Cross-Linking Connectivity in Bone Collagen Fibrils: The COOH-Terminal Locus of Free Aldehyde[†,‡]

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ABSTRACT: Quantitative analyses of the chemical state of the 16^c residue of the α1 chain of bone collagen were performed on samples from fetal (4–6-month embryo) and mature (2–3 year old) bovine animals. All of this residue could be accounted for in terms of three chemical states, in relative amounts which depended upon the age of the animal. Most of the residue was incorporated into either bifunctional or trifunctional cross-links. Some of it, however, was present as free aldehyde, and the content increased with maturation. This was established by isolating and characterizing the aldehyde-containing peptides generated by tryptic digestion of NaB^3H_4-reduced mature bone collagen. We have concluded that the connectivity of COOH-terminal cross-linking in bone collagen fibrils changes with maturation in the following way: at first, each 16^c residue in each of the two α1 chains of the collagen molecule is incorporated into a sheet-like pattern of intermolecular iminium cross-links, which stabilizes the young, nonmineralized fibril as a whole. In time, some of these labile cross-links maturate into pyridinoline while others dissociate back to their precursor form. The latter is likely due to changes in the molecular packing brought about by the mineralization of the collagen fibrils. The resultant reduction in cross-linking connectivity may provide a mechanism for enhancing certain mechanical characteristics of the skeleton of a mature animal.

The mechanical properties of bone derive from the properties of its two major constituents, type I collagen and mineral (Glimcher, 1976). The collagen is present in the form of axially periodic fibrils (see Figure 1). These are produced by bone cells through the elaboration of rod-shaped molecules which have the ability to aggregate spontaneously into fibrillar form (Bruns et al., 1979). The molecules within the fibrils are packed in parallel and are axially offset with respect to one another by distances equal to multiples of the axial repeat distance of the fibril (Hodge & Petruska, 1963; Golub & Katz, 1977). During fibrillogenesis, an enzymatically induced process of cross-linking occurs and stabilizes the fibril. Soon after this, the fibrils start mineralizing, becoming, in a manner of days, impregnated by very fine apatitic crystals (Robinson & Watson, 1952, 1955; Glimcher, 1976; Katz & Li, 1973a,b; Weiner & Traub, 1986). The mineralization process then continues over months and perhaps years to a point that the fibrils are fully permeated and encased in mineral. Coincident with the mineralization of type I collagens, changes occur in the shape of the fibrils (Lee & Glimcher, 1989; Landis et al., 1991), and in the geometry of the organization of the molecules within a fibril (Bonar et al., 1985; Sachar et al., 1987; Bigi et al., 1988; Katz et al., 1989).

We have been studying the stereochemical aspects of cross-linking in a variety of connective tissue collagens with the goal of characterizing the molecular organization within fibrils and have now extended this approach to mineralized tissues. We have reported some initial findings on fetal bone (Yamauchi et al., 1989) and present here more results of a study of the bovine bone system. Somewhat comparable findings on bovine dentin will be presented in a separate publication.

The formation of covalent cross-links between collagen molecules is initiated by the enzymatic oxidative deamination of ϵ-amino groups on specific peptidyl lysine and hydroxylysine in the COOH- and NH_2-terminal nonhelical portions of the molecule (Tanzer, 1976). The aldehydes then spontaneously react with specific peptidyl hydroxylysine residues on juxtaposed neighboring molecules in a fibril to form iminium cross-links (see Figure 2) (Yamauchi et al., 1986a). These can mature into nonreducible cross-links by differing pathways which are tissue specific (Eyre et al., 1984; Yamauchi & Mechanic, 1988). As shown below, almost all of the COOH-terminal telopeptide aldehydes of bone collagen are derivatives of Hyl and are referred to here as Hyl^{ald} (2-hydroxy-5-amino-5-carboxypentanal). These react with Hyl-87 in the triple-helical portion of the collagen molecule to form bifunctional reducible cross-links, referred to as deH-DHLNL[1] (dehydrodihydroxylysinonorleucine). These cross-links are present in either an aldimine or ketoamine form (Mechanic et al., 1974). Upon reduction, both forms produce the same product, which is referred to as DHLNL. The trifunctional cross-link pyridinoline (Pyr) is thought to be a direct maturational product of deH-DHLNL, either through the reactions between two vicinal ketoamine cross-links (Eyre

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[1] Abbreviations: cpm, counts per minute; deH, dehydro; DEAE, diethylaminoethyl; DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; dpm, disintegrations per minute; EDTA, ethylenediaminetetraaccetic acid; HLNL, hydroxylysinonorleucine; HNL, hydroxynorleucine; HPLC, high-performance liquid chromatography; Hyl^{ald} or hydroxylysine aldehyde, 2-hydroxy-5-amino-5-carboxypentanal; Lys^{ald} or lysine aldehyde, 5-amino-5-carboxypentanal; LP, lysyl analogue of pyridinoline; PDL, periodontal ligament; Pyr, pyridinoline; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TPCK, 1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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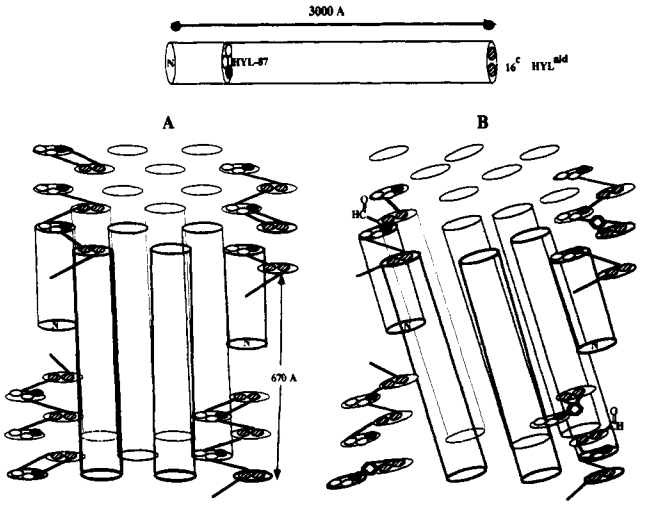

