremely sensitive and specific marker for detecting cross-linked peptides in SDS–polyacrylamide gels. Figure 6A shows the densitometric and fluorometric scanning patterns of the total CNBr peptides derived from pepsin-soluble type II collagen. The two fluorescent peaks had estimated molecular weights of 12 500 and 10 500. The enriched fractions from Bio-Gel P-30 gave several peptide bands by staining, but

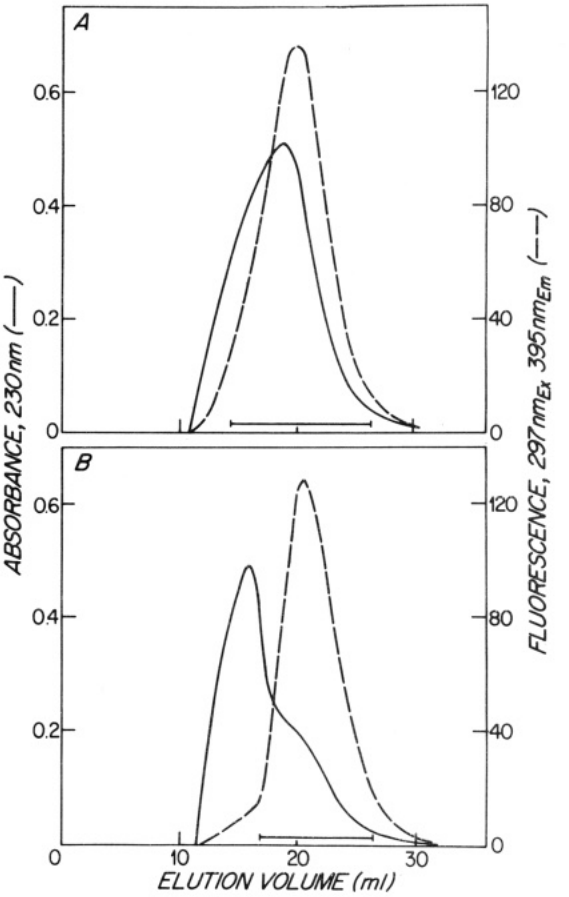

FIGURE 5: Molecular sieve chromatography of fluorescent material pooled from CM-cellulose as shown in Figure 4. The column (Bio-Gel P-30, 100–200 mesh, 1.8 cm × 14 cm) was eluted with 0.1 M acetic acid containing 10% (v/v) 2-propanol. (A) Fraction A; (B) fraction B.

each showed only one band of fluorescence (Figure 6B,C) corresponding to the peptides of molecular weights 12 500 and 10 500 seen in the total digest (Figure 6A).

Because the polyacrylamide gel was itself fluorescent, it was not possible to determine the fluorescence spectra of HP peptides within the gel. Nevertheless, the optimal wavelengths (λ) for detecting the fluorescent peptides on scanning the gel were λ_{ex} = 297 nm and λ_{em} = 395 nm when the gel was equilibrated in 10% acetic acid and λ_{ex} = 330 nm and λ_{em} = 395 nm immediately after electrophoresis with the gel still at pH 9. After elution from the gel, the excitation and emission spectra of the peptides were virtually identical with those of the purified HP amino acid.

The pure, fluorescent peptides isolated by preparative electrophoresis were subjected to UV irradiation as described under Materials and Methods. The cross-linked peptides CB9,7HP and CB12HP respectively released smaller peptides that ran identically with CB9,7 and CB12 on SDS–polyacrylamide electrophoresis (Figure 7). Control experiments showed that under these conditions of irradiation, the single-chained peptides CB8 and CB9,7 were unaffected and had not suffered peptide bond cleavage.

