Molecular Site Specificity of Pyridinoline and Pyrrole Cross-links in Type I Collagen of Human Bone*

Dennis A. Hanson† and David R. Eyre‡§

From the University of Washington, Departments of †Orthopaedics and §Biochemistry, Seattle, Washington 98195

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Compared with soft tissue collagens, bone type I collagen displays a distinctive pattern of covalent cross-linking, with evidence of preferred sites of molecular interaction and a prominence of both immature, divalent cross-links and mature, trivalent cross-links in the adult tissue. In this study the site-specificity of the mature cross-links in human bone collagen was examined. Peptides containing fluorescent pyridinoline cross-links and Ehrlich’s-reactive pyrrole cross-links were isolated from a bacterial collagenase digest of demineralized bone matrix. The digest was fractionated by molecular sieve chromatography, monitoring for peptide absorbance, pyridinoline fluorescence, pyrroles by Ehrlich’s reagent, and immunoassay for cross-linked N-telopeptides. Individual cross-linked peptides were resolved by ion-exchange and reverse-phase HPLC. Structures were established by NH2-terminal microsequencing, cross-link analysis, electrospray mass spectrometry, and immunoassay. Two, about equally occupied, sites of pyridinoline cross-linking were identified, N-telopeptide to helix and C-telopeptide to helix. Pyrroles were alternative cross-linking products at the same sites, but concentrated (85%) at the N-telopeptide end. Only one combination of chains was cross-linked by pyridinolines at the C-telopeptide to helix site, [α(I)]N-telopeptide [α(I)]helix. Several peptide combinations arose from the N-telopeptide to helix site, but the main source of pyridinolines was from the locus, α(I)N-telopeptide [α(I)]helix. Pyridinolines linking two α(I) N-telopeptides were a minor component. Pyrroles were concentrated at the locus, [α(I)]N-telopeptide [α(I)]helix. The cross-link ratio of hydroxylysylpyridinoline to lysylpyridinoline differed between N-telopeptide and C-telopeptide sites, and between the individual interchain combinations. Cross-linked N-telopeptides accounted for two-thirds of the total lysylpyridinoline in bone. N-telopeptide pyridinoline fluorescence was quenched on chromatography, so that reliance on peptide fluorescence alone can underestimate the level of N-telopeptide pyridinoline cross-linking.

The chemistry of lysine-mediated intermolecular cross-linking in the fibrillar collagens is understood in some detail (1–4). The three principal collagens, types I, II, and III, have four cross-linking sites at equivalent locations in their molecules, one in each telopeptide and two others at sites in the triple-helical domains at or close to residues 87 and 930. The initial intermolecular cross-links in new fibrils are borohydrade-reducible residues that are condensation products between aldehyde side chains formed on the telopeptides by lysyl oxidase, and hydroxylysine or lysine residues in helical domains of adjacent molecules. Both N-telopeptide-to-helix and C-telopeptide-to-helix cross-links occur (5–12). However, as young connective tissues mature, borohydrde-reducible cross-links disappear, progressing to more stable, non-reducible compounds (13). The best characterized and most widely distributed mature cross-links are the fluorescent compound, hydroxylysylpyridinoline (HP) (14) and its deoxy analog, lysylpyridinoline (LP; Ref. 15), also referred to as pyridinoline (Pyr) and deoxypyridinoline (16), respectively. HP is widely distributed in the collagen of most internal, vertebrate connective tissues, whereas LP, although widely distributed, features most prominently in bone and dentin. The pattern of cross-linking in bone collagen is also strikingly different from that of soft tissue collagens in other respects, including retention of a significant pool of the borohydrde-reducible, initial cross-links throughout adult life (4). The distinctive pattern of cross-linking interactions in bone collagen may be a result of or, as proposed, a necessary requirement for its mineralization (11, 12).

In recent years, the pyridinoline residues HP and LP have become widely adopted in clinical studies as urinary markers of bone resorption. The molecular sites of origin of HP and LP in human bone collagen are, therefore, important to define. Pyridinoline residues have been proposed to form by a condensation reaction between two divalent keto-amine cross-links (17), with the resulting structure effectively incorporating one divalent cross-link and one hydroxysine aldehyde (18). The pyridinoline residue, therefore, links three collagen α-chains, two telopeptide and one helical domain, each in a separate collagen molecule (17–19). Thus, in the homotrimeric type II collagen of bovine articular cartilage, there are only two molecular sites of pyridinoline cross-linking, between the same residues that initially formed borohydrde-reducible cross-links in the immature collagen (18, 20). Reports on type I collagen of bone, dentin, and tendon, however, present inconsistent results on the molecular sites of the pyridinolines, with respect to the α-chains involved and the functional importance of these residues in stabilizing the collagen fibrils (10, 12, 21–24).