FIGURE 1: Illustration of the effects of mineralization and maturation on the packing and cross-linking of bone collagen molecules. In the top panel, the type I collagen molecule is represented as a flexible cylinder, about 3000 Å long. The NH_2-terminal end and the carboxyl-terminal aldehydes are so labeled. The position of the two α1 chain Hyl-87 residues are indicated by open circles, the single α2 chain Hyl-87 by a solid circle and the 16^c aldehydes of the α1 chains by shaded cricles. One axial period of a collagen fibril is sketched in the bottom panels. Note the gaps between the NH_2 and COOH ends of molecules that account for about half of an axial period, and which are the putative sites for the mineral crystallites at early stages of the mineralization of collagen. The values for the axial periods shown, 670 Å for nonmineralized collagen (in panel A) and 650 Å for bone (in panel B), are based on the neutron diffraction values reported by Bonar et al. (1985). We have represented this by a tilting of the molecular segments away from the fibril axis in panel B. Panel A illustrates the stoichiometry of bifunctional cross-linking (the solid lines) observed for the PDL and predentin. Note that there are two linkages at the COOH ends for each molecule with the involvement of Hyl-87 of α1 chains and Hyl-87 of the α2 chain being in the proportion of 3/1; panel B illustrates cross-linking in mature bone. The appearance of pyridinoline in mature matrices is represented by the hexagon connecting α1-Hyl-87 and two 16^c-Hyl^{ald}. Pyr occurs in tendons as well as bone collagen. However, the presence of free COOH-terminal aldehyde, represented by HC=O, has been to date observed only in bone and dentin.

& Oguchi, 1980, Eyre et al., 1984) or through the reactions between a vicinal ketoamine and Hyl^{ald} (Robins & Duncan, 1983) or between a vicinal ketoamine and iminium cross-link (Yamauchi & Mechanic, 1988).

The results that we report here are based on an approach used to characterize the stoichiometry of cross-linking in the periodontal ligament (PDL) (Yamauchi et al., 1986a). The experimental procedure consisted of reducing the collagen with standardized NaB^3H_4, completely digesting the collagen with trypsin, and isolating the digestion products. The identity of the various radioactive peaks was then established by means of amino acid and sequence analyses, and their quantification, by radioassay. The relevant findings for the PDL were that the 16^c residues of the COOH telopeptides were completely converted to aldehyde, that about $^2/_3$ of the aldehyde was Hyl^{ald} and $^1/_3$ Lys^{ald}, and that each aldehyde went on to form the iminium cross-links deH-DHLNL and deH-HLNL (dehydrohydroxylysinonorleucine), respectively. In order to study the cross-linking connectivity in bone collagen fibrils, we have extended the protocol by fractionating and quantifying Pyr and aldehyde-containing fragments. Our initial findings were that fetal bone had a cross-linking stoichiometry approaching that of the PDL; i.e., almost every molecule was connected to two other molecules by means of COOH-terminal linkages (Yamauchi et al., 1989). However, we will show here that a decrease in the COOH-terminal connectivity occurs in mature animals due in part to a splitting of deH-DHLNL cross-links. This may be the consequence of changes in the structure of collagen fibrils brought about by mineralization.

Materials and Methods

Preparation of Collagen. Fresh femoral bone samples of 4–6-month embryos and 2–3 year old cows were obtained from a local slaughterhouse. The samples from five different animals in each age group were pooled. A 9 year old bovine bone was also obtained. After removal of surrounding soft tissues, cartilage, and bone marrow, the bones were cut into small pieces. The bone pieces were defatted in methylene chloride and methanol solution (2:1) overnight at 4 °C and then pulverized to fine powder under liquid nitrogen using a Spex Freezer Mill (Spex Inc., Metuchen, NJ). Five grams of fetal bone powder and 10 g of mature bone powder were taken and demineralized with 0.5 M EDTA containing a cocktail of protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 25 mM ϵ-aminocaproic acid, and 5 mM benzamide hydrochloride hydrate), pH 7.4, for 3 weeks at 4 °C with 4–5 changes. The EDTA residues were washed with cold distilled water thoroughly and lyophilized.

Reduction with NaB^3H_4. The demineralized fetal bone (1.2 g) and mature bone (2.1 g) were suspended in 0.15 M TES buffer, pH 7.4, and reduced with standardized NaB^3H_4 by the method described previously (Fukae & Mechanic, 1980; Yamauchi et al., 1986a). The specific activity of the NaB^3H_4 was determined by the method described previously (Yamauchi et al., 1986a) and it was 9.23×10^7 dpm/μmol. The reduced samples were dialyzed against cold distilled water until the dialyzate counted less than 200 cpm/mL and were then lyophilized.