The amino acid compositions of cross-linked peptides CB9,7HP and CB12HP are given in Table I. They are consistent with CB9,7 and CB12 contributing the major portions of CB9,7HP and CB12HP, respectively, and with short telopeptide sequences of 12–17 residues being attached to each of the two remaining arms of the trivalent cross-linking residue in each peptide. The electrophoretic mobilities of the cross-linked peptide (Figure 6) also indicate a size increase

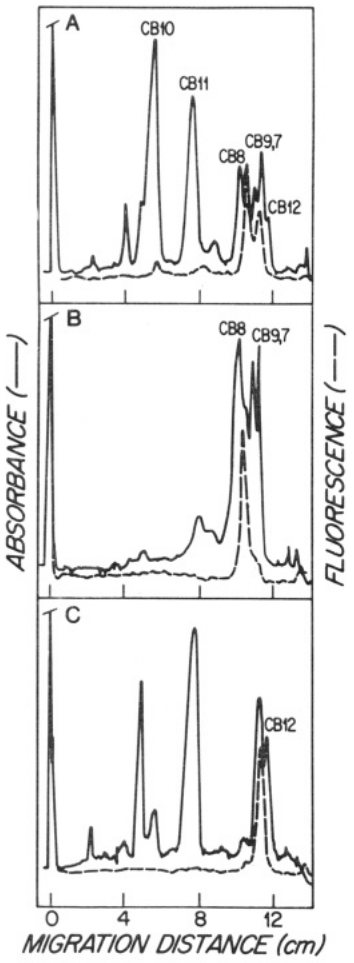

FIGURE 6: Densitometry and fluorometry profiles of CNBr peptides from pepsin-soluble collagen fractionated by slab-gel electrophoresis in SDS–10% polyacrylamide. Gels were scanned for fluorescence before being stained as described under Materials and Methods. (A) CNBr digest of total pepsin-soluble type II collagen. (B) Pooled material shown in Figure 5A. (C) Pooled material shown in Figure 5B.

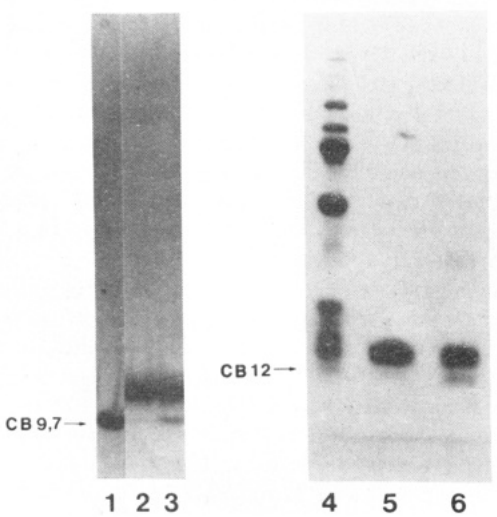

FIGURE 7: Electrophoretic analysis of the effects of UV irradiation on HP cross-linked peptides from pepsin-soluble type II collagen. Isolated CNBr peptides (see Materials and Methods) were irradiated at 366 nm in 0.43 M Tris-HCl, pH 9.18, for 16 h. Lane 1, standard, peptide CB9,7; lane 2, peptide CB9,7HP; lane 3, peptide CB9,7HP after UV irradiation; lane 4, total CNBr peptides of pepsin-soluble type II collagen; lane 5, peptide CB12HP; lane 6, peptide CB12HP after UV irradiation. Samples in lanes 1–3 were analyzed in SDS–10% polyacrylamide while samples in lanes 4–6 were analyzed in SDS–12% polyacrylamide.

Table I: Amino Acid Composition of Fluorescent Peptides CB12HP and CB9,7HP from Pepsin-Solubilized Type II Collagen

	CB12HP	CB12[a]	CB9,7HP	CB9,7[b]
3-Hyp			1 (0.7)	1
4-Hyp	12 (11.8)	12	10 (9.7)	10
Asp	7 (6.9)	4	9 (9.2)	5
Thr	2 (1.5)	1	4 (3.8)	2
Ser	5 (5.3)	4	8 (7.8)	4
Glu	11 (11.2)	6	10 (9.7)	9
Pro	9 (8.9)	6	13 (13.0)	13
Gly	36 (36.0)	28	42 (42.0)	36
Ala	8 (7.8)	5	11 (10.9)	8
Val	3 (2.9)	2	2 (2.1)	2
Ile	1 (0.8)	0	3 (2.9)	1
Leu	5 (4.8)	2	5 (5.0)	3
Tyr	2 (1.8)	1	1 (0.7)	0
Phe	2 (2.0)	1	3 (2.8)	1
Hyl	2 (2.3)	3	1 (0.9)	2
Lys	3 (3.4)	3	2 (2.1)	2
His	2 (1.9)	1	1 (1.3)	1
Arg	6 (5.6)	4	6 (5.8)	6
Hse[c]		1		1
HP	1 (0.6)		1 (0.4)	
	117[d]	84[d]	133[d]	107[d]

