Intermolecular Cross-Linking and Stereospecific Molecular Packing in Type I Collagen Fibrils of the Periodontal Ligament†

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ABSTRACT: A trypsin digest of denatured NaB^3H_4-reduced native bovine periodontal ligament was prepared and fractionated by gel filtration and cellulose ion-exchange column chromatography. Prior to trypsin digestion, a complete acid hydrolysate was subjected to analyses for nonreducible stable and reducible intermolecular cross-links. Minute amounts of the former and significant amounts of the reduced cross-links dihydroxylysinonorleucine (1.1 mol/mol of collagen), hydroxylysinonorleucine (0.9 mol/mol of collagen), and histidinohydroxymerodesmosine (0.6 mol/mol of collagen) were found. The covalent intermolecular cross-linked two-chained peptides that were isolated were subjected to amino acid and sequence analyses. The structures for the different two-chained linked peptides were α1CB4-5(76–90)[Hyl-87] × α1CB6-(993–22^c)[Lys^{ald}-16^c], α1CB4-5(76–90)[Hyl-87] × α1CB6(993–22^c)[Hyl^{ald}-16^c], α2CB4(76–90)[Hyl-87] × α1CB6(993–22^c)[Lys^{ald}-16^c], and α2CB4(76–90)[Hyl-87] × α1CB6(993–22^c)[Hyl^{ald}-16^c]. The cross-link in each peptide was glycosylated. This is the first characterization by sequence analysis of a cross-link involving Hyl-87 in an α2 chain in collagen. A stoichiometric conversion of residue 16^c aldehyde to an intermolecular cross-link in each of the COOH-terminal nonhelical peptide regions of both α1 chains in a molecule of type I collagen was found. The ratio of α1 to α2 intermolecularly cross-linked chains involved was 3.3:1, indicating a stereospecific three-dimensional molecular packing of type I collagen molecules in bovine periodontal ligament.

Connective tissues owe their stability, physical and mechanical properties, and cohesiveness to covalent intermolecular cross-links in collagen fibrils. The cross-linking is initiated by the enzymatic oxidative deamination of ϵ-amino groups on specific peptidyl residues of Lys and Hyl (Gallop et al., 1972; Siegel, 1976). The aldehydes that are produced are 5-amino-5-carboxypentanal (Lys^{ald})[1] and 2-hydroxy-5-amino-5-carboxypentanal (Hyl^{ald}). These then condense with ϵ-amino groups on specific residues of peptidyl Lys and Hyl on juxtaposed molecules to form iminium intermolecular cross-links. Chemical specificity is dictated by the staggered packing of collagen molecules in fibrils (Tanzer & Mechanic, 1970). The first stages of collagen cross-linking begin, upon fibril formation, in the COOH-terminal nonhelical peptides of the α1 chains with the conversion of Lys-16^c to its respective aldehyde and formation of iminium cross-links (Fukae & Mechanic, 1980). With maturation, nonreducible stable cross-links evolve through a series of condensations (Eyre et al., 1984).

The periodontal ligament (PL) is the soft connective tissue situated between the surface of the tooth and the bone forming the wall of the tooth socket. Its function is to support the tooth and dissipate the forces generated by mastication. This is achieved through fibrillar collagen, the most abundant organic material in the PL (Shuttleworth & Smalley, 1983).

An exceptionally high rate of turnover of this protein in the PL has been found (Sodek, 1977). Because the existing cross-linking studies on PL have not been concordant and were inconsistent (Pearson et al., 1975; Kuboki et al., 1981a; Plecash & Bentley, 1982), we have carried out a quantitative cross-link analysis on PL. We report here the characterization of the major two-chained intermolecular cross-linked peptides resulting from a tryptic digest of the $NaBH_4$-reduced bovine PL. The peptides were all from type I collagen, even though type III (Butler et al., 1975) and type V (unpublished results) are in this tissue. The stoichiometry of COOH-terminal cross-linking indicates a stereospecific chemistry, most probably due to a stereospecific molecular packing in the fibrils.

Materials and Methods

Preparation of Insoluble Collagen. Bovine PL was obtained as described by Butler et al. (1975). All operations were carried out at 4 °C. After removal of the small fragments of alveolar bone adherring to the tissue, the central third of the PL was removed carefully so as to avoid contamination from the surrounding tissues. The samples were pooled and suspended in about 20 volumes of ice-cold distilled water containing 0.01 M EDTA, 0.005 M NEM, 0.0005 M PMSF, and 0.025 M ϵ-aminocaproic acid, homogenized with a Polytron homogenizer, and centrifuged. The residue was washed with the same solution repeatedly and centrifuged at 5000*g* for 30 min each time until the supernatant was clear. The residue

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[1] Abbreviations: Lys^{ald}, 5-amino-5-carboxypentanal; Hyl^{ald}, 2-hydroxy-5-amino-5-carboxypentanal; PL, periodontal ligament; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; DHNL, dihydroxynorleucine; HNL, hydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; HHL, histidinohydroxylysinonorleucine; HAH, hydroxyaldolhistidine; PYR, pyridinoline; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PTH, phenylthiohydantoin; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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was next twice suspended in 10 volumes of ice-cold 1.0 M NaCl–0.05 M Tris-HCl, pH 7.5, solution and centrifuged at 10000*g* for 60 min. The tissue was washed 3 times with ice-cold distilled water to free the insoluble collagen of salt and lyophilized.