Cross-Link Analysis. The reduced fetal and mature bone collagens were hydrolyzed in 6 M HCl under N_2 for 24 h at 115 °C. An aliquot of the hydrolysate was subjected to amino acid analysis to determine Hyp. The hydrolysates in amounts containing 300 nmol of hydroxyproline were applied to the cross-link analyzer. Cross-link analyses were performed on a Varian 5500 liquid chromatography equipped with a stainless steel column (Interaction AA911) filled with a cation-exchange resin (Yamauchi et al., 1986a). A slightly modified buffer and gradient system was used than described previously (Mechanic, 1974). Radioactivity was measured with an on-line FLO-ONE Beta instrument (Radiomatic Instrument and Chemical Co., Tampa, FL). Pyr and its lysyl analogue (LP) were determined by a fluorescence flow monitor (Shimadzu Instrument Co., Japan) calibrated by an apparently pure Pyr cross-linked peptide isolated from bovine Achilles' tendon. Excitation and emission were set at 330 and 390 nm, respectively.

The NaB^3H_4-reduced cross-links as well as their aldehyde precursors were identified on the basis of their chromatographic elution positions on the cross-link analyzer by using two different gradient systems. The elution positions for these compounds were established by using the standards prepared from reduced bovine dentin and Achilles' tendon, skin, and periodontal ligament collagens (Mechanic & Tanzer, 1970; Mechanic et al., 1971; Yamauchi et al., 1986a, 1987).

DHNL (dihydroxynorleucine, reduced Hyl^{ald}) in the reduced bone collagen as well as in the isolated peptide was confirmed by chromatographic comparison with the standard prepared from Achilles' tendon collagen (Mechanic et al., 1971). The structure of DHNL was originally determined by mass spectrometry and by its synthesis (Mechanic & Tanzer, 1970).

The cross-link analyses for fetal and 2–3 year old bovine bones were performed in triplicate, and a single analysis was

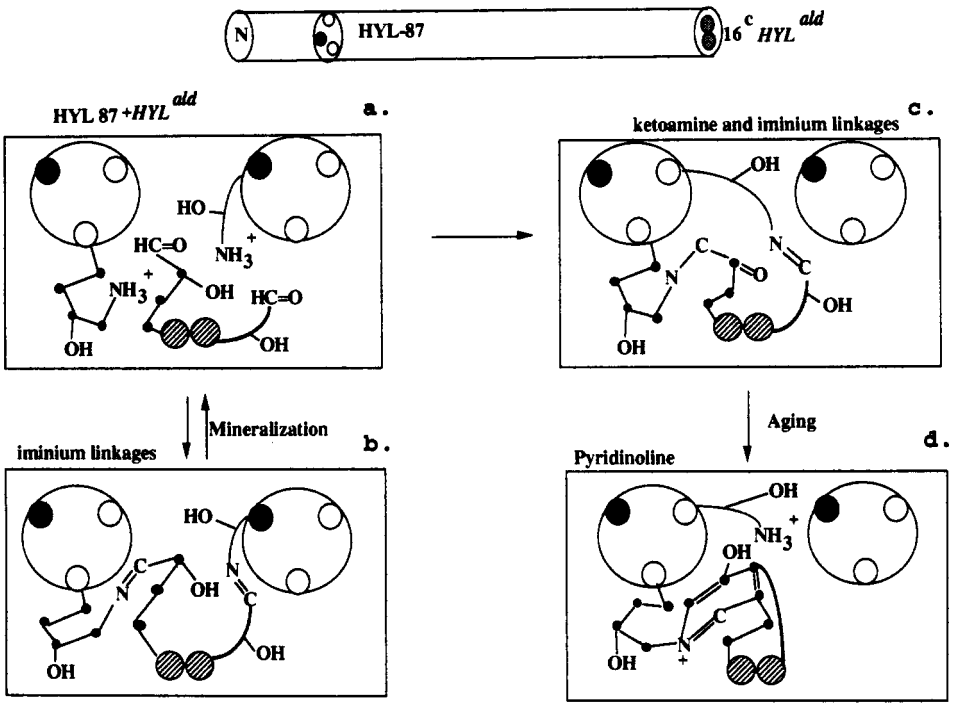

FIGURE 2: Illustration of the COOH-terminal cross-linking chemistry in bone collagen fibrils. In the top panel, the type I collagen molecules are represented as cylinders with the position of Hyl-87 residues in α1 chains indicated by open circles, the Hyl-87 residues of α2 chains be a closed circle, and the two α1 chain 16^c-Hyl^{ald} residues by shaded circles. The reaction stereochemistry between three molecules, as viewed in cross section, is illustrated in the four lower panels. Panel a indicates the initial reactant states of two of the six Hyl-87 and the two 16^c-Hyl^{ald}. The aldehydes react first to form two iminium linkages either with Hyl on different molecules (panel b) or on the same molecule (panel c). An iminium bond can rearrange to form a ketoamine linkage as indicated in panel c. Note that for molecules having the (same) azimuthal orientation indicated, the proportion of the cross-links involving α1-Hyl-87 to α2-Hyl-87 is about 3/1. In both mineralized and nonmineralized collagens two vicinal bifunctional linkages can spontaneously maturate into pyridinoline (panel d). This is most likely a pure aging effect. The presence of free aldehyde in mature bone and dentin suggests, however, that some of the iminium linkages dissociate back to their reactant states as a result of mineralization.

performed on material obtained from 9 year old bovine.