[a] Values in this column from sequence data (Butler et al., 1976). [b] Data from Eyre & Muir (1975). [c] Recoveries of homoserine were particularly low and inaccurate due to the elution of interfering substances from the polyacrylamide in the region of homoserine lactone. [d] Total.

of about 25–30 residues over the single-chained peptides, CB9,7 and CB12, again suggesting attached telopeptide sequences on the other two arms.

The rather low recovery of HP residues from the purified cross-linked peptides may be due to destruction during isolation. The HP ring is highly unstable to UV light, and it was noticed that fluorescence was progressively lost during purification. Some ring opening, but retained trivalent cross-linking function, may therefore have occurred. Chemical modification of a fraction of HP during CNBr digestion could have caused further apparent losses. Also, contaminants that eluted with the peptides from the polyacrylamide gel may have resulted in some destruction during acid hydrolysis. Control experiments with collagen samples hydrolyzed in the presence of sample buffer (0.05 M Tris-HCl, pH 6.8, containing 10% glycerol and 1% SDS) destroyed 40% of the HP as measured by a reverse-phase HPLC procedure (Eyre et al., 1984).

Though the present data provide no direct evidence, it is strongly suspected that the other two short peptide chains in each cross-linked structure are two amino-terminal telopeptides in CB9,7HP and two carboxy-terminal telopeptides in CB12HP. Because of the likelihood of variable degradation of the telopeptide sequences during pepsin extraction (Miller, 1972), there could be variability in the lengths of these attached peptides. This might be a source of the slight heterogeneity in the HP-cross-linked peptides seen on chromatography on CM-cellulose (Figure 4) and on reverse-phase HPLC (data not shown).

Insoluble Collagen. Electrophoretic profiles of fluorescent CNBr peptides from Gdn·HCl-insoluble cartilage collagen (Figure 8A) differed consistently from those of pepsin-soluble type II collagen (Figure 6A). The fluorescent band corresponding to CB12HP was diminished, and a new fluorescent band migrating slightly faster than CB11 appeared. Peptides CB9,7, CB12HP, and CB9,7HP derived from the insoluble collagen were slightly larger than those of pepsin-soluble collagen, each by about three to five amino acid residues as judged their relative mobilities. This fits the findings of Miller (1972) that pepsin removes CB14 and a few residues at the

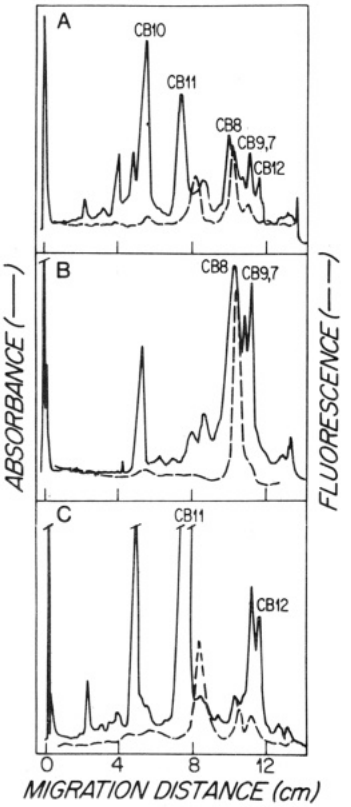

FIGURE 8: Densitometry and fluorometry profiles of CNBr peptides from insoluble cartilage collagen fractionated by slab-gel electrophoresis in SDS–10% polyacrylamide. (A) CNBr digest of total insoluble collagen. (B) Pooled fraction A from Figure 9. (C) Pooled fraction B from Figure 9.