Scott et al. (25–27) originally proposed the existence of pyrrole cross-links in collagens, based on an observed reactivity of tissues and isolated peptides with Ehrlich’s reagent (p-dimethylaminobenzaldehyde) to give a cherry-pink color. Several connective tissues, including bone and skin and purified cross-linked peptides from types III and IV collagens, gave this reaction (27). A cross-linked peptide isolated from type III

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† To whom correspondence should be addressed: University of Washington, Orthopaedic Research Laboratories, Box 356500, Seattle, WA 98195-6500.

‡ The abbreviations used are: HP, hydroxylysyl pyridinoline; LP, lysyl pyridinoline; CB, cyanogen bromide; mAb, monoclonal antibody; C-and N-telopeptides, short sequences that lack the (GXY) collagen repeat and form the carboxyl and amino ends of the fibril-forming collagen α-chains (collagen types I, II, III, etc.); HPLC, high performance liquid chromatography.

§ Towhom correspondenceshouldbe addressed: University of Washington, Departments of Orthopaedics and Biochemistry, Seattle, Washington 98195.

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Collagen revealed both pyridinolines and reactive pyrroles (27). Kuypers et al. (28–30) pursued the origin and nature of the Ehrlich’s reactivity in bovine tendon and muscle collagens, and showed definitively that the source of the pyrrole was in type I collagen at the N-telopeptide to helix and C-telopeptide to helix cross-link sites. They proposed (29) that the Ehrlich’s chromogen was a tri-substituted pyrrole that was formed from ketoamine and aldime cross-links in an analogous fashion to pyridinoline cross-link formation, except that one aldehyde donor came from a telopeptide lysine and the other from a telopeptide hydroxlysine, rather than from two hydroxlysine aldehydes. A recent report concluded that pyrrole cross-links were as abundant as pyridinolines in human bone collagen (31).

In the present study, the interpeptide sites of mature cross-links in human bone collagen were sought. The approach was to resolve peptides chromatographically from a bacterial collagenase digest of the mature tissue protein. Pyridinoline-containing and Ehrlich’s chromogen-reactive peptides were traced by their respective fluorescence and p-dimethylaminobenzaldehyde reactivity. Isolated peptides were structurally identified by NH₄-terminal sequencing, pyridinoline cross-link analysis, electrospray mass spectrometry, and immunoreactivity with a monoclonal antibody specific to collagen type I cross-linked N-telopeptides (19).

**Materials and Methods**

**Preparation of Bone**—Human cortical bone (male, aged 25 years, banked tissue, Northwest Tissue Center, Seattle, WA) was excised from the mid-shaft of the femur, washed at 4 °C in phosphate-buffered saline containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 2 mM EDTA, 5 mM benzamidine) and defatted at 4 °C with methanol/chloroform (1:3, v/v). Bone was then manually splintered, desiccated, and powdered under liquid N₂ in a SPEX mill (SPEX Industries). Bone powder was demineralized at 4 °C with two changes of excess 0.5 mM EDTA, 0.05 M Tris-HCl, pH 7.5, over 2 weeks. The demineralized bone powder was washed thoroughly with cold distilled H₂O, lyophilized, and stored at −20 °C before use.

**Digestion with Bacterial Collagenase**—Demineralized bone matrix was suspended in 0.1 M CaCl₂, 0.05 M Tris-HCl, pH 7.4, containing 0.001% (w/v) thiomersalate (Sigma Ultrex) was added to each tube, capped with Teflon-lined screw caps, and incubated for 24 h. After hydrolysis, samples were acidified to 1% (v/v) HClO₄ (25). After 5 min, samples were read for absorbance at 572 nm using a spectrophotometer.

**Reverse-phase HPLC of Peptide Hydrolysates for Pyridinoline Cross-links**—Peptide samples to be hydrolyzed were dried in new glass Kimax tubes with screw tops on a rotary evaporation unit. One ml of 6 M HCl (Sigma Ultra) was added to each tube, capped with Teflon-lined screw top, and hydrolyzed for 24 h at 110 °C. After hydrolysis, samples were dried and dissolved in 1% (v/v) n-heptafluoroctyl acrylic acid (Pierce sequential grade) and analyzed by reverse-phase HPLC as described (4).

**DEAE-5PW Anion-exchange HPLC of Peptides**—Lyophilized pools of P-10 fractions were chromatographed on a Bio-Gel DEAE-5PW column (7.5 × 75 mm, Bio-Rad) using a Rainin two-pump system, collecting 1-min fractions. The two buffers were identical in composition, 0.02 M Tris-HCl in 10% (v/v) acetonitrile, pH 7.5, except buffer B contained 0.5 M NaCl. The initial column conditions were 2% buffer B at 1.0 ml/min. Samples were dissolved in buffer A and eluted using a two-stage gradient: buffer B held at 2% for the first 2 min then increasing linearly to 40% over the next 28 min. Effluent was monitored for UV absorbance (220 nm) and for fluorescence (excitation, 330 nm; emission, 395 nm) (4).