Digestion with Trypsin. Two grams of reduced mature bone and 1.15 g of reduced fetal bone were digested with TPCK–trypsin (Worthington) as described previously (Yamauchi et al., 1981; Kuboki et al., 1981; Yamauchi et al., 1986a). Approximately 99% of the collagen was recovered in the supernatant for both samples.

Molecular Sieve and Ion-Exchange Chromatography. Gel filtration chromatography was carried out with 200–400-mg portions of the trypsin digest each time, on a Sephadex G-50 superfine column (1.9 × 206 cm) that had been equilibrated with 2 M guanidine hydrochloride and 0.05 M Tris-HCl, pH 7.5, by the method described previously (Yamauchi et al., 1986a). Five-milliliter fractions were collected and assayed for absorbance at 230 nm, fluorescence, and radioactivity. The radioactive fraction (IV, Figure 2) of mature bone collagen was then chromatographed on ion-exchange columns.

Ion-exchange column chromatography was carried out basically as reported previously (Yamauchi et al., 1986a). A DEAE-cellulose column (1.7 × 13 cm, Whatman DE-52) was equilibrated with 0.01 M NH_4HCO_3 containing 5% 2-propanol (Fukae & Mechanic, 1980) at 42 °C. Elution was carried out with a 500-mL linear gradient between 0.01 M NH_4HCO_3 and 0.2 M NH_4HCO_3 at a flow rate of 120 mL/h. A phosphocellulose column (1.7 × 11 cm, Whatman P11) was equilibrated with 0.001 M sodium formate containing 5% 2-propanol, pH 3.8. Elution was carried out with a 400-mL linear gradient between 0.001 M sodium formate, pH 3.8, and the same buffer containing 0.5 M NaCl at a flow rate of 120 mL/h. The radioactive peaks were collected and dialyzed against cold distilled water using 1000 molecular weight cutoff tube (Spectrum Med. Inc.) and lyophilized.

High-Performance Liquid Chromatography. The dried peptides were dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to reversed-phase column C18 (Vydac) using a VISTA 5500 HPLC system (Varian) in a similar manner as described before (Yamauchi et al., 1986b). The column was equilibrated with 0.1% TFA. The samples were eluted with a 0–30% linear gradient of acetonitrile in 0.1% TFA for 40 min at a flow rate of 0.8 mL/min at room temperature. The effluent was monitored for UV absorbance at 230 nm, and fractions of 0.5 mL were collected. Twenty microliters of each fraction was taken for measurement of radioactivity in a liquid scintillation counter.

Characterization of the Purified Peptides. Samples were hydrolyzed in 6 N HCl under N_2 at 115 °C for 24 h. The hydrolysates were applied to an amino acid analyzer (Varian 5500 liquid chromatography, AA911 column, Interaction) as described previously (Yamauchi et al., 1986a). No corrections were made for losses during hydrolysis.

An aliquot of the hydrolysate of the isolated peptide was also applied to the cross-link analyzer in order to confirm the purity of the NaB^3H_4-reduced compounds and to quantify by radioassay based on the specific activity of NaB^3H_4.

RESULTS

Characterization of the Cross-Linked Tryptic Peptides. Typical chromatographs of the tryptic digest of fetal and mature (2–3 year old) bone collagens are shown in Figure 3. These display about five peaks which are either radioactive or fluorescent (labeled GI–GV). Peaks GI and -II were the main fluorescent peaks, and the cross-link analyses showed that these arose from a rich content of Pyr. Amino acid analyses of apparently pure fractions indicated that these

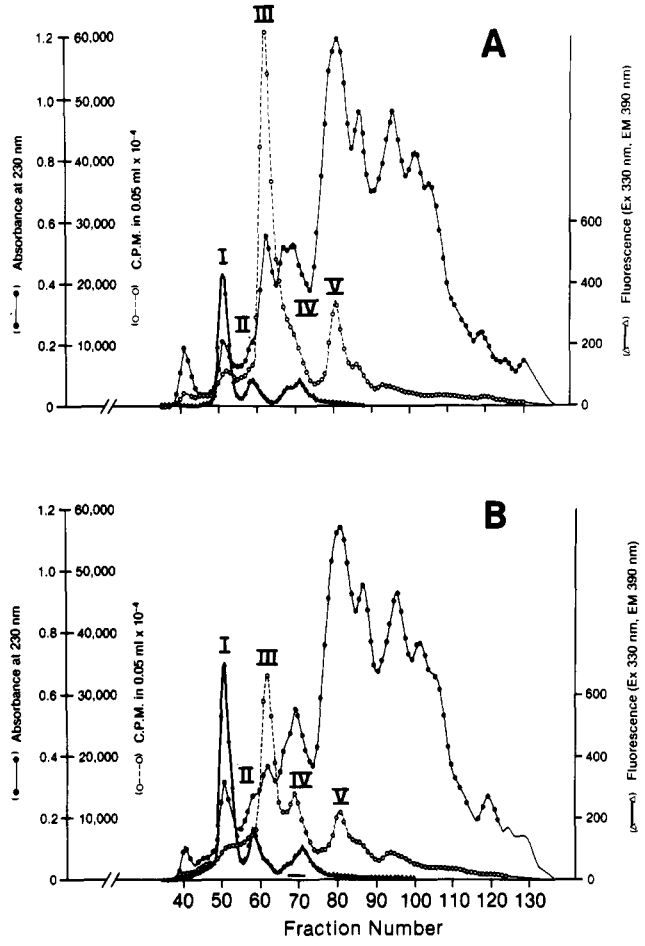

FIGURE 3: Sephadex G-50 Superfine gel filtration chromatographic profile of tryptic digest of NaB^3H_4-reduced fetal and mature bovine bone collagens. (A) Fetal bone collagen; (B) mature bone collagen. Fluorescent or radioactive fractions are denoted by the Roman numerals, and fractions pooled (GIV) are denoted by the bar.