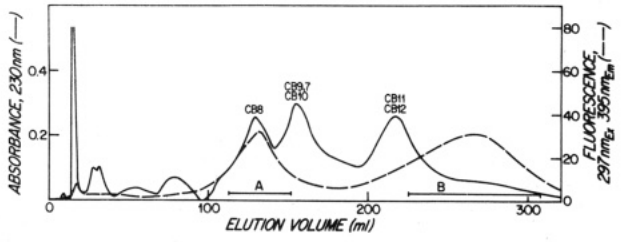

FIGURE 9: CM-cellulose chromatography of CNBr peptides of Gdn·HCl-insoluble cartilage collagen. The conditions are described in the legend to Figure 4. The fractions indicated by the bars were pooled for further analysis.

carboxy terminus of CB7 in chick type II collagen. The HP-cross-linked, CB peptides of the insoluble collagen were isolated preparatively by using procedures similar to those used for the pepsin-soluble collagen.

A CM-cellulose chromatograms of the CNBr peptides of insoluble cartilage collagen is shown in Figure 9. Although the UV absorbance profile is very similar to that of pepsin-soluble collagen, the distribution of fluorescent material was shifted. A larger proportion of the total fluorescence eluted in the later peak, and both fluorescent peaks were retained longer on the column relative to the main UV-absorbing peaks. Material in the earlier fluorescent peak (fraction A, Figure 9) showed a fluorescent peptide closely resembling CB9,7HP in mobility on electrophoresis (Figure 8B) after P-30 chromatography (not shown). On UV irradiation, it also released a peptide that on electrophoresis had a mobility identical with that of CB9,7 (Figure 10). Electrophoresis after P-30 chromatography showed that the second fluorescent peak to elute from CM-cellulose (fraction B, Figure 9) contained the fluorescent peptide not seen in the pepsin-soluble collagen (Figure 8C). Its estimated molecular weight (22000) matches

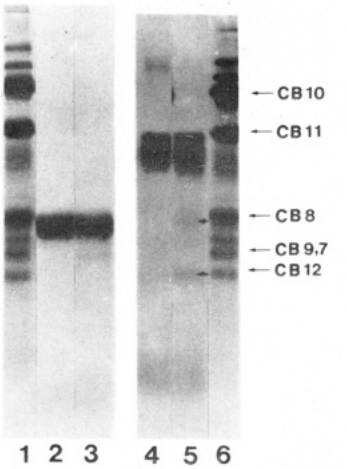

FIGURE 10: Electrophoretic analysis of the effects of UV irradiation on HP-cross-linked peptides from insoluble cartilage collagen. The major fluorescent bands shown in Figure 8B,C were excised from the gel. The peptides were eluted and irradiated at 254 nm in 10% acetic acid for 16 h and then run in a SDS–15% polyacrylamide slab gel. Lanes 1 and 6, total CNBr peptides of insoluble collagen; lanes 2 and 3, the fluorescent peptide shown in Figure 8B before and after UV irradiation, respectively; lanes 4 and 5, the major fluorescent peptide shown in Figure 8C before and after UV irradiation, respectively. In lane 5, the upper arrowhead indicates the suspected peptide CB9,7,14 × CB14 and the lower arrowhead CB12.

that of CB9,7,14 × CB14 × CB12, which could be derived by incomplete CNBr cleavage of the C-terminal telopeptide donors to the cross-link (illustrated in Figure 11). On UV irradiation, this peptide was partially cleaved into two smaller peptides that on electrophoresis had apparent molecular weights equal to CB12 and CB9,7,14 × CB14 (Figure 10, lane 5, and Figure 11). The peptides released by UV irradiation are indicated by arrowheads. The preparation shown in lanes 4 and 5 of Figure 10 is still contaminated with a small amount of peptide CB11 and a trace of another peptide running higher in the gel. This latter peptide appeared to be a dimer of the cross-linked peptide that formed when the sample was freeze-dried. We have no chemical explanation of why this artifactual dimer should occur, but it was clear on analysis of many different preparations that its intensity was unrelated to that of the eventual UV cleavage product of the cross-linked peptide.