**Reverse-phase HPLC of Peptides**—Lyophilized pools of DEAE-5PW fractions were fractionated by reverse-phase HPLC on an Aquapore RP-30 column (C8, 5 μm beads, 4.6 mm × 25 cm, Brownlee Labs) using a Rainin two-pump system. Samples were dissolved in 1% (v/v) trifluoroacetic acid and eluted using a linear gradient (usually 0–45%) of acetonitrile/1-propanol (3:1, v/v) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml/min. Effluent was monitored for UV absorbance (220 nm) and fluorescence (excitation, 297 nm; emission, 395 nm) (4). One-min fractions were collected and those corresponding to fluorescent peaks were pooled for analysis.

**Inhibition Enzyme-linked Immunosorbent Assay Using mAb 1H11**—Immunosassay of cross-linked α2(I) N-telopeptide was essentially performed as described previously (19). Briefly, dried samples were dissolved in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 100 μl was applied to separate wells of a 96-well plate. A 100 μl volume of 5% (v/v) acetonitrile was added, and all samples were incubated at room temperature for 24 h. After the incubation period, all samples were washed, and a 100 μl volume of 1:50 (enzyme:substrate, w/w) peroxidase-conjugated mAb 1H11 was added to each well and incubated for 48 h. All samples were washed, and 50 μl of 2% (v/v) hydrogen peroxide and 0.001% (v/v) thiomersalate in 25 μl of 1 M H₂O₂ was added to each well. After 20 min, the absorbance was measured at 490 nm.

**Table I**

| Cross-linking site | HP | LP | HP + LP | HP/LP molar ratio |
|-------------------|----|----|---------|------------------|
| Fractions A       | C-telopeptides | 56 | 35 | 2.0 |
| Fractions B       | C-telopeptides | 14 | 14 | 1.0 |
| Fractions C       | C-telopeptides | 30 | 50 | 0.6 |
| Total bone digest | Both sites | 100 | 100 | 1.0 |
directly to a well of a previously prepared antigen plate. After all wells of the plate contained either sample, dilutions of a standard, or buffer, 100 μl/well of appropriately diluted 1H11 ascites was quickly added. After 1 h at room temperature, the plate was washed with 0.9% (w/v) NaCl, 0.05% Tween 20 (Sigma), and patted dry. Bound mAb 1H11 was determined by sequential addition of peroxidase-conjugated goat anti-mouse Ig (Jackson ImmunoResearch Laboratories), wash buffer, and TMB substrate solution (0.1 mg/ml tetramethylbenzidine in 0.1M sodium acetate, pH 5.0, containing 0.01% (w/v) H2O2). Color reaction was stopped with 1.5 M H2SO4 and absorbance was read at 450 nm using a Titertek Multiskan PLUS plate reader. Relative immunoreactivity values for samples were calculated from a semi-log plot of the relative concentration of a standard versus absorbance.

NH2-terminal Protein Sequencing—A Porton model 2090E instrument with on-line reverse-phase HPLC detection of phenylthiohydantoin-derivatives was used to obtain NH2-terminal sequence data from the enriched cross-linked peptides.

Electrospray Mass Spectrometry—Individual purified peptides were structurally identified from their molecular masses deduced from their ions on electrospray mass spectrometry using a triple quadrupole instrument (Sciex Model API III).

RESULTS

Fig. 1 shows the UV absorbance and fluorescence profiles of a collagenase digest of human bone collagen fractionated by molecular sieve chromatography. Two prominent fluorescent peptide peaks, labeled A and C, with a shoulder labeled B in between, were recovered, eluting between the void volume (60 ml) and total bed volume (200 ml) of the column. Most (85%) of the Ehrlich’s chromogen (pyrrole) reactivity, with a peak of absorbance at 572 nm, coeluted with fluorescent peak C. Peak C was also shown previously to be the major source of reactivity to mAb 1H11, an antibody which specifically recognizes cross-linked type I collagen N-telopeptides that contain a cleaved S-amino acid α2(I)N-telopeptide sequence, cross-linked in a ternary peptide complex (19). Fractions corresponding to pools A, B, and C (Fig. 1) were combined and freeze-dried for further analysis. (The peak of Ehrlich’s reactivity evident at

FIG. 2. Ion-exchange HPLC resolution of peptide pools A (A), B (B), and C (C) described in the legend to Fig. 1. The column (75 × 7.5 cm DEAE-5PW, Bio-Rad) was eluted with a 0–0.2 M NaCl gradient (see “Materials and Methods”) continuously monitoring for peptide absorbance and pyridinoline fluorescence. Collected fractions were assayed for reactivity with p-dimethylaminobenzaldehyde. Pool C alone gave a measurable reaction and the quantitative results are shown in the lower panel of C. Fraction pools labeled 1–4 were dried for reverse-phase HPLC.