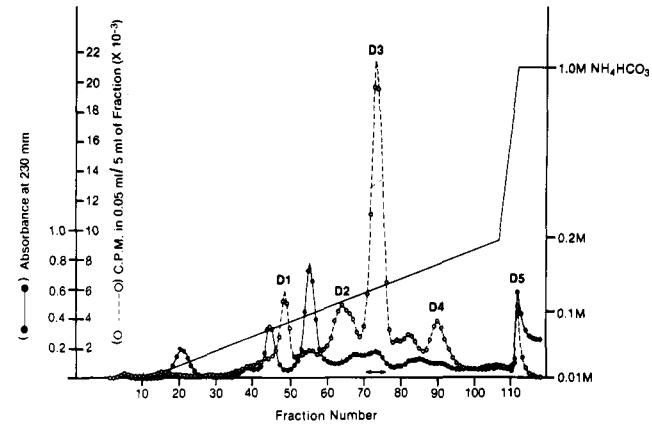

FIGURE 4: DEAE-cellulose chromatography of fraction GIV from Figure 3. Radioactive fractions are denoted as D1–D5. DHNL was found to be rich in fraction D3, the predominant radioactive peak. Fractions denoted by the double arrow bar were pooled and was rechromatographed on the same column under the same conditions. See Materials and Methods for details.

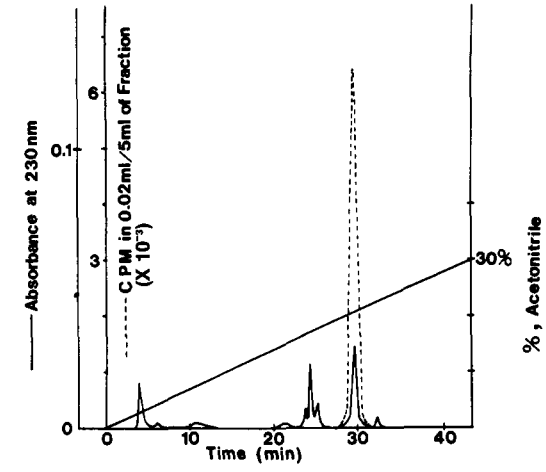

FIGURE 5: Final purification of the DHNL-containing peptide by C18 reversed-phase HPLC. The D3 fraction (Figure 4) was further fractionated by phosphocellulose column chromatography, and a single radioactive peak, which contained DHNL, was obtained (see Results). This fraction was applied to a C18 column. The radioactive peak (28–30-min) fractions were pooled and subjected to amino acid and cross-link analyses. See Materials and Methods for details.

Pyr-containing peaks were the tryptic and chymotryptic digestion products, respectively, from COOH-terminal containing Pyr cross-linked peptides (Kuboki et al., 1981; Yamauchi et al., 1989) (data not given).

The principal radioactive peaks were GIII–GV. Peak III, the predominant radioactive peak, eluted at the same position as the DHLNL-containing tryptic peptide isolated from PDL [α1(I)(993–22^c) × α1(I)(76–90)] (Yamauchi et al., 1986a). The cross-link analysis showed that it consisted of more than 95% DHLNL cross-linked fragments. Further chromatographic studies using fetal bone collagen have shown that this peak consisted of two trypsinated products: one derived from a cross-link between the α1(I)Hyl^{ald}-16^c and Hyl-87 on an α1(I) chain, and the other from the α1(I)Hyl^{ald}-16^c residue and a Hyl-87 on an α2(I) chain, in the proportion of 3.3/1 (Yamauchi et al., 1989). These findings are similar to that reported for the PDL (Yamauchi et al., 1986a). However, unlike the PDL, only a trace amount of COOH-terminal Lys^{ald}-derived cross-linked peptide was present.

A significant amount of deH-HLNL was identified in the peak V fraction. The elution position of this peak was identical to that of the deH-HLNL-containing tryptic peptide, α1-(I)(1^N–9) × α1(I)(928–933), isolated from the PDL (Yamauchi et al., 1986a). This indicates that the majority of deH-HLNL of bone collagen also derives from Lys^{ald} located at the NH_2-terminal telopeptide of the molecule.

Peak IV contained mostly DHNL-containing peptides, and as shown below, it appears to be a tryptic COOH-terminal peptide which contained unreacted aldehyde. This peak was more distinct in mature bone collagen than in fetal bone (Figure 3). The characterization of GIV involved the purification steps of ion-exchange and reversed-phase chromatography which are shown in Figures 4 and 5. Five radioactive peaks in the DEAE chromatography were designated as D1–D5 (Figure 4). DHNL was found to be most abundant in D3, the predominant radioactive peak. The other minor radioactive peaks contained small amounts of DHLNL and HLNL. Fraction D3 was next rechromatographed on the same column under the same conditions (data not shown) and the resulting single peak subjected to phosphocellulose chromatography. The single radioactive peak was obtained from this chromatography (data not shown), and it was further purified by HPLC. The chromatograph is shown in Figure 5.