In some CNBr digests, a small amount of another fluorescent peptide of even higher molecular weight (32000) was detected (Figure 8A, small peak at 5.5 cm). This has the expected mobility for CB9,7,14 × CB9,7,14 × CB12. In support of its identity as a partial cleavage product of CNBr digestion, the band was especially enriched in samples cleaved with CNBr for only 4 h instead of 24 h. This presence of two partial cleavage products of molecular weights 22000 and 32000 provides indirect evidence that the HP-cross-linked peptides are three-chained structures.

Discussion

The spectroscopic and chemical identification of hydroxypyridinium residues in purified α chains and derived CB peptides demonstrates conclusively that the compound is a natural cross-linking component of collagen and is not, for example, formed artifactually during acid hydrolysis (Elsden et al., 1980). In addition, essentially all the HP in cartilage is contained in type II collagen as judged by the similar concentrations in moles of HP per mole of collagen found in purified type II as in whole cartilage.

The scanning of polyacrylamide gels for HP fluorescence proved to be a powerful analytical technique for locating the

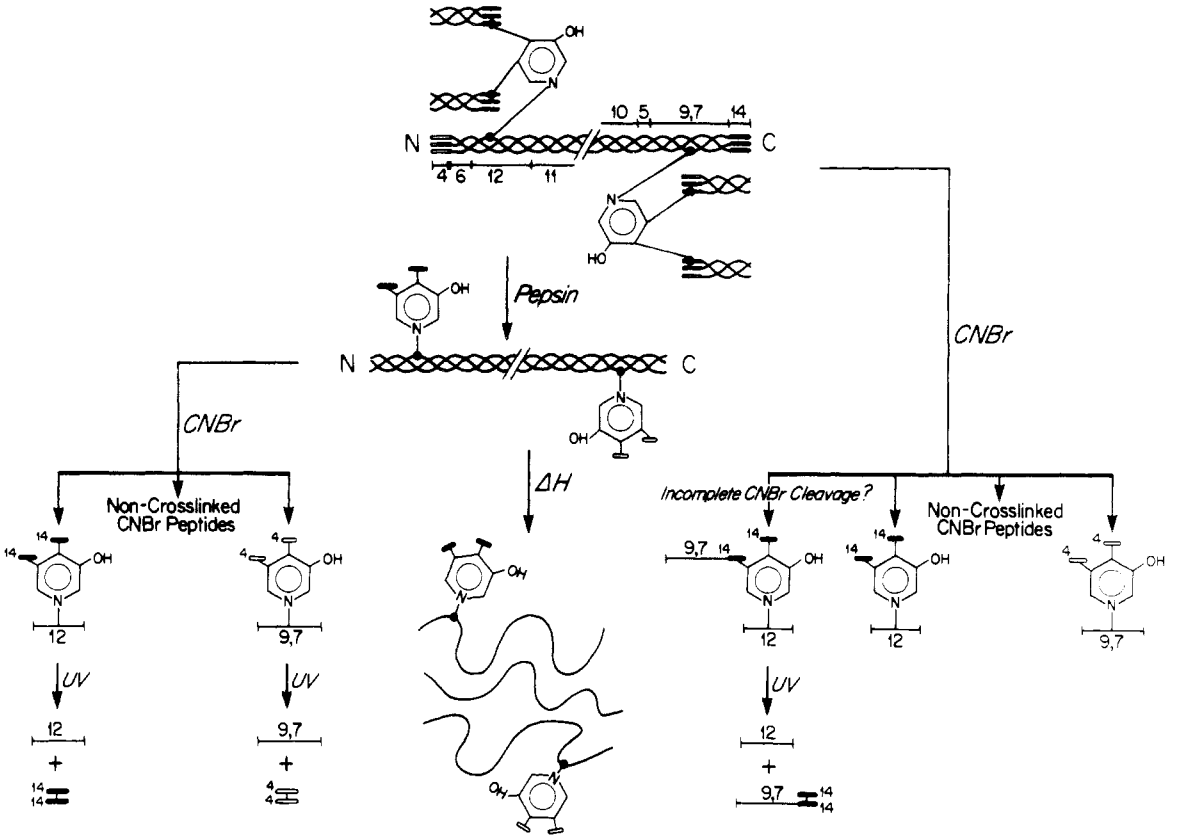

FIGURE 11: Diagram showing the two suspected intermolecular loci of HP cross-links in type II collagen and summarizing how the various cross-linked CNBr peptides are thought to have arisen in the present study. The indicated linkage of three collagen molecules by each HP residue is speculative and based on a postulated mechanism of cross-link formation (Eyre, 1980) rather than current structural knowledge. The two telopeptide sequences proposed to be attached to each HP residue might also be present in chains of the same collagen molecule.

HP cross-links in collagen peptides. The cross-linked peptides were separated in a single run with excellent resolution. They can be located and recovered from the gel for further characterization by using very small samples. A peptide containing 60 pmol of HP is readily detected in a 1.5-mm-thick slab gel using an 8-mm-wide sample well. The amount of sample can be scaled down further by using a thinner gel or a narrower sample well. The procedure should be useful for the rapid, semiquantitative comparison of HP cross-link distributions in the collagens of various types of connective tissue.

Though the full amino acid sequence of the α1(II) chain has not been published, the HP cross-linking sites can be predicted from the CNBr peptide map of α1(II) (Miller & Lunde, 1973; Miller et al., 1973; Bornstein & Traub, 1979) and by homology to known sites of telopeptide aldehydes in type I collagen. Previous studies on type II collagen of chick cartilage showed intermolecular cross-links between peptides CB4 and CB9 (Miller, 1971; Miller & Robertson, 1973). Recently, it has been demonstrated that an HP-cross-linking site in bovine type I collagen of tendon (Fujimoto, 1980) and of dentin (Kuboki et al., 1981) probably involves the interaction of a hydroxylysine residue located in α1(I)CB5 with an aldehyde residue in the telopeptide region of α1(I)CB6.