Reduction with NaB^3H_4. The dried, insoluble PL collagen (2 g) was suspended in 0.15 M TES buffer, pH 7.5, and reduced with NaB^3H_4 by the method described previously (Fukae & Mechanic, 1980).

Specific Activity of NaB^3H_4. The specific activity of the NaB^3H_4 was determined from the amount of radioactivity recovered from HLNL in the apparently homogeneous two-chained cross-linked peptide α1CB0,1(1^N–9)[Lys-9^N] × α1CB6(928–933)[Hyl-930][2] (see below). Equal aliquots of the hydrolysate of this peptide were subjected to amino acid and radioactive cross-link analyses. It was ascertained that 3.5 nmol of HLNL contained 26 500 dpm of tritium. This indicated that the specific activity of the NaB^3H_4 was 7.57 × 10^6 dpm/μmol.

Cross-Link Analysis. An aliquot of the reduced insoluble PL collagen was hydrolyzed in vacuo with 3 N HCl for 48 h at 115 °C. The hydrolysate was taken to dryness 3 times, with water being added each time. About 0.5 mg was analyzed for cross-links on a Varian HPLC liquid chromatograph fitted with a stainless steel amino acid column (AA911, Interaction) and linked to an on-line flow liquid scintillation monitor (Flo-one β, Radiomatic Instruments and Chemical Co.). Cross-link standards were prepared separately (see below) and used to identify the elution positions of radioactive peaks. Determination of Hyp was used to calculate collagen content for all operations.

The nonreducible stable mature cross-links were quantified as follows: approximately 200 mg of reduced PL collagen, which had been hydrolyzed with 6 N HCl, was dissolved in 0.5 mL of 20% acetic acid and filtered on a Bio-Gel P-2 column (1.2 × 100 cm, −400 mesh), essentially by a method used for the bulk separation of the cross-links from the amino acids of collagen (Housley & Tanzer, 1981). The column was equilibrated with the same solvent.

Digestion with Trypsin. A tryptic digest was obtained by procedures described previously (Kuboki et al., 1981b; Yamauchi et al., 1981; Becker et al., 1975). The reduced PL (2 g) was suspended in 0.2 M NH_4HCO_3, pH 7.9, solution, brought to 65 °C with stirring and kept there for 20 min to denature the collagen. This was cooled to 37 °C and treated with TPCK-trypsin (1% w/w, Worthington) for 4 h. The solution was heated once again to 60 °C for 10 min, which resulted in an almost clear solution. This was cooled to 37 °C and retreated with trypsin (0.5% w/w) for 2 h with stirring. The solution was filtered and lyophilized. Over 98% of the starting material was recovered in the supernatant of the trypsin digest. Amino acid analysis indicated that the residual material essentially consisted of noncollagenous material.

Molecular Sieve and Ion-Exchange Chromatography. Gel filtration chromatography was carried out, with 200-mg portions of the trypsin digest each time, on a column (1.9 × 230 cm) filled with Sephadex G-50 superfine that had been equilibrated with 2 M guanidine hydrochloride–0.05 M Tris-HCl, pH 7.5, at room temperature. The column was eluted with the same solution at a flow rate of 15 mL/h and monitored continuously at 230 nm; 5-mL fractions were collected (Yamauchi et al., 1981). The most abundant radioactive fraction (VII, Figure 3) was then chromatographed on a DEAE-cellulose column (1.7 × 17 cm, Whatman DE-52) that was equilibrated with 0.01 M NH_4HCO_3 containing 1% 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. All the buffers for cellulose ion-exchange chromatography contained 1% 2-propanol. The fractions DI and DII (Figure 4) were rechromatographed separately with a shallower 400-mL linear gradient between 0.03 M NH_4HCO_3 (200 mL) and 0.15 M NH_4HCO_3 (200 mL) on the same column. In one of the two isolations performed, each fraction, DI and DII, was then fractionated on a phosphocellulose (Whatman P-11) column (1.7 × 15 cm) maintained at 42 °C. The column was equilibrated with 0.001 M sodium acetate, pH 3.8. A 400-mL linear gradient between 0.001 M sodium acetate, pH 3.8 (200 mL), and the same buffer which was 0.5 M in NaCl (200 mL) was used to elute the column at 120 mL/h. The fractions were desalted on a Bio-Gel P-2 column (4.0 × 50 cm, 50–100 mesh) eluted with 0.1 M acetic acid.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in vacuo, after flushing with N_2, at 115 °C for 24 h. The hydrolysates were analyzed for their amino acid compositions on a Hitachi 835 amino acid analyzer and a Varian 5560 liquid chromatograph configured as an amino acid analyzer (AA911 column, Interaction) using ninhydrin with color development at 135 °C in a stainless steel reaction coil. Temperature was maintained to ±0.05 °C with a thermostated silicone oil bath. The ninhydrin color was monitored at 570 nm except that wavelength programming was used to detect Hyp and Pro at 440 nm. Standards of DHLNL, HLNL, and HHMD prepared from reduced dentin (Mechanic et al., 1971) and skin (Mechanic, 1974) were used to identify the positions of these cross-links in the elution pattern. The residue composition obtained from amino acid analysis and sequencing of the tryptic peptide α1CB0,1(1^N–9)[Lys-9^N] × α1CB6(928–933)[Hyl-930] (see below) obtained from the same tryptic digest as described above was used to obtain the color factor for HLNL. The same color factor was assigned for DHLNL. Analyses were performed in duplicate.