The amino acid composition of the purified tryptic peptide is given in Table I along with the literature values expected for the tryptic peptide α1(I)(993–22^c) (Galloway, 1982). The composition of both are in excellent agreement except for the complete absence of Hyl from the isolated peptide. Instead, DHNL (reduced form of Hyl^{ald}) was confirmed in the hydrolysate of the peptide. The DHNL, which elutes between

Table I: Amino Acid Composition of Aldehyde-Containing Peptide

	peptide found		lit. value,[a] 993–22°
Hyp	4	(4.0)[b]	4
Asp	3	(3.0)	3
Thr	1	(0.9)	1
Ser	2	(1.8)	2
Glu	3	(3.3)	3
Pro	11	(10.6)	10
Gly	11	(11.2)	11
Ala	3	(3.0)	3
Val	0	(0.0)	0
Met	0	(0.0)	0
Ile	0	(0.0)	0
Leu	2	(2.1)	2
Tyr	1	(0.6)	1
Phe	1	(1.0)	1
His	1	(1.0)	1
Hyl	0	(0.0)	1
Lys	0	(0.0)	0
Arg	1	(1.0)	1
DHNL[c]	1	(1.1)	
total	45		44

[a] Obtained from sequence of Galloway (1982). [b] Values in parentheses are actual values found. [c] Calculated by radioassay (see Materials and Methods in the text).

aspartic acid and threonine on our amino acid analysis system, was present as about 1 mol/mol of peptide (see below).

In addition to amino acid analysis, an equal amount of the hydrolysate was subjected to cross-link analysis in order to confirm the concentration of DHNL in the peptide. The chromatogram indicated that most of the radioactivity of this peptide was derived from DHNL (about 90% of the total count). A minute amount of HNL was present in this peptide. On the basis of the count of radioactivity, specific activity of NaB^3H_4, and amino acid analysis, it was determined that 1.1 residues of DHNL was present per peptide.

These results strongly suggest that the isolated peptide is the tryptic peptide derived from α1(I)(993–22^c) with DHNL at the 16^c position.

Quantification of the COOH-Terminal Cross-Link Distribution. Typical chromatographs showing the relative distribution of the reducible aldehydes and cross-links in fetal and mature bone collagens are given in Figure 6. The quantification of the various peaks was obtained by integrating the respective peaks and converting the numbers into a per mole of collagen basis using the specific activity of NaB^3H_4 and a Hyp value of 300 residues per collagen molecule. These are presented in Table II under the category of "raw" data. Since almost all DHLNL, DHNL, and Pyr involve α1(I)-16^c–Hyl^{ald}, the total 16^c residue content of bone collagen was calculated as the sum of DHLNL, DHNL, and twice the Pyr content (i.e., each Pyr contained two 16^c residue values). The contents for fetal and mature bone thus calculated ranged from 2.24 to 2.49 and from 1.68 to 2.16 mol/mol of collagen, respectively (analyses were done in triplicate). The respective

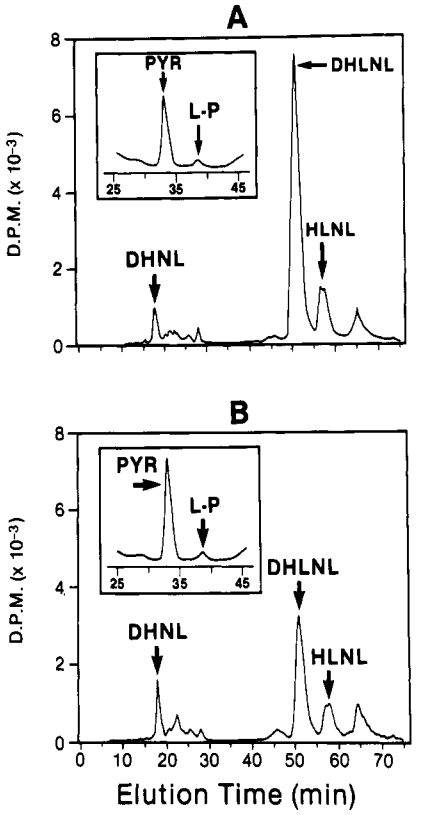

FIGURE 6: Chromatographic profiles of cross-links and their precursors of fetal and mature bone collagens. (A) Fetal bovine bone collagen; (B) 2–3 year old bovine bone collagen. Samples were hydrolyzed in 6 N HCl, and hydrolysates in amounts containing 300 nM of hydroxyproline were applied to the cross-link analyzer. See Methods and Materials in the text for details.

means were 2.34 and 1.84 mol/mol of collagen. The difference between these means was not statistically significant within the approximate 6% precision of the evaluations.

These estimates for the 16^c content of fetal and adult bone are very close to the "theoretical value" of 2.0 mol/mol of collagen which comes from the assumptions that all Hyp-containing peptides in bone are in the form of intact collagen molecules, each having two α1(I) chains with intact COOH-terminal telopeptides. This agreement signifies that, in the elaboration of bone collagen, the 16^c lysine residues of the α1(I) chains of bone collagen have been stoichiometrically converted to the Hyl and then to the aldehyde state.