Figure 11 illustrates the two locations for HP cross-links within the helix of the type II collagen molecule indicated by the results and speculates how the residues might cross-link three collagen molecules. Quantitatively, the two helical sites are about equally important in type II collagen of bovine articular cartilage. The exact sites in peptides CB12 and CB9,7 are probably the known hydroxylysines at residues 87 and 930 already identified as major locations for reducible cross-links in type I, II, and III collagens (Bornstein & Traub, 1979; Tanzer & Waite, 1982; Kuhn, 1982). These triple-helical residues are presumed to contribute the ring nitrogen side arms of the trivalent 3-hydroxypyridinium cross-links and are probably the hydroxylysines liberated on UV irradiation (Koob et al., 1983; Lamy et al., 1977). We suspect that two amino-terminal telopeptides are attached to the other two side arms of the HP residue in CB9,7 and two carboxy-terminal telopeptides in CB12. This is consistent with four main cross-linking residues along the α1(II) chain, two hydroxylysines in the triple helix and one hydroxyallysine in each telopeptide. more work, including sequence analysis, is needed to confirm the identity of the telopeptide components. Sequencing alone, however, will not distinguish whether the two telopeptide arms originate in one or two collagens molecules, and this question will be hard to resolve.

The present results indicate that all the HP cross-links in type II collagen of bovine articular cartilage are present at head to tail interaction sites between molecules staggered by four D periods (i.e., 4 × 670 Å). Each HP residue can potentially link three different collagen molecules, two of which must be in register laterally. This is consistent with the proposal that neighboring pairs of reducible cross-links interact within fibrils to form HP cross-links (Eyre, 1980). It requires that the molecular packing arrangement always provides head to tail cross-linked molecules (4D stagger) with a lateral neighbor that is in register (0D stagger) and close enough for telopeptide side-chain interactions. The data at present, however, cannot rule out only two collagen molecules linked by the HP residue, i.e., two adjacent telopeptides in one molecule linked to the helix of a second molecule.

Added in Proof

While this paper was in press, a paper appeared (Robins & Duncan, 1983) reporting comparable findings, also on

CNBr-derived peptides from the bovine type II collagen molecule, and concluding that the HP residues were located at essentially the same two basic sites as found here.

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