FIG. 3. Reverse-phase HPLC isolation of the pyridinoline-containing peptide in fraction 15 from Fig. 2A (A). The column (C8, RP-300, Brownlee Labs) was eluted with a linear solvent gradient (0–45%) in 0.1% (v/v) trifluoroacetic acid (see “Materials and Methods”). NH2-terminal sequencing results and the electrospray mass spectrum (B) identify the structure shown, in which two α1(I) C-telopeptides are cross-linked to a heptapeptide containing α1(I)Hy7ε embodied in a hydroxylsylpyridinoline cross-link to which galactose is attached.
about 220 ml was of low molecular weight and did not behave as a cross-linked collagen peptide.)

Table I shows the results of pyridinoline cross-link analysis by HPLC for the three major pools fractionated by molecular sieve chromatography. Two points emerge. First, total pyridinolines are about equally distributed between N-telopeptide and C-telopeptide sites. Monitoring fluorescence alone (Fig. 1) can underestimate the N-telopeptide contribution because of fluorescence quenching, observed also for cross-linked N-telopeptides from urine (36). The tyrosine residues in the N-telopeptides may be responsible for the quenching. Dilution in organic solvent can unmask the quenched fluorescence (36). Second, two-thirds of the LP is at the N-telopeptide site and one-third at the C-telopeptide site. The HP/LP ratio of the cross-linked N-telopeptide pool from the bone digest is similar to that of the pool immunopurified from human adolescent urine using mAb 1H11 (19).

Fig. 2 shows the profiles resulting from anion-exchange chromatography (DEAE-5PW) of peptide pools A, B, and C from Fig. 1. Pool A gave a series of fluorescent, pyridinoline-containing peaks (Fig. 2A). Pool B gave one main and one smaller fluorescent peak (1 and 2 in Fig. 2B). Pool C gave two main fluorescent peaks (3 and 4 in Fig. 2C), collected as shown by the bars. Also shown in Fig. 2C is the profile of pyrroline reactivity by p-dimethylaminobenzaldehyde reagent given by pool C. Individual peptides were resolved by reverse-phase HPLC. Purified pyridinoline-based cross-linked peptides were characterized by HP and LP analysis, reactivity with mAb 1H11, direct NH₃-terminal sequencing, and electrospray mass spectrometry.

Fig. 3 shows the reverse-phase HPLC elution profile for fraction 15 from Fig. 2A and the structure of the isolated peptide determined by sequence analysis and mass spectrometry. All flanking fluorescent subcomponents in Fig. 2A gave very similar elution profiles with essentially one peak in this same region of the chromatogram (not shown). All contained primarily HP, were not mAb 1H11 reactive, and gave two running sequences. The dominant one was of the α1(I) C-telopeptide (32) beginning at phenylalanine. The second sequence was seven residues in length, at about half the molar yield of the C-telopeptide sequence, and came from the α1(I) N-helix cross-linking site at residue 87 (33). The counterpart peptide containing the α2(I) chain cross-linking site at residue 87 (34) was not found in any of the DEAE-5PW column fractions (Fig. 2A). The most prominent cross-linked peptide from this series of peaks (fraction 15, Fig. 2A) was analyzed by electrospray mass spectrometry yielding a mass greater by 162 daltons than the mass of the peptide sequences and cross-link alone (Fig. 3). This finding can be explained if a galactose residue is attached to the hydroxyl group on the ring-nitrogen side arm of the hydroxylysyl pyridinoline residue. This is consistent with galactosyl hydroxylysine being the main form of glycoside on human bone collagen (35) and a prior study which showed a glycosylated ketoamine cross-link in a peptide from this same, α1(I) residue 87 to C-telopeptide, site in bone collagen (7). The different peak fractions seen in Fig. 2A appear to be both post-translational variants (LP, HP, and glycosylated HP) and different proteolytic cleavage products of this same peptide. Based on amino acid compositions (data not shown),
FIG. 5. Electrospray mass spectra and predicted structures of the most prominent cross-linked N-telopeptides containing pyr-ridinoline, isolated by reverse-phase HPLC and identified as peaks a–h in Fig. 4. Structures are based on NH2-terminal sequence and molecular ion data.
the heterogeneity was also due in part to missing tyrosine residues from the COOH termini of the telopeptides.

Pools 1 and 2 (Fig. 2B) each gave two main fluorescent peptides on reverse-phase HPLC, labeled a, b, c, and d, respectively (Fig. 4, A and B). Shown in Fig. 4, pool 3 gave one major (e) and one minor (f) fluorescent peak (Fig. 4C) and pool 4 gave a similar pattern with peptides labeled g and h. It was evident in the original chromatograms, however, on aligning the coincidence of UV absorbance and fluorescence for peptides e and g, that the fluorescence of g was sharper and more concentrated in the first half of the UV peak. The second half aligns more with the pyrrole reactivity given by Ehrlich’s reagent. This suggested two forms of the same peptide that differed only in their cross-linking residue, later borne out by amino acid compositions, sequencing, and mass spectrometry (see below).

Fig. 5 summarizes the mass spectra and predicted structures of peptides a–h resolved from the pool of pyridinoline-cross-linked N-telopeptides. Peptides a–d (from Pool B, Fig. 1) are all slightly larger versions of peptides e–h, explaining their earlier elution on molecular sieve chromatography. For each peptide, molecular ions that differ by 16 mass units and represent less complete proteolysis products from the same molecular site of cross-linking. For each peptide, molecular ions that differ by 16 mass units and represent HP and LP versions, are evident in a similar ratio to that measured directly by analysis of HP and LP after acid hydrolysis (Table II). In addition, a molecular ion 16 mass units greater than that of the predicted HP ion was consistently observed. This we interpret to be the photolyzed form of HP produced during UV exposure on chromatography (4). In structures shown with NH2-terminal glutamine residues, the glutamine was present as pyroglutamic acid which blocked NH2-terminal sequencing of that chain component. Deblocking with pyroglutaminase was partially effective with some peptides, helping confirm the identities, and mass spectrometry established the basic structures. From the data, it is not possible to predict which way round the a1(I)N and a2(I)N-telopeptides are placed on the pyridinium ring. Interestingly, none of the peptide structures identified had galactose or glucosylgalactose attached, based on the mass spectral data, in contrast with the C-telopeptide structures. From the HP/LP ratios of the individual peptides, reported in Table II, lysine a1930 is less hydroxylated than lysine a2933 as evidenced by the consistently lower HP/LP ratio found in peptides recovered from this site.

Fig. 6 shows the mass spectrum and an interpreted structure for the pyrrole-containing peptide found in the later portion of peak g (Fig. 4D). The mass spectrum is consistent with a mixture of forms of peptide g that differ only in the mass of the cross-linking residue. We interpret these as the HP and LP containing structures (LP, m/z 1426.1; HP, m/z 1434.1 shown in Fig. 5G), the UV photolysis product of HP (m/z 1441.6) and two additional molecular ions (m/z 1437.1 and 1445.1) that differ from each other by mass 16 and are larger by mass 22 than the HP- and LP-containing ions. The latter two ions can be accounted for by the pyrrole structure shown, if the ring were oxidatively cleaved and two water molecules were added.

In order to verify further the origins of the major peptides and their identities, the antibody mAb 1H11 was used in an enzyme-linked immunosorbent assay inhibition format (19) to monitor reverse-phase HPLC elution profiles. Fig. 7 shows the results for a DEAE-SPW column fraction that is especially enriched in pyrroles as defined by Ehrlich’s reactivity. The analyzed peptide pool was comparable to a mixture of pools 2 (Fig. 2B) and 4 (Fig. 2C) that combines peptides c, d, g, and h (Fig. 5). The results indicate that both pyridinoline and pyrrole versions of the cross-linked combination, a1(I)N,a2(I)N,a2(I)N-helix (peptides c and g), bind to mAb 1H11, which recognizes specifically the cleaved a2(I)N N-telopeptide sequence QYDGKGVG when it is cross-linked to either a1(I)N or a second a2(I)N in a trivalent complex. The two less abundant fluorescent peptides (equivalent

**TABLE II**

**The molar ratios of HP to LP determined on isolated cross-linked N-telopeptides**

Peptides a–h were isolated by chromatography as shown in Fig. 4. Their structures were determined by sequence analysis and electrospray mass spectrometry and are shown in Fig. 5 (A–H). HP and LP were assayed by reverse-phase HPLC after acid hydrolysis, expressing the results as a molar ratio.

| Peptide | Intermolecular origin | HP/LP molar ratio |
|---------|-----------------------|------------------|
| a       | α1(I)Nα2(I)Nα1(I)N   | 1.6              |
| b       | α1(I)Nα2(I)Nα1(I)N   | 1.3              |
| c       | α1(I)Nα2(I)Nα1(I)N   | 2.8              |
| d       | α1(I)Nα1(I)Nα1(I)N   | 1.8              |
| e       | α1(I)Nα2(I)Nα1(I)N   | 1.7              |
| f       | α1(I)Nα2(I)Nα1(I)N   | 2.5              |
| g       | α1(I)Nα2(I)Nα1(I)N   | 4.6              |
| h       | α1(I)Nα1(I)Nα1(I)N   | 1.9              |

**FIG. 6.** Electrospray mass spectrum and predicted structure of the pyrrole-containing peptide from Fig. 4D, fraction 40. Molecular ions 1426.1 and 1434.1 are believed to be from the LP- and HP-containing versions of the same peptide (see Fig. 5G); 1441.6, a photolysis product of the HP structure; and 1437.1 and 1445.1, hydrated ring-cleavage products of the respective pyrrole structures.
to d and h in Fig. 4), which are both based on two α1(1)N telopeptides lack this epitope and so are not recognized by the antibody.

**DISCUSSION**

Human bone collagen has a characteristic ratio of the two forms of pyridinoline cross-link, HP and LP, with a higher proportion of LP than any other connective tissue (37). This reflects post-translational specificity in the biosynthesis of bone type I collagen. Before the present study, the quantitative distribution of the cross-linking residues at the two principal cross-linking sites in the molecule was unknown. The results establish that HP and LP residues are not evenly distributed and that each type of cross-link, HP, LP, or the pyrrole structures, is formed preferentially at a particular site and between a particular combination of the α1(I) and α2(I) domains that potentially can interact at each site.

The findings indicate that, in total, the pyridinoline cross-links are about equally distributed between N and C ends of the type I collagen molecule. The ratio of HP/LP of 3.6:1 and their combined concentration of 0.29 mol of pyridinoline per mol of collagen (Table I) is typical of previously published values (37). It should be noted, however, that this concentration in moles/mol of collagen is lower than that of pyridinolines in most non-mineralized tissues that use this cross-linking pathway. The low pyridinoline content of bone collagen is explained in part by an arrested maturation of the initial, bivalent cross-links to pyridinolines once the bone matrix mineralizes. Thus, in bone, throughout life the borohydride-reducible cross-links are more abundant than pyridinoline residues (37). Although the continuous turnover of bone will contribute a pool of immature tissue, the extreme overabundance of immature cross-links (37) and observations from maturation experiments in vitro (15) imply that the major reason is an arrested maturation pathway in the mineralized collagen. In soft tissues, in contrast, the initial (borohydride-reducible) cross-links rapidly disappear as tissues mature (13).

Pyridinolines clearly stabilize type I collagen fibrils of human bone at both ends of the molecule, much like type II collagen in articular cartilage (18, 20). Analysis of the individual peptide fractions, however, showed that LP is not evenly distributed, with about two-thirds at the N-telopeptide to helix site (Table I). The strongest reactivity with p-dimethylaminobenzaldehyde, coincident with the pool of the cross-linked N-telopeptides, monitored for their fluorescence (Fig. 4; Ref. 19), indicates that the pyrrole cross-links are also more concentrated at the N-telopeptide than the C-telopeptide site.

Various studies have provided direct evidence for an α1(1)N→α1(1)Nα1(2) intermolecular site of pyridinoline cross-linking in type I collagen using different digestion methods (10–12, 21–24). One report was unable to confirm this location based on analyses of CNBr (CB)-derived collagen peptides from bovine bone, dentin, and tendon (38). Instead, it was concluded that pyridinoline involved α2(I)CB3,5, implying a linkage between N-telopeptides and the carboxyl-terminal domain of the α2(I) chain. A later study, however, concluded that α2(I)CB3,5 did not participate in pyridinoline cross-linking in rat bone collagen (24). Pyridinoline residues were found in peptides that were derived from both N-telopeptide and C-telopeptide cross-linking sites, but without sequence data it was not possible to specify the individual chain origins. A lack of N-telopeptide-based cross-links was reported in mineralized type I collagen of bovine bone and turkey tendon (12, 22).

We can conclude from the present results that C-telopeptide-based pyridinolines link exclusively α1(I) chains, including the donor site in the triple helix. In types I, II, and III collagens, the helical domain cross-linking sites in α1(I), α1(II), and α1(III) chains share a common sequence motif, GXXGHR, in which

**FIG. 7. Reverse-phase HPLC of pyrrole and pyridinoline cross-linked N-telopeptides, monitored for their α2(I) N-telopeptide immunoreactivity with mAb 1H11.** A preparation similar to fractions 27 and 28 shown in Fig. 2C was run, except that peptide pools equivalent to A and B (Fig. 1), containing both long and short N-telopeptide forms, were combined. The sample, therefore, contained primarily peptides c, d, g, and h (Figs. 4 and 5).

Lys (or Hyl) is the cross-linking residue. The α2(I) chain differs somewhat. In vertebrates, α2(I) lacks a cross-linking Lys in its C-telopeptide and, in human α2(I), Asn replaces Arg three residues after cross-linking Lys9833 and Ile replaces His two residues after cross-linking Lys87 (34, 39). Borohydride-reducible divalent cross-links have been found between the α1(I) C-telopeptide and α2(I) residue 87 in bovine periodontal ligament (11) and bone (12). If human bone resembles bovine bone in this respect, the absence of pyridinoline cross-linked peptides involving α2(I) residue 87 implies that the histidine located two residues COOH-terminal to the cross-linking lysine at α1(I) residue 87 is crucial for pyridinoline formation. Perhaps the mechanism of maturation requires the neighboring histidine side chain, with a near neutral pKₐ, either to stabilize or be directly involved in a transition state, comparable to an active site in an enzyme. Histidines occur rarely in the collagen triple-helix, so their conserved placement adjoining cross-linking lysines is probably important. Interestingly, the α2(I) N-helical cross-linking domain does contain a histidine, but five residues COOH-terminal to the cross-linking lysine (39). This latter residue was shown to participate directly in the mature intermolecular cross-link, histidinohydroxyllysinosonorleucine, produced on the cross-linking pathway from telopeptide lysine aldehydes that dominates in skin collagen (40).

A greater diversity of interchain pyridinoline linkages was found at the N-telopeptide site, which theoretically can involve α1(I) and α2(I) telopeptides and α1(I) and α2(I) helical sequences. The combinations α1(1)Nα2(1)Nα1(1)N and α1(1)Nα2(1)Nα2(1)N were dominant, shown to be the origin of peptides b, c, e, and g, and at a respective molar ratio of about 2 to 1. This establishes that α1(1)CB6 and α2(1)CB3,5 are both triple-helical sites of pyridinoline cross-linking in human bone collagen. Apparently, even though the human α2(I) C-helical cross-linking domain differs somewhat from the sequence motif found in α1(I), α1(II), and α1(III) (39), pyridinolines do form linking N-telopeptides to α2(I) residue 933. It is notable with respect to the above discussion, the presence of α2(I)His935. The findings indicate that pyrrole cross-links are most abundant at the N-telopeptide to helix site and in particular when linked to α2(I) Lys9833, consistent with findings on bovine ten-
This would suggest that the replacement of Arg by Asn, at α2(I)Asn936, three residues COOH-terminal to the cross-linking Lys933 favors the interactions forming a pyrrole. The known lability and reactivity of pyrroles and their potential for oxidation and hydrolytic ring opening explains the difficulty in isolating and characterizing these structures. Indeed, during the course of the present chromatographic isolations, the majority of the Ehrlich’s reactivity was lost, with major decreases observed after each step. Nevertheless, based on the known chemistry of collagen cross-link formation, the chromatographic coincidence of fluorescences, Ehrlich’s reactivity, and immunoreactivity in purified peptide fractions and the mass spectra, the data in combination provide compelling evidence that the structure shown in Fig. 6 is the source of the pyrrole reactivity in bone. It appears to be most concentrated at the N-telopeptide cross-linking site in the collagen.

Fig. 8 summarizes a cross-linking pathway that can give either the pyridinoline cross-links or pyrrole cross-links at essentially the same intermolecular cross-linking sites, consistent with the present findings and the observations and proposal of Kuypers et al. (29). The chemistry, similar to HP and LP formation, also predicts lysyl and hydroxylsyl versions of the pyrrole cross-links. The degree of hydroxylation of telopeptide lysines will control whether a pyridinoline or a pyrrole forms. The present results would suggest a lower degree of hydroxylation of the N-telopeptides than the C-telopeptides in bone collagen. This is consistent with previous observations on chick and rat bone collagen which showed about 50% hydroxylation of the N-telopeptide lysine residues (41).

The structures of the major pyridinoline cross-linked peptides identified in this study are summarized in Figs. 3 and 5. Additional chain combinations are possible and may exist in lower yield. For example, a pyridinoline-containing peptide embodying the telopeptide interaction α2(I)Nα2(I)S was isolated as a minor, but significant component from urine (19). Such a peptide was not found in the current direct analyses of bone collagen, but could have been missed since its NH₂ termini would be blocked to sequencing. It is also noteworthy that none of the cross-linked N-telopeptides had galactose or glucosylgalactose attached to the cross-link, whereas the main HP-containing cross-linked C-telopeptide had a galactose on its side chain hydroxyl group from the mass spectral results. It is possible, although unlikely, that sugar residues were removed during digestion with crude bacterial collagenase. Cross-linked N-telopeptides isolated from urine also lacked any attached sugar residues (19).

In conclusion, the results define in more detail how bone collagen differs markedly from other tissue collagens in its pattern of telopeptide cross-linking interactions. The favored telopeptide combinations found in the mature cross-links of bone collagen, α1(I)Nα1(I)S and α1(I)Nα2(I)S, may provide further insights on stereospecific features of the packing of type I collagen molecules in bone collagen fibrils. The molecular packing of type I collagen in bone is known to exhibit unique properties, including evidence that the pattern of cross-linking and the interchain placement of bonds may alter as the collagen mineralizes (22, 42). This may result in a bone-specific arrangement of bonds, including the favoring of the α2(I)N-telopeptide participating in mature cross-links in general at the N-telopeptide site and the favoring of the α2(I)N-telopeptide formation at pyrrole cross-link formation.

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REFERENCES

1. Tanzer, M. L. (1973) Science 180, 561–566
2. Bailey, A. J., Robins, S. P., and Balian, G. (1974) Nature 251, 105–109
3. Eyre, D. R., Paz, M. A., and Gallop, P. M. (1984) Annu. Rev. Biochem. 53, 717–748
4. Eyre, D. R. (1987) Methods Enzymol. 144, 115–139
5. Kang, A. H. (1972) Biochemistry 11, 1828–1835
6. Miller, E. J., and Robertson, P. B. (1973) Biochem. Biophys. Res. Commun. 54, 432–439
7. Eyre, D. R., and Glinscher, M. J. (1973) Biochem. Biophys. Res. Commun. 52, 663–671
8. Heinkel, W., Rauterburg, J., and Stirtz, T. (1976) Eur. J. Biochem. 69, 223–231
9. Kuboki, Y., Takagi, T., Shimokawa, H., Oguchi, H., Sasaki, S., and Mechanic, G. L. (1981) Connect. Tissue Res. 9, 107–114
10. Kuboki, Y., Tsuzuki, M., Sasaki, S., and Mechanic, G. L. (1982) Connect. Tissue Res. 102, 119–126
11. Yamashita, M., Katz, E. P., and Mechanic, G. L. (1986) Biochemistry 25, 4987–4992
12. Yamashita, M., Katz, E. P., Otsubo, K., Terakado, K., and Mechanic, G. L. (1989) Connect. Tissue Res. 21, 159–169
13. Robins, S. P., Shinokomaki, M., and Bailey, A. J. (1973) Biochem. J. 131, 721–730
14. Fujimoto, D., Akiba, K., and Nakamura, N. (1977) Biochem. Biophys. Res. Commun. 76, 1124–1129
15. Eyre, D. R. (1981) Dec. Biochem. 22, 51–55
16. Ogawa, T., Ono, T., Tsuda, M., and Kawanishi, Y. (1982) Biochem. Biophys. Res. Commun. 107, 1252–1257
17. Eyre, D. R., and Oguchi, H. (1986) Biochem. Biophys. Res. Commun. 92, 403–410
18. Robins, S. P., and Duncan, A. (1983) Biochem. J. 215, 175–182
19. Hanson, D. A., Weis, M. A. E., Bollen, A. M., Maslan, S. L., Singer, F. R., and Eyre, D. R. (1992) J. Bone Miner. Res. 7, 1251–1258
Cross-linking in Human Bone Collagen

20. Wu, J.-J., and Eyre, D. R. (1984) *Biochemistry* **23**, 1850–1857
21. Fujimoto, D. (1980) *Biochem. Biophys. Res. Commun.* **93**, 948–953
22. Yamauchi, M., and Katz, E. P. (1993) *Connect. Tissue Res.* **29**, 82–98
23. Henkel, W., Glanville, R. W., and Greifendorf, D. (1987) *Eur. J. Biochem.* **165**, 427–436
24. Robins, S. P., and Duncan, A. (1987) *Biochem. Biophys. Acta* **914**, 233–239
25. Scott, J. E., Hughes, E. W., and Shuttleworth, A. (1981) *Biosci. Rep.* **1**, 611–618
26. Kemp, P. D., and Scott, J. E. (1988) *Biochem. J.* **252**, 387–393
27. Scott, J. E., Qian, R., Henkel, W., and Glanville, R. W. (1983) *Biochem. J.* **209**, 263–264
28. Horgan D. J., King, N. L., Kurth, L. B., and Kuypers, R. (1990) *Arch. Biochem. Biophys.* **281**, 21–26
29. Kuypers, R., Tyler, M., Kurth, L. B., Jenkins, I. D., and Horgan, D. J. (1992) *Biochem. J.* **283**, 129–136
30. Kuypers, R., Tyler, M., Kurth, L. B., and Horgan, D. J. (1994) *Meat Sci.* **37**, 67–89
31. Risteli, J., Eriksen, H., Risteli, L., Mansell, J. P., and Bailey, A. J. (1994) *J. Bone Miner. Res.* **9**, Suppl. 1, S186
32. Bernard, M. P., Chu, M.-L., Myers, J. C., Ramirez, F., Eikenberry, E. P., and Prockop, D. J. (1988) *Biochemistry* **165**, 1471–1478
33. D'Alessio, M., Bernard, M., Preterous, P. J., de Wet, W., and Ramirez, F. (1988) *Gene (Amst.)* **67**, 105–115
34. Kuivaniemi, H., Tramp, G., Chu, M.-L., and Prockop, D. J. (1988) *Biochem. J.* **252**, 633–640
35. Pinnell, S. R., Fox, R., and Krane, S. M. (1971) *Biochem. Biophys. Acta* **229**, 119–122
36. Eyre, D. R. (1995) *Acta Orthop. Scand.* **66**, 166–170
37. Eyre, D. R., Dickson, I. R., and Van Ness, K. (1988) *Biochem. J.* **175**, 1–7
38. Light, N., and Bailey, A. J. (1985) *FEBS Lett.* **182**, 503–508
39. Bernard, M. P., Myers, J. C., Chu, M.-L., Ramirez, F., Eikenberry, E. P., and Prockop, D. J. (1983) *Biochemistry* **22**, 1139–1145
40. Mechanic, G. L., Katz, E. P., Henmi, M., Noyes, C., and Yamauchi, M. (1987) *Biochemistry* **26**, 3500–3509
41. Barnes, M. J., Constable, B. J., Morton, L. F., and Kodicek, E. (1971) *Biochem. J.* **125**, 433–437
42. Veis, A. (1993) *J. Bone Miner. Res.* **8**, suppl. 2, 3493–3497
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Dennis A. Hanson and David R. Eyre

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