Changes in Cross-Linking Patterns with Maturation. A comparison of panel A with panel B in Figure 3 shows that, with maturation, the relative amount of reducible COOH-terminal cross-links (peak GIII) decreased while the amounts of the COOH-terminal free aldehyde (peak GIV) and Pyr (peaks GI and -II) increased. In order to compare the distribution of states of the 16^c residues in fetal and mature bone collagen on the same basis, we have normalized all of the data

Table II: Cross-Link Distribution in Fetal and Adult Bovine Bone Collagen

	mol/mol of collagen[a]					
	DHNL	DHLNL	Pyr	total 16 C[b]	HLNL	LP
fetal						
raw	0.16 (0.01)	1.72 (0.15)	0.23 (0.02)	2.34 (0.13)	0.45 (0.03)	0.02
normal	0.14 (0.01)	1.47 (0.13)	0.20 (0.02)	2.00 (0.11)	0.37 (0.03)	0.02
adult						
raw	0.24 (0.03)	0.86 (0.03)	0.39 (0.11)	1.84 (0.26)	0.36 (0.02)	0.04
normal	0.26 (0.03)	0.93 (0.02)	0.42 (0.12)	2.00 (0.28)	0.39 (0.02)	0.04
maturation change	0.12 [0.02]	(−)0.54 [0.08]	0.22 [0.08]	0 [0.17]		

[a] Values in parentheses are SD, $N = 3$. Values in brackets are SE of difference in means. [b] DHNL + DHLNL + 2 × Pyr.

in Table II to the theoretical COOH-terminal 16^c content of 2 mol/mol. This was done by multiplying the respective raw data values by the ratio of the theoretical to experimental total 16^c residue content. It can thus be seen that about 70% of the 16^c residues of fetal bone collagen are present as bifunctional cross-links with 30% as free Hyl^{ald} and trifunctional cross-link, Pyr. In the adult animal the bifunctional cross-links are depressed to a 46% level of the 16^c residue, and now Pyr and Hyl^{ald} together account for 54% of this residue.

The level of DHNL obtained on a single cross-link analysis of bone collagen from a 9 year old animal was similar to that of the adult bone.

The changes in composition which occur upon the maturation of fetal to adult bone are listed in Table II in the row titled "maturation change". It should be noted that there is almost a perfect correspondence between the loss in DHLNL and the increase in DHNL plus 2 times Pyr. This is evidence for an accurate "conservation of mass" type accounting of the states of the 16^c residue in fetal and adult bone.

An additionally significant feature of the results of Figure 3 and Table II is the relative paucity of either NH_2-terminal involved reducible (GV) or NH_2-terminal Pyr cross-linked trypsin digests or their aldehyde derivative contents (Yamauchi et al., 1989).

Discussion

This study has demonstrated that significant amounts of unreacted COOH-terminal aldehyde are present in both fetal and adult bone collagen. This appears to be a characteristic of the collagen from mammalian mineralized tissues, since similar results have been obtained in our study of bovine dentin collagen. This is in contrast to the situation in such nonmineralized bovine type I collagen tissues as predentin or PDL where, using an identical protocol, virtually all of the COOH-terminal aldehyde was found to be tied up in cross-links. The occurrence of free aldehyde in mineralized collagen may have important physiological implications with regard to the mechanical properties of bone which we will discuss below. A related finding of importance is the change of the COOH-terminal aldehyde content with animal maturation. As can be seen in the "raw data" in Table II, the DHNL content increases from 0.16 to 0.24 mol/mol which is a 50% change. The estimation of aldehyde increase based on the normalized data is even higher (0.12 mol/mol), which is close to a 100% change from the fetal state. This is probably related to the respective levels of mineral maturation in the fetal and adult tissues.

Findings have been accumulating that bone collagen fibrils undergo a change in structure upon mineralization. In fish bones, the fibrils bulge out upon mineralization (Lee & Glimcher, 1989), and in fully mineralized bone the axial period, as measured by neutron diffraction, is reduced to 650 Å and the equatorial spacing to about 13 Å (Bonar et al., 1985); in mineralizing turkey tendon, the axial periodicity of the mineral disposition as measured by X-ray diffraction remains constant, but the intermolecular spacing becomes reduced to 12.4 Å (Bigi et al., 1988), and finally the segmental motion of bone collagen molecules are reduced upon mineralization (Sackar et al., 1987).

At present our understanding of how the structure of bone collagen changes with mineralization is based on circumstantial evidence. Since type I collagens do not have an isotropic lateral structure (Katz & Li, 1973b; Golub & Katz, 1977), it is likely that mineral particles, in their growth, deform the fibrils in an anisotropic fashion following the paths of least resistance defined by the cross-linking patterns (Katz et al., 1989). As illustrated in Figure 1, bifunctional cross-links tend to tie groups of molecules together laterally into sheets. The lateral paths of least resistance for crystal growth therefore are first parallel to and then normal to these cross-linked molecules. There are a number of ways that a nascent crystallite could deform collagen. It could simply be by mechanical forces (a growing crystallite could literally push the collagen segments out of its way), or it could be by osmotic forces (Katz et al., 1986; Volpi and Katz, in press). It was the possibility that these forces might be enough to cause deH-DHLNL cross-links to dissociate back to the aldehyde and Hyl-87 state which motivated this investigation into the free aldehyde content of bone collagen. It would appear that the findings presented here are compatible with this idea. More definitive findings indicating that the free aldehyde content is a direct result of mineralization is presented in our studies of predentin and dentin collagen in a separate publication.