The amounts of glycosylated cross-links in the peptides were obtained by amino acid analysis after 2 N NaOH hydrolysis in polypropylene tubes under N_2 at 115 °C for 24 h.

Amino Acid Sequence Analysis. Amino acid sequences were determined commercially on an Applied Biosystems Model 470A gas-phase protein sequenator. The phenylthiohydantoin derivatives of the amino acids were identified by reversed-phase chromatography.

RESULTS

Quantification of Stable Nonreducible Cross-Links. The stable nonreducible mature cross-links from aliquots of hydrolysates of PL were obtained from the Bio-Gel P-2 column. Quantification of the cross-links was performed on the amino acid analyzer. The ninhydrin color factors for the stable cross-links were obtained from the amino acid compositions of apparently pure peptides containing HHL (Yamauchi et al., 1982) and PYR. The PYR peptide was isolated from a tryptic digest of bovine achilles tendon in a similar manner to its previous isolation from dentin (Kuboki et al., 1981b); sequence and amino acid analysis demonstrated its structure to be α1CB6(993–22^c)[Hyl-16^c] × α1CB6(993–22^c)[Hyl-16^c] × α1CB4-5(76–90)[Hyl-87]. Approximately 0.02 mol of HHL and PYR per mole of collagen was present. The cor-

[2] Designations of the cross-linked peptides in this paper are made in terms of the CNBr fragments obtained from collagen: numbers in parentheses correspond to the residue number assigned in the sequence in Galloway (1982); residues in brackets are the residues that participate in the cross-link.

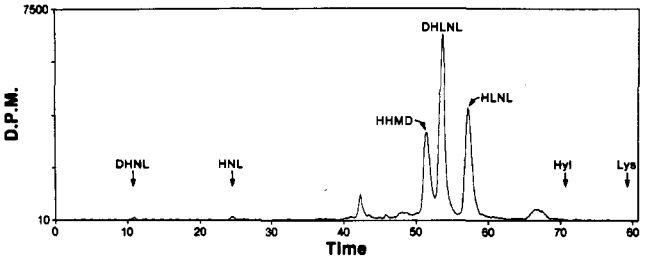

FIGURE 1: Typical chromatographic profile from the stainless steel amino acid column (Interaction AA911 column). Buffer flow rate was 0.6 mL/min, and scintillation fluid flow rate was 6.0 mL/min. See Materials and Methods for other details.

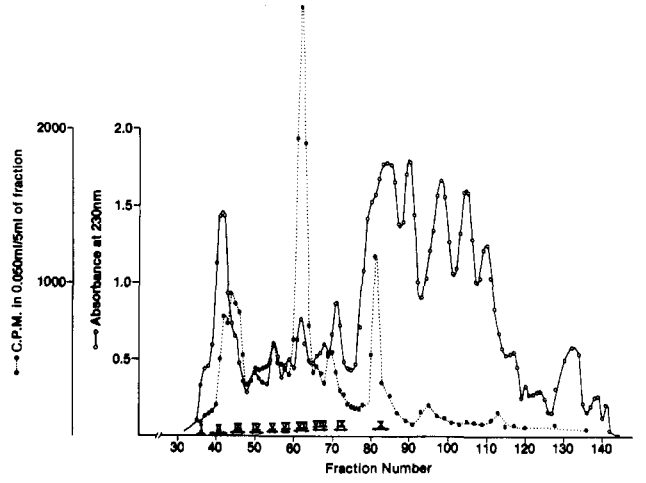

FIGURE 2: Typical Sephadex G-50 superfine gel filtration chromatographic profile of a tryptic digest of heat-denatured NaB^3H_4-reduced native bovine periodontal ligament. Fractions pooled are denoted by the double-arrow bar underneath the Roman numerals that denote the fraction number. See Materials and Methods for other details.

rection of the structure of HAH (Housley et al., 1975) and its determination as HHL will be the subject of another report.

Cross-Link Analysis. The radioactive elution profile of a complete hydrolysate of reduced whole bovine PL collagen is depicted in Figure 1. The percentage radioactivity was 0.4% for DHNL, 0.7% for HNL, 24% for HHMD, 42% for DHLNL, and 32% for HLNL. The ratio of DHLNL:HLNL was 1.3.

Isolation of Cross-Linked Peptides. The molecular sieve pattern of the tryptic digest of NaB^3H_4-reduced PL collagen is depicted in Figure 2. This pattern was reproducible throughout the 10 runs for the preparation of the starting materials for ion-exchange chromatography. On the basis of the absorbance at 230 nm and the radioactive content, ten peaks were designated (I–X). Amino acid analyses indicated that HHMD was found to be most abundant in fractions II and III but was present in decreasing amounts as the peaks were more retarded. Peaks IV–VIII contained DHLNL and HLNL with the most abundant amounts appearing in VII. Peak X contained HLNL and little if any DHLNL. The stable nonreducible cross-links HHL and PYR appeared in fractions III and IV. Fraction VII, the most prominent radioactive peak is the focus of this paper.

The chromatographic pattern of fraction VII on DEAE-cellulose is depicted in Figure 3. When the fractions labeled DI and DII were rechromatographed separately on the same column with a shallower gradient, they eluted as single symmetrical peaks with DI eluting at a lower salt concentration than DII (data not shown). The patterns from the phosphocellulose column for fractions DI and DII are shown in Figures 4 and 5, respectively. Fraction DI contained one major ra-

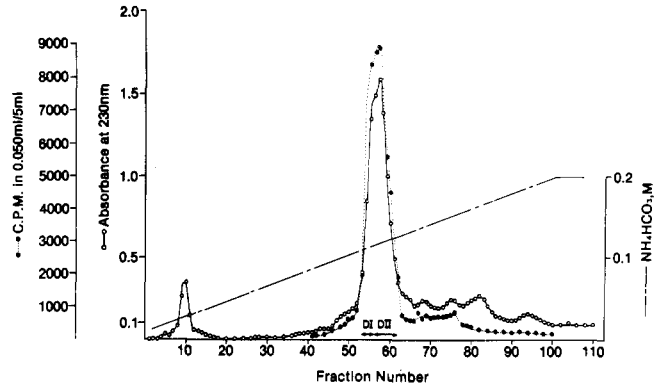

FIGURE 3: DEAE-cellulose chromatographic profile of fraction VII from Figure 3. Fractions pooled are denoted by a double-arrow bar below the Roman numerals that denote the fraction number. See Materials and Methods for other details.

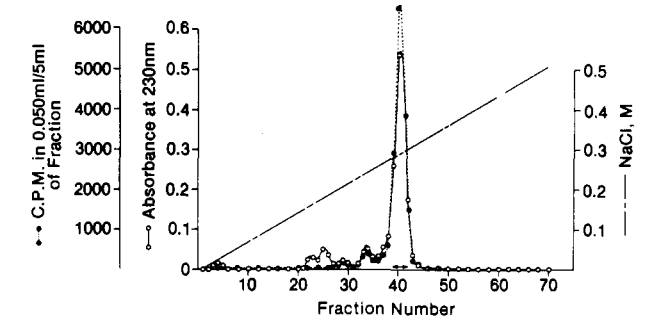

FIGURE 4: Chromatographic elution pattern of fraction DI from Figure 4 on phosphocellulose column. Fraction denoted by double-arrow bar under the peak was subjected to amino acid composition and sequencing analyses. This fraction is denoted as DI-P in the text. See Materials and Methods for other details.

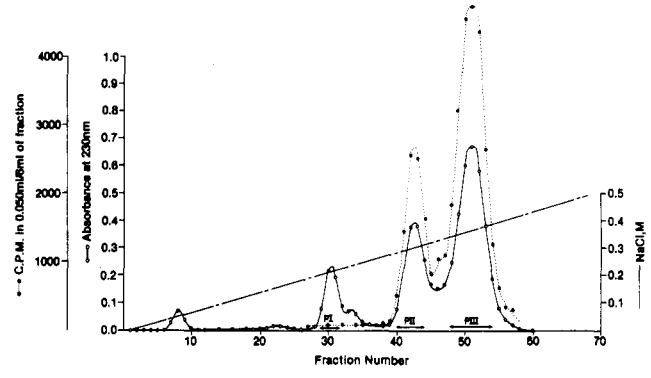

FIGURE 5: Chromatographic separation of fraction DII from Figure 4 on phosphocellulose column. Fractions denoted by the double-arrow bars were pooled and subjected to amino acid composition and sequence analyses. Fraction PII is peptide DII-PII and fraction PIII is peptide DII-PIII in the text. See Materials and Methods for other details.

dioactive peak (DI-P) and some minor peaks (Figure 4). Fraction DII contained only two radioactive peaks and some minor UV-absorbing peaks (Figure 3). The peaks containing radioactivity were labeled PII and PIII and designated as DII-PII and DII-PIII. Amino acid compositions (see below) indicated near homogeneity for the cross-linked moieties.

Sequence Structures and Amino Acid Compositions. The amino acid compositions for the two-chained cross-link peptides isolated are shown in Table I. The compositions for DI-P and DII-PIII are virtually identical except that the former contains one residue of HLNL while the latter contains one residue of DHLNL. In addition, DII-PIII is slightly contaminated with DI-P as evidenced by an HLNL content of 0.2 residue. The complete absence of Lys and Hyl, along with the presence of

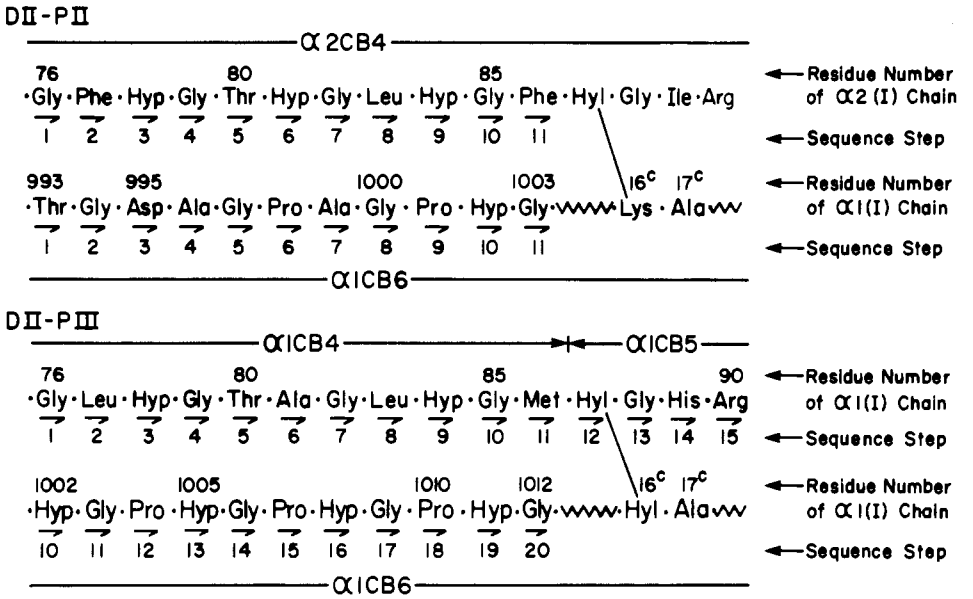

FIGURE 6: Sequence analyses of two of the three two-chained peptides reported here. Entire sequence of bottom peptide not shown in order to conserve space. The half-arrow signifies the residue found with the number below it designating the sequence cycle in which it was found. After cycle 15, in peptide DII-PIII only one residue at each cycle was found. In the latter, only Pro was found at cycle 12 in an amount that corresponded to one residue. Aside from this cycle, two amino acid derivatives were found in each of the first 15 cycles while two derivatives were found in the 11 sequence cycles performed on peptide DII-PII (upper two-chained peptide). The bar indicates the residues that are cross-linked.

Table I: Amino Acid Compositions of Cross-Link Peptides

	α2CB4(76–90) × α1CB6(993–22c)		α1CB4-5(76–90) × α1CB6(993–22c)		
residue	peptide DII-PII	lit. value[a]	peptide DII-PIII	lit. value[a]	peptide DI-P
Hyp	5 (5.3)[b]	7	5 (5.2)	6	5 (5.2)
Asp	3 (3.0)	3	3 (3.2)	3	3 (3.2)
Thr[c]	2 (1.8)	2	2 (1.9)	2	2 (2.0)
Ser[c]	2 (1.8)	2	2 (1.7)	2	2 (2.0)
Glu	3 (3.0)	3	3 (3.3)	3	3 (2.9)
Pro	11 (11.0)	10	11 (11.4)	10	11 (10.6)
Gly	16 (16.4)	16	17 (17.4)	16	17 (16.9)
Ala	3 (3.1)	3	4 (4.3)	4	4 (4.0)
Val	0 (0.0)	0	0 (0.0)	0	0 (0.0)
Met	0 (0.0)	0	1 (0.5)	1	1 (1.0)
Ile	1 (0.6)	1	0 (0.0)	0	0 (0.0)
Leu	3 (3.1)	3	4 (4.3)	4	4 (4.2)
Tyr	1 (0.7)	1	1 (0.8)	1	1 (1.1)
Phe	3 (2.8)	3	1 (1.1)	1	1 (1.1)
His	1 (1.0)	1	2 (2.0)	2	2 (2.0)
Hyl	0 (0.0)	1	0 (0.0)	1	0 (0.0)
Lys	0 (0.0)	1	0 (0.0)	1	0 (0.0)
Arg	2 (1.6)	2	2 (2.1)	2	2 (1.9)
total	56 [58][d]	59	58 [60][d]	59	58 [60][d]
DHLNL[e]	0.6		0.9		0.0
HLNL[f]	0.4		0.2		1.0

[a] Obtained from sequence of Galloway (1982). [b] Values in parentheses are actual values found. [c] Not corrected for hydrolysis. [d] Values in brackets include amino acid residues in cross-links. [e] DHLNL, dihydroxylysinonorleucine; made up of two residues of Hyl. [f] HLNL, hydroxylysinonorleucine; made up of a residue of Hyl and a residue of Lys.

one cross-link residue, indicated that each peptide preparation was not contaminated with un-cross-linked peptides. The compositional features of DI-P and DII-PIII strongly suggest that both represent identical portions of the cross-linked peptide α1CB4-5(76–90) × α1CB6(993–22c) (Galloway, 1982). This was confirmed by amino acid sequencing of DII-PIII, whose sequence and structure is shown in the lower part of Figure 6. The sequence analysis also indicated that residue Hyl-87 in each case was linked to Lys-16c or Hyl-16c. Despite the overall similarity in composition, DII-PII eluted earlier than DII-PIII from the phosphocellulose column (Figure 5); however, DII-PII possessed distinct differences in amino acid composition from that of its chromatographic mate (DII-PIII) and peptide DI-P (see Table I). The peptide had one residue of Ile, Met was absent, and there were two more residues of Phe and one residue less each of His and Leu. The composition of DII-PII resembled that of a two-chained cross-linked peptide, α2CB4(76–90) × α1CB6(993–22c). Sequence analysis confirmed that the cross-link involved Hyl-87 of the α2 chain and Lys-16c of the α1 chain. The peptide contained 0.6 residue of DHLNL and 0.4 residue of HLNL, which is indicative of partial hydroxylation of Lys-16c. Twenty sequence cycles were performed on peptide DII-PIII, and 11 were completed on DII-PII; these are shown diagrammatically in Figure 6. When the PTH derivatives of the amino acid residues were identified by reversed-phase liquid chromatography, only two derivatives in nearly equal amount appeared at each cycle of the sequenator for each of the cross-linked peptides. The one exception occurred in cycle 12 for peptide DII-PIII; this cycle only gave the derivative for Pro, which approximated the amount of only one residue. Therefore, cycle 12 defined the position of the cross-linking residue in the two-chained peptide. Since the amino acid composition for peptide DI-P was virtually identical with that of peptide DII-PIII except for the cross-linking residue and DI-P and DII-PIII came from the same molecular weight fraction (Figure 2), no sequence analysis was performed on peptide DI-P.

Cross-Link Residue Molecular Distribution. Under the conditions we used for chromatography, recoveries of collagen chains and peptides from the gel filtration and cellulose ion-exchange columns were quantitative (Fukae & Mechanic, 1980). Thus, area integration of radioactivity of peaks from the cellulose ion-exchange columns allowed calculation of the molar amounts of each cross-link peptide present per mole of total collagen. The amount of α1CB4-5(76–90)[Hyl-87] × α1CB6(993–23c)[Lys-16c] was 0.4 (0.40) mol while that of its counterpart α1CB4-5(76–90)[Hyl-87] × α1CB6(993–23c)[Hyl-16c] was 0.9 (0.92) mol. In the same manner, there were 0.2 (0.24) mol of α2CB4(76–90)[Hyl-87] × α1CB6-(993–23c)[Hyl-16c] and 0.2 (0.16) mol of α2CB4(76–90)-

Table II: Peak Area Integration of Cross-Linked $\alpha 1$ and $\alpha 2$ Chain Peptides[a]

isolation	width at half-height (vol) × height of peak (cpm): (A) $\alpha 1[16^c] \times \alpha 2[87]^b$	(B) $\alpha 1[16^c] \times \alpha 1[87]^b$	ratio, B/A
1	71 350	228 290	3.20
2	50 290	175 000	3.48
			3.34 ± 0.14[c]

[a] From cellulose ion-exchange chromatographic profiles. [b] Brackets contain the number of the residue that is cross-linked. [c] Average.

Table III: Glycosyl Derivatives of Hyl-87 Involved in Cross-Links in Peptides Isolated

peptide	cross-link	% mono-*O*-glycosyl derivative: *O*-Glc-Gal	*O*-Gal	total
DII-PII	DHLNL	97	3	100
	HLNL	89	11	100
DII-PIII	DHLNL	85	15	100
	HLNL	100	0	100
DI-P	HLNL	90	10	100

[Hyl-87] × α1CB6(993–23^c)[Lys-16^c] per mole of total collagen, respectively. These values are minimum estimates for the amount of each cross-link peptide in the type I collagen, since PL collagen consists of approximately 75% type I, 20% type III (Butler et al., 1975), about 5% type V (unpublished results), and a small amount of a unique collagenous component (Yamauchi et al., 1986).

The ratio of the cross-links involving $\alpha 1$ and $\alpha 2$ chains, respectively, is 3.34 ± 0.14. This value was the average of two independent isolations (Table II) and was obtained directly from the radioactive counts in the respective chromatographic peaks.

Glycosylation of Cross-Link Residues. In our amino acid analysis system using the Varian 5560 liquid chromatograph, the derivatives *O*-Glc-Gal- and *O*-Gal-DHLNL and -HLNL are easily separated from each other and from the two cross-links themselves as well as from the other amino acids. Alkaline hydrolysis of the three two-chained cross-linked peptides indicated that all the cross-link residues were glycosylated (Table III). Both *O*-Glc-Gal and *O*-Gal cross-link residues were present in each peptide, with the *O*-Glc-Gal derivatives predominating.

Discussion

There have been conflicting findings about the cross-linking patterns obtained from NaB^3H_4-reduced PL. The results of Pearson et al. (1975) and Plecash and Bentley (1982) are similar to that observed for developing bone or dentin and mature dentin (Mechanic et al., 1971). The Kuboki et al. (1981a) findings are comparable to results from skin (Mechanic, 1974). The distribution of reducible cross-links that we observe (DHLNL:HLNL of 1.3 and HHMD:HLNL of 0.75) is distinctive. The disparity in findings from different laboratories might be attributable to species differences or differences in chromatographic procedures, or, more likely, to sampling procedures. In this regard, we took special precautions to avoid contamination of the PL samples with type I collagen containing bone or tooth tissues. In contrast to all other collagen-containing tissues, PL collagen has relatively abundant reducible cross-links (Bailey, 1969; Fujii & Tanzer, 1974; Cannon & Davidson, 1977; Nielsen et al., 1983; Light & Bailey, 1979) and only small amounts of mature, nonreducible cross-links (Housley et al., 1975; Fujimoto et al., 1978; Eyre et al., 1981, 1984; Walters & Eyre, 1983). This undoubtedly reflects the exceptionally high rate of collagen turnover in this tissue (Sodek, 1977). Rapid turnover would not permit collagen fibrils to "age" and thus form mature cross-links. The function of carbohydration of specific Hyl residues may be to inhibit the formation of more complex cross-links, and thereby prevent maturation and aging (Yamauchi et al., 1982). Decreased turnover of a collagenous tissue has been associated with aging. Henkel et al. (1976) have shown that the degree of glycosylation did not depend on site in the molecule but was tissue-dependent. The complete glycosylation of the Hyl residues of the COOH-terminal cross-links of the PL collagen (Table III) thus might be required to maintain the tissue in an immature state and thereby allow it to turnover more rapidly. We speculate that the glycosyl groups might prevent mature cross-linking by sterically hindering reacting species.

The two-chained cross-linked peptides that we isolated have amino acid compositions that show one residue of Glu less than reported previously (Rauterberg et al., 1972). Our results are consistent with nucleotide sequencing data (Fuller & Boedtker, 1981).

Cross-links between residue 9^N and helical residue 930 and between 16^c and helical residue 87 of the $\alpha 1$ chain have been found in a variety of tissues such as rat tail tendon (Kang, 1972), bovine bone (Eyre & Glimcher, 1973), rabbit skin, bone, and tendon (Henkel et al., 1976), bovine skin (Becker et al., 1975; Yamauchi et al., 1982), and bovine dentin (Kuboki et al., 1981b; Scott, 1980). Involvement of the $\alpha 2$ chain in cross-linking has been suggested in earlier studies (Fujii et al., 1975; Scott et al., 1976; Scott, 1980; Light & Bailey, 1985). The results on the PL collagen are the first that characterize by sequence analysis a helical locus for cross-linking of the $\alpha 2$ chain of type I collagen. This locus is Hyl-87, which is compatible with the molecular packing model that was first derived from electron microscopic studies (Hodge & Petruska, 1963).

The stoichiometry of the intermolecular cross-linking reactions between Lys^{ald}-16^c and the Hyl-87 residues of the $\alpha 1$ and $\alpha 2$ chains of the type I collagen allows an insight into the elaboration and the geometry of the molecular packing of type I collagen fibrils of the PL. We recovered 1.72 mol of type I collagen cross-links per total mole of PL collagen. Of this, 0.6 and 1.16 mol involved a 16^c Lys^{ald} and Hyl^{ald}, respectively. Since type I collagen in this tissue is about 75% of the total collagen, we can conclude (1) only $^2/_3$ of the 16^c Lys was hydroxylated by lysylhydroxylase but (2) the conversion of 16^c residues to aldehydes by lysyl oxidase was complete. In the temporal sequence of cross-linking, residues at position 16^c are the first to be converted to aldehyde (Fukae & Mechanic, 1980) and to participate in the initial cross-linking of collagen, which is intermolecular. This indicated that lysyl oxidase binds initially to the COOH-terminal nonhelical peptides in fibrils. The complete oxidation of the 16^c residues is compatible with this hypothesis.

Concepts of the three-dimensional molecular packing in collagen fibrils derive from the packing model of Hodge and Petruska (1963). Here, rod-like molecules are packed in parallel but are longitudinally staggered with respect to one another by a multiple of *D*, the axial repeat distance of a collagen fibril (67 nm). The collagen molecules are laterally disposed on a hexagonal or pseudohexagonal lattice (Katz & Li, 1972a,b, 1973a, 1981; Fraser et al., 1983). However, the precise geometry between all the parts of the molecules has not yet been established. We have calculated the stoichiometry of COOH-terminal cross-linking for all of the hexagonal

packing schemes that are being considered for type I collagens. The calculations were based on the condition that each molecule in the array forms two COOH-terminal cross-links, which appears to be true for PL type I collagen. A 2:1 ratio of α1- to α2-derived cross-links is associated with any hexagonal array in which the collagen molecules have a randomized angular (azimuthal) orientation with respect to one another. This same distribution of cross-links would also be present if the dynamics of the cross-linking chemical reactions were independent of structural considerations, that is to say that each Hyl-87 had the same probability of reaction with each Lys^{ald}-16^{c}. The ratio of 2:1 then establishes the base line for considerations of the stereospecific aspects of the cross-linking reactions. For this reason, the α1 and α2 cross-linked peptide stoichiometry of 3.3 observed for the type I collagen of the PL (Table II) is of special significance. It strongly suggests that the collagen molecules are packed into fibrils with a specific angular orientation.

This ratio is associated with a number of super lattice models that have been proposed to characterize the X-ray diffraction effects for rat tail tendon (MacFarlane, 1971; Katz & Li, 1972a,b, 1973a,b; Hulmes & Miller, 1979; Trus & Piez, 1980; Fraser et al., 1983).

Additional evidence for a stereospecific collagen orientation in a fibril comes from the analysis of the spatial pattern and stoichiometry of noncovalent intermolecular side-chain interactions in fibrillar aggregates (Hoffman et al., 1978; Piez & Trus, 1978; Li et al., 1981; Katz & Li, 1981). Thus, besides X-ray diffraction and physical chemical findings, the stoichiometry of the COOH-terminal cross-linking chemistry of the type I collagen of the PL now provides chemical evidence that collagen molecules take up specific azimuthal orientations with respect to one another in the fibril. As the cross-links between molecules occur after fibrillogenesis, they represent an effect rather than a cause of the molecular orientation. The orientation is most probably the results of the same kind of noncovalent forces that cause the *D* staggering of molecules in a self-assembly process that produces axially periodic fibrils from aperiodic molecules.

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Genetic Regulation of Testosterone 15α-Hydroxylase (Cytochrome P-450$_{15\alpha}$) in Renal Microsomes of Female Mice†

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ABSTRACT: P-450$_{15\alpha}$ is a form of cytochrome P-450 purified from liver microsomes of female 129/J mice that is specific for oxidation of testosterone to its 15α-hydroxylated product. Testosterone 15α-hydroxylase activity that was inhibited by anti-P-450$_{15\alpha}$ antibody was approximately 50 times higher in renal microsomes from 129/J than in BALB/cJ females. Western blots of renal microsomes using anti-P-450$_{15\alpha}$ antibody showed the presence of immunoreactive protein with a molecular weight identical with that of hepatic P-450$_{15\alpha}$ in 129/J but not in BALB/cJ female mice. To investigate the genetic basis for the strain differences in this activity, the distribution of P-450$_{15\alpha}$-dependent testosterone 15α-hydroxylase activity in renal microsomes from individual females of 129/J and BALB/cJ, of F1 offspring of these strains, and of F1 back-crosses to the progenitor strains were determined. The results were consistent with a sex-related autosomal dominant regulation of the higher activity in 129/J females by a single locus, designated Rsh (regulation of steroid hydroxylase). The amounts of immunochemically cross-reactive P-450$_{15\alpha}$ protein were linearly correlated with testosterone 15α-hydroxylase activities in renal microsomes from Rsh heterozygotes and homozygotes. At least twice as much mRNA, which hybridized with the cDNA clone for hepatic P-450$_{15\alpha}$, was detected in 129/J and 129CF1/J compared to BALB/cJ female kidneys. The evidence suggests a pretranslational regulation of the P-450$_{15\alpha}$ isozyme in the female mouse kidney by the Rsh locus. More than 90% of the renal testosterone 15α-hydroxylase activity in the female mice was inhibited by anti-P-450$_{15\alpha}$ while the activity in renal microsomes from 129/J and BALB/cJ males was decreased in the presence of the antibody only to approximately 40% of the control levels. This indicates the presence of another isozyme besides P-450$_{15\alpha}$ in the kidney of male mice. The total and P-450$_{15\alpha}$-dependent testosterone 15α-hydroxylase activities in renal microsomes were higher in male than in female mice; the opposite is true in hepatic microsomes from the same mice.

As with several other liver enzymes and proteins such as drug oxidases, monoamine oxidase, prolactin receptor, and major mouse urinary protein (MUP),[1] steroid hydroxylase activities in microsomes exhibit a marked sexual dimorphism is rodents (Roy & Chatterjee, 1983; Colby, 1980). For instance, testosterone 16α-hydroxylase activities are predominant in males while testosterone 15β- and 15α-hydroxylase activities are predominant in females (Einarsson et al., 1973; Levin et al., 1975; Gustafsson & Ingelman-Sundberg, 1974; Harada & Negishi, 1984a,b). These hydroxylase activities are catalyzed by specific forms of cytochrome P-450, the terminal oxidase of a membrane-bound monooxygenase system which also consists of NADPH-cytochrome P-450 reductase, cytochrome b_5, and NADH-cytochrome b_5 reductase (Sato & Omura, 1978).

It has been reported that hepatic testosterone 15α-hydroxylase activity is severalfold higher in females than in males of some mouse strains such as 129/J and AKR/J, but not in BALB/cJ (Ford et al., 1979; Hawke & Neims, 1983; Harada & Negishi, 1984a). A form of cytochrome P-450 specific for hydroxylation of testosterone at the 15α-position (P-450$_{15\alpha}$) was recently purified from liver microsomes of untreated female 129/J mice by this laboratory (Harada & Negishi, 1984a,b). A specific inhibitory antibody elicited against the purified P-450$_{15\alpha}$ in rabbits was used to elucidate the female-predominant expression of this isozyme in mouse liver. Furthermore, cDNAs encoding P-450$_{15\alpha}$ were cloned and utilized to demonstrate that the hepatic level of P-450$_{15\alpha}$ mRNA was 6 times higher in female than in male 129/J mice (Burkhart et al., 1985).

Alcohol dehydrogenase, β-glucuronidase, and ornithine decarboxylase are known to be expressed predominantly in kidneys of male rather than female rats (Paigen et al., 1975;

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[1] Abbreviations: MUP, mouse urinary protein; Rsh, regulation of steroid hydroxylase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.