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Isolation and Structure of a Cross-Linked Tripeptide from Calf Bone Collagen†

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ABSTRACT: A cross-linked tripeptide has been isolated from alkaline hydrolysates of NaB^3H_4-reduced calf bone collagen. The peptide contains dihydroxylysinonorleucine, the most abundant cross-link in bone collagen, and it has a single N-terminal proline and a single C-terminal valine. These amino acids are in peptide linkage with the cross-link, in a trans configuration with respect to the secondary amine.

The majority of the borohydride-reducible intermolecular cross-links in collagen have been characterized with regard to their covalent structures (Tanzer, 1967, 1973, Bailey, 1967). Most of these compounds reflect condensation products between the carbonyl moiety of the collagen aldehyde, α-aminoadipic δ-semialdehyde, and other amino acids in collagen. The relative abundance of each cross-link varies with the tissue source of the collagen and with the chronologic age of the animal. For example, it is generally agreed that the mineralized collagens of adult animals are particularly rich in the cross-link, dihydroxylysinonorleucine (Mechanic and Tanzer, 1970). This compound appears to be present in two different forms prior to borohydride reduction, namely the aldimine and its ketoamino rearrangement form (Eyre and Glimcher, 1973a; Tanzer, 1973). It may also contain *O*-glycosidic galactose and glucose as the disaccharide, galactosyl glucose, linked to one of the hydroxyl groups of the cross-link (Eyre and Glimcher, 1973a,b).

In the present study, we have examined which amino acids are adjacent to the dihydroxylysinonorleucine found in mineralized bone collagen. A tripeptide, prolyldihydroxylysinonorleucylvaline, has been isolated and its primary structure has been determined.

Materials and Methods

Preparative Steps. Calf tibiae were cleaned, cut into small pieces, and powdered in a liquid nitrogen cooled Spex freezer/mill. The powdered bone was demineralized with

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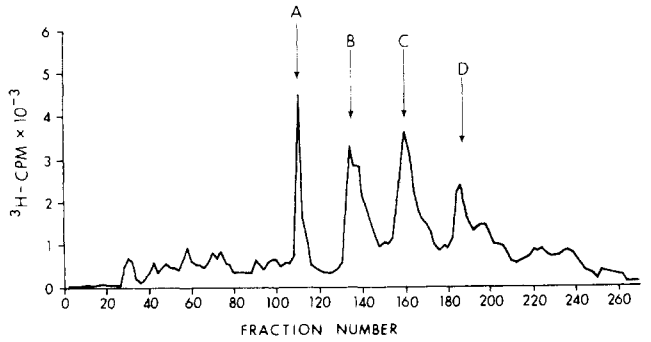

FIGURE 1: Ion-exchange chromatography on a column of Dowex 50-X8, 200–400 mesh (2.5 × 60 cm), using a linear gradient between 0.25 *M* potassium formate (pH 2.9) and 0.75 *M* potassium acetate buffer (see text for details).

0.8 *M* EDTA (pH 7.5) at 4°C by shaking for several weeks in frequently replenished solvent. The bone powder was then washed exhaustively with deionized water and lyophilized.

The dry powder (15 g) was suspended in 500 vol (v/w) of potassium phosphate buffer (pH 7.6), $\mu = 0.15$, and hydrated at 5°C for several days. The sample was transferred to a 37°C water bath and reduced with a mixture of 30 mg of NaB^3H_4 and 420 mg of $NaBH_4$, previously dissolved in 5 ml of 0.01 *N* NaOH. Following incubation at 37°C for 1 hr the protein suspension was filtered on Miracloth, washed exhaustively with deionized water, and then lyophilized.

The tritiated protein was hydrolyzed in 2 *N* KOH at 107°C by refluxing for 24 hr, and then filtered on glass wool. The filtrate was chilled and 60% $HClO_4$ was added dropwise to adjust the pH to 6.0; the $KClO_4$ precipitate was removed by centrifugation (10,000 rpm, 30 min) at 4°C. Norit A charcoal was added to the supernatant fluid which was then filtered on a sintered glass filter, previously covered with Celite; the filtrate was dried in a rotary evaporator. The dry hydrolysate was redissolved in distilled water and refiltered on a Millipore filter (0.45 μ, 44 mm).

The solution was fractionated by preparative ion-exchange chromatography, an initial purification step as described (Tanzer et al., 1973). The hydrolysate, adjusted to pH 2, was applied to a column (2.5 × 60 cm) of Dowex 50-X8, 200–400 mesh, equilibrated with 0.25 *M* potassium formate (pH 9) and maintained at 50°C. After running 600 ml of the equilibrating buffer, elution was achieved using a linear gradient provided by 800 ml of the buffer and 800 ml of limiting buffer (0.75 *M* potassium acetate), in a constant-level device at a flow rate of 130 ml/hr. The effluent from the column was collected in 11-ml fractions and monitored for radioactivity using a toluene-based scintillation fluid containing Beckman Biosolv-3. The peak areas were pooled and titrated with 60% $HClO_4$ in the manner described above. The supernatant fluid was concentrated by rotary evaporation and applied to a Dowex 50-X8 column (H^+ form) and the radioactive compounds were eluted with 2 *M* NH_4OH. This solution was dried by rotary evaporation at 40°C, then the residue was dissolved in 0.1 *M* acetic acid and applied to a column (0.9 × 60 cm) of Bio-Gel P-2, 50–100 mesh, in 0.1 *M* acetic acid at room temperature. The effluent from the column was collected in 1.5-ml fractions and monitored for radioactivity, and pooled fractions were dried. The radioactive compounds obtained from gel filtration were dissolved in 0.01 *M* HCl and purified on a column (2.5 × 95 cm) of Bio-Rad Aminex A-5, which was maintained at 50°C and equilibrated with 0.2 *M* pyridine acetate (pH 3.5). The column was developed with a linear gradient between the equilibration buffer and 0.8 *M* pyridine acetate (pH 5.2) over a total volume of 1600 ml. Flow rate was maintained at 130 ml/hr, and the effluent from the column was collected in 10-ml fractions.

Amino Acid Analysis. Amino acid analyses of isolated radioactive compounds were carried out by using a Beckman Model 116 amino acid analyzer. Amino acid composition was determined after 3 *N* and 6 *N* HCl hydrolysis for 24 hr at 110°C. The NaB^3H_4-reduced cross-link compound was identified on the basis of its elution position as previously established (Tanzer et al., 1973).

Sequential Analysis from Amino Terminus. The sequence of amino acids from the amino terminal end of the isolated peptide was determined using the technique of sequential Edman degradation combined with the dansylation procedure of Gray (1967).

The hydrolyzed samples were dissolved in 60% pyridine and the dansyl-amino acids were separated with a system of four solvents (Hartley, 1970) on polyamide sheets (Cheng-Chin Trading Co. Ltd.) according to the method of Woods and Wang (1967). A standard mixture of 20 reference dansyl-amino acids was run simultaneously on the reverse side of the polyamide sheets. Prior to sequence studies of the peptide, purified NaB^3H_4-reduced dehydrodihydroxylysinonorleucine (dihydroxylysinonorleucine) was dansylated and its migration behavior was evaluated in the solvent systems. Separation was obtained by two-dimensional chromatography: (I) 1.5% formic acid in water, (II) benzene–acetic acid (9:1 v/v). Sometimes solvents III, ethyl acetate–acetic acid–methanol (20:1:1), and IV, 0.05 *M* Na_3PO_4 in 25% aqueous ethanol, were applied in the same direction after solvent II to obtain better migration of the slowly moving cross-link derivative.

Carboxyl Terminal Analysis. The hydrazinolysis method, as described by Akabori et al. (1952) and by Schroeder (1972), was used for carboxyl terminal analysis. The free carboxyl terminal amino acids were established on a Beckman Model 116 amino acid analyzer. The samples were treated with 0.5 ml of anhydrous hydrazine in sealed tubes at 80°C for 12 and 100 hr. After removing excess hydrazine in a vacuum desiccator over concentrated H_2SO_4, the residue was dissolved in 0.01 *N* HCl and immediately applied to the amino acid analyzer.

Results

Isolation of a Cross-Linked Tripeptide. The isolation of a cross-linked tripeptide whose amino acid sequence is described in detail below was accomplished by sequential ion-exchange and gel filtration chromatography. The initial step was to fractionate the alkaline hydrolysate of bone collagen using Dowex 50-X8, eluting with potassium formate and potassium acetate buffers (Tanzer et al., 1973). Four major peaks were detected (Figure 1) and pooled separately. Following titration with 60% $HClO_4$ at 4°C, centrifugation to remove $KClO_4$, and the removal of residual salts from the supernatant by ion-exchange displacement, peak C (Figure 1) was applied to a column of Bio-Gel P-2. In this fractionation, two major peaks were detected (Figure 2); the first to emerge was chromatographed on Bio-Gel Aminex A-5 resin and it separated into one major and several minor radioactive peaks (Figure 3). The major component appeared to be a single peptide as described below.

Amino Acid Composition. Amino acid analyses of the

Table I: Amino Acid Composition of Isolated Cross-Linked Peptide.

	μmoles (× 10^{-2})		Molar Ratios	
	a	*b*	*a*	*b*
Proline	0.51	2.88	1.0	1.2
Valine	0.51	2.88	1.0	1.2
DIOHLNL	0.52[c]	2.42[c]	1.0	1.0

[a] After hydrolysis in 3 *N* HCl for 24 hr. [b] After hydrolysis in 6 *N* HCl for 24 hr. Approximately six times as much peptide was hydrolyzed compared to *a*, accounting for the difference in μmoles × 10^{-2}. [c] Based on a molar color yield of DIOHLNL purified from calf bone collagen (see text for details).

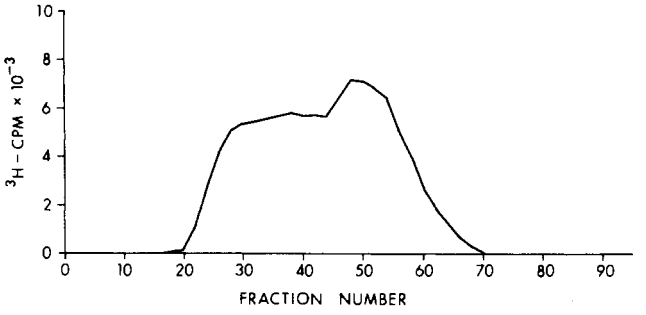

FIGURE 2: Gel filtration of peak C (Figure 1) on Bio-Gel P-2, 50–100 mesh (0.9 × 60 cm), in 0.1 *M* acetic acid.

isolated peptide were performed. The amino acid composition is shown in Table I. The NaB^3H_4-reduced cross-link which is present in this peptide was identified as dihydroxylysinonorleucine by ion-exchange chromatography under two different conditions (Tanzer et al., 1973). The cross-link content was determined by its actual color yield. This constant was obtained from 15 mg of dihydroxylysinonorleucine which had been isolated from calf bone collagen and found to be pure by nuclear magnetic resonance spectroscopy. Compared to a molar color yield of 53.8/nm for leucine the ninhydrin color yield for dihydroxylysinonorleucine was 72.6/nm. The intact, nonhydrolyzed peptide could be detected on the amino acid analyzer and neither it nor any ninhydrin reactive unknown peaks were present following each type of acid hydrolysis.

Sequence Results. Prior to the sequential analysis of this peptide, the migration behavior of dansyl-dihydroxylysinonorleucine (NaB^3H_4-reduced form of dehydrodihydroxylysinonorleucine) on polyamide thin layer was established. On two-dimensional chromatography, two bright spots were observed. These were cut out and eluted with 10% dodecyl sulfate and the eluates were monitored for radioactivity. Reference spots (blanks) were cut from other regions of the chromatogram. The brightest spot, containing 87% of the eluted radioactivity, remained in the vicinity of the origin (Figure 4). The second spot, containing some 13% of the radioactivity, moved appreciably in all four solvent systems. Additional chromatography using solvents III and IV sequentially showed that the major product barely migrated from the location shown in Figure 4. The dansyl-dihydroxylysinonorleucine, observed near the origin, is probably the derivative obtained from the dansylation reaction with only one α-amino group of the cross-link. The minor product is probably α-didansyl-dihydroxylysinonorleucine which was absent in the studies on the cross-linked peptide (see below).

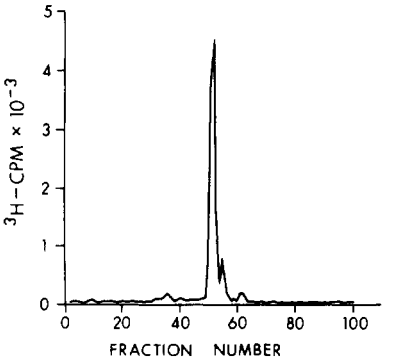

FIGURE 3: Ion-exchange chromatography of the material contained in fractions 20–44 (Figure 2) on Bio-Rad Aminex A-5 (2.5 × 95 cm). A linear gradient between 0.2 *M* pyridine acetate (pH 3.5) and 0.8 *M* pyridine acetate (pH 5.2) was applied (see text for details).

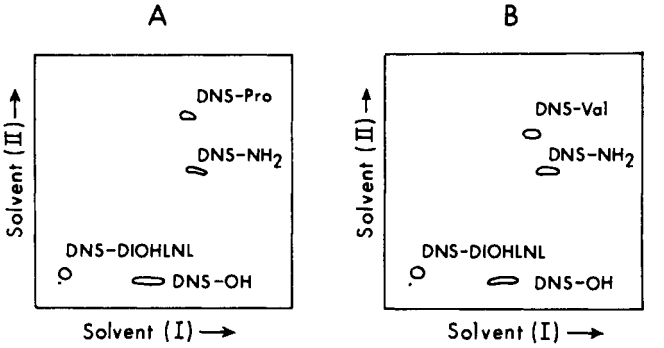

FIGURE 4: Thin-layer chromatography of the dansylated amino acids found in the intact peptide (A) and in the peptide after one cycle of Edman degradation (B).

After hydrolysis of the dansylated peptide, dansyl-proline and dansyl-dihydroxylysinonorleucine (the major product located near the origin) were detected (Figure 4A). After completion of the first Edman degradation, dansyl-valine and dansyl-dihydroxylysinonorleucine were detected (Figure 4B); no dansyl-amino acids were detected after a second and a third Edman degradation. The butyl acetate extractions following the completion of the Edman degradations were dried and treated with dansyl-Cl to verify that the remaining peptide had not been extracted at this point. All such dansylation reactions indicated no peptides were present.

Hydrazinolysis was carried out to determine the carboxyl terminal amino acid. Only valine was released from the peptide after treatment with anhydrous hydrazine at 80° for 12 and 100 hr. The quantity of valine increased significantly after heating for 100 hr.

The amino acid composition, the determination of amino and carboxyl termini, and the Edman degradations indicate that the total structure of this peptide is that shown in Figure 5.

Discussion

The tripeptide described in this report, prolyldihydroxylysinonorleucylvaline, was isolated from NaB^3H_4-reduced calf bone collagen after incomplete alkaline hydrolysis. Apparently the amino acid composition and the primary struc-

[1] Abbreviations used are: DNS or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DIOHLNL, dihydroxylysinonorleucine; BuAc, butyl acetate; PTC, phenylthiocarbamyl.

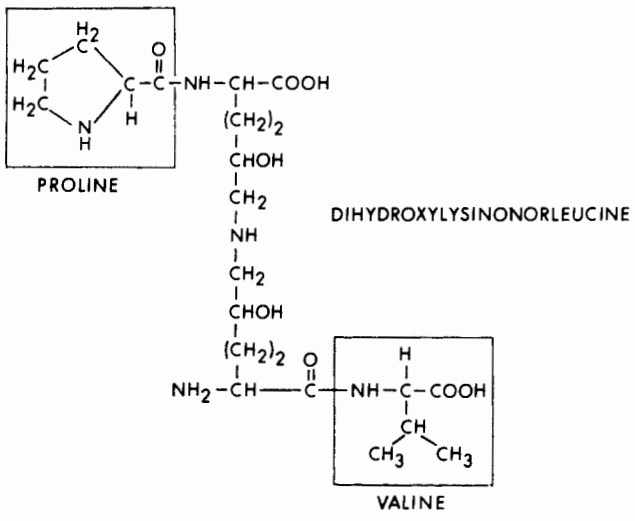

FIGURE 5: Primary structure postulated for the isolated cross-linked peptide.

ture of this peptide impose considerable resistance to hydrolysis under the conditions described. In this regard, a study on the effects of alkaline hydrolysis on bovine elastin showed that valylproline was one of the major products and that this peptide accounted for at least 3% of the total elastin (Cannon et al., 1967). Another consideration, in the present case, is that after mild hydrolysis of the cross-linked peptide in 2 *N* HCl for 2 hr at 110°C, glucose and galactose were detected by thin-layer chromatography on cellulose using the solvent system, ethyl acetate–pyridine–water (10:4:3) (unpublished results). Possibly, the presence of carbohydrate moieties in *O*-glycosydic linkage to a hydroxyl group of the cross-link may be a factor in the stability of the peptide to alkaline hydrolysis. The ready hydrolysis of the peptide in mineral acid, which releases the carbohydrate, would be consistent with this concept.

The content of dihydroxylysinonorleucine in this peptide was determined by its color yield after reaction with ninhydrin. The molar color yield of dihydroxylysinonorleucine has been previously assumed to be twice the molar color yield of leucine (Eyre and Glimcher, 1973a,b) as found for the other cross-links, lysinonorleucine (Franzblau et al., 1969), lysinoalanine (Bohak, 1964), and hydroxylysinonorleucine (DeLuque et al., 1970). The molar color yield of dihydroxylysinonorleucine indicates that the two NH_2 groups of this compound do not react with ninhydrin to yield two leucine equivalents. In fact, it is well known that both cystine and lanthionine, each containing two NH_2 groups, have a color yield approximately equimolar to leucine (Spackman et al., 1958; Zacharius and Talley, 1962). Amino acid analyses of the peptide were performed under two different conditions (Table I) to evaluate the effects of acid hydrolysis on the cross-link. No obvious peaks of break-down products, including the possibility of lactone formation from dihydroxylysinonorleucine (Tanzer et al., 1970) were present. Although slightly different contents of dihydroxylysinonorleucine were observed under these conditions, the molar ratios are supported by the studies of the primary structure of the peptide; the results indicated that there was one residue each of proline, valine, and dihydroxylysinonorleucine.

The composition of the peptide indicates that it is not related to the larger peptides, containing dihydroxylysinonorleucine, which have been isolated from collagenase digests

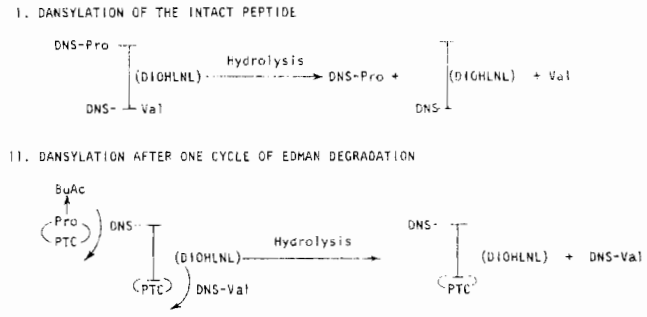

FIGURE 6: Interpretation of the results obtained from the dansyl-Edman method.

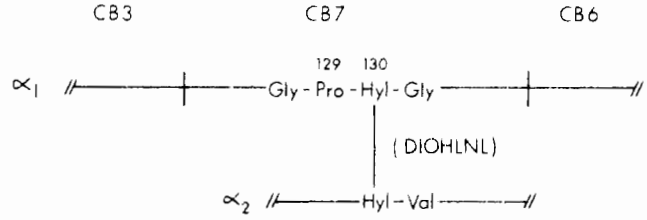

FIGURE 7: The hypothetical location of the intermolecular cross-link, dihydroxylysinonorleucine, between $\alpha 1$ and $\alpha 2$ chains of bone collagen. The numbers (129, 130) show the amino acid residues from the N-terminus of $\alpha 1$-CB7.

of calf and chicken bone collagen (Eyre and Glimcher, 1973a,b). Thus, it would represent another site of intermolecular cross-link formation in bone collagen (see below). It is noteworthy that this peptide seems to contain a substantial proportion of the radioactivity in the original hydrolysate, as shown by its abundance in Figures 1–3.

The postulated primary structure of the tripeptide was mainly derived from sequence studies using the dansyl-Edman method. The interpretation of the results is shown schematically in Figure 6, which illustrates the basis for placing the proline and valine residues on opposite sides of the cross-link and in a "trans" configuration with relation to the secondary amine. Thus, dansylation of the intact peptide, followed by acid hydrolysis, released dansyl-proline and dansyl-dihydroxylysinonorleucine (Figure 6,I). Following one cycle of Edman degradation, the new amino termini were detected as dansyl-valine and dansyl-dihydroxylysinonorleucine (Figure 6,II); a second cycle of Edman degradation showed no detectable amino acids. If valine were at the COOH group of the cross-link opposite to the one shown in Figure 6, it would have appeared as a new amino terminus following the second Edman degradation, not after the first one.[2] The location of valine as the carboxyl terminal amino acid was independently established by hydrazinolysis. Thus, all of the data are consistent with regard to the tripeptide.

Recent studies on calf and rat skin collagen have elucidated the entire amino acid sequence of the α_1 chains (Gallop et al., 1972; Hulmes et al., 1973), while the amino acid sequence of the α_2 chain has not been fully elucidated except for α_2-CB1, α_2-CB2, and small amino terminal parts of the other large cyanogen bromide peptides (Gallop et al., 1972; Fietzek et al., 1974). Examination of the available amino acid sequences of these α_1 and α_2 chains shows that

[2] Figure 6 has been simplified for clarity by omitting the reaction of the secondary amine of the cross-link with