We have illustrated our interpretation of the effect of mineralization on bone collagen cross-linking in Figures 1 and 2. Before mineralization, the COOH-terminal cross-linking of type I collagen is represented exclusively as a pattern of bifunctional cross-links. This is in accordance with PDL and predentin results. After mineralization, the cross-link pattern is influenced by the presence of both Pyr and Hyl^{ald}. This is in accordance with the dentin findings (Yamauchi et al., in preparation). In contrast to dentin, however, the cross-linking results of bone have to be viewed in terms of a time-averaged remodeling phenomenon. This is because bone is turned over throughout the life of the animal. Fetal bone is but a transitory tissue which is rapidly and completely replaced during its development, whereas in the mature animal, only a small fraction of the bone at any one time is actively turning over. This means that the average chronological age of a matrix element in fetal bone is much younger than that of the mature animal. Therefore, the average level of mineralization of matrix element of fetal bone is less than that in the mature animal. We believe that this latter is the most probable explanation for the increase in free aldehyde with maturation seen in Table II.

The mechanism of collagen cross-link formation in bone illustrated in Figure 2 is based on our findings on bone and dentin as well as results reported by Eyre et al. (1980, 1984) on the mechanism of Pyr formation. Collagen cross-linking involves a number of stereospecific steps. The first is a formation of Schiff base cross-links. The Schiff base formation is most probably a reversible reaction between the 16^c residues and the Hyl-87 resides in the triple-helical regions of adjacent molecules. The stereospecificity of the reaction is manifested by the relative involvement of the α1(I)-Hyl-87 and α2(I)-Hyl-87. These form bifunctional cross-links in the proportion of 3.3/1, whereas a nonspecific reaction stoichiometry would be 2/1 (Yamauchi et al., 1986a). It is this step that is the one most likely to be influenced by any distortions in the molecular packing resulting from mineralization and which would generate the free 16^c aldehyde in bone and dentin. The next step depicted in cross-link maturation involves the Amadori rearrangement of the Schiff base into a ketoamine form of the cross-link (Mechanic et al., 1974), and then either the condensation of two vicinal ketoamine cross-links or a ketoamine and a Schiff base into a Pyr. As Eyre (1981) has noted, when either bone or demineralized bone matrix is incubated in saline, the Pyr content of bone increases spontaneously in time, indicating the Pyr formation is a chemical rather than a cell-mediated event. This author also found that the DHLNL content progressively decreased by 2 for every

Pyr formed. This was one of the reasons we assumed in our calculations that a Pyr contained two 16^c residues. The close agreement then of the total 16^c contents of both fetal and mature bones with the theoretical 2.0 mol/mol value, as well as the "maturation change" of Table II, would indicate that, in vivo as well, two 16^c aldehyde residues were incorporated into every Pyr. The change in Pyr content with maturation is also consistent with the relative chronological ages of the matrix elements in the two tissues.

At this time we can only speculate about the physiological significances of the presence of aldehyde in bone collagen. Like the presence of Pyr, it manifests a change in the cross-linking that occurs with maturation of bone collagen. As we noted above in context from Table II, the absolute number of molecular segments connected by cross-links drops upon mineralization. When measured from a PDL and predentin state, the drop in collagen connectivity in fully mineralized collagen is even more dramatic. Such a transformation is most appropriate for the two functions the collage fibril evidently has in the mineralization process. The first is to provide a template for the orderly precipitation of mineral. The second is to define the domains of the final mineral–collagen composite. A lability in the spatial patterning of cross-linking stabilizing the collagen structure could be the way nature provides for this. In particular, the intermolecular cross-linking having initially a sheetlike pattern would provide a mechanical coherency most suitable for a stable template for growing mineral particles, while a cross-linking pattern having more lateral discontinuity would enable a fibril to change more easily its molecular organization and shape in order to accommodate the final mass of mature mineral crystals.

In contrast to the stoichiometric cross-linking at the COOH-terminal portion of the molecule, the NH_2-terminal situation seems quite different. Our preliminary study indicated that the majority of the deH-HLNL (0.43 mol/mol of collagen in fetal and 0.36 mol/mol in adult; see Table II) derived from N-terminal Lys^{ald} (see Results). In soft connective tissues such as skin, periodontal ligament, and rat tail tendon, 2–3 mol/mol of collagen of aldehyde-derived cross-links are recoverable from this portion of the molecule (unpublished data). It has been proposed that the NH_2 terminus might be the nucleation site of mineralization (Berthet-Colominus et al., 1979). Thus the low density of aldehyde-derived cross-linking at the NH_2 termini might be important in that regard (Yamauchi et al., 1989).

Since intermolecular cross-links are the means by which connections between molecules at the atomic levels become translated into tissue cohesion at the microscopic level, one could argue that the loss of connectivity with mineralization would allow a bone fibril to swell and thereby incorporate extra mineral into its structure. In this sense, the loss of connectivity at the COOH-terminal ends of the molecules also reflects an adaptive capability of the bone to deal with the skeletal demands of the heavier mature animal.

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Registry No. Pyr, 63800-01-1; deH-DHLNL, 35761-40-1; deH-HLNL, 31504-13-9; LP, 90032-33-0; DHNL, 38886-80-5; HLNL, 21895-67-0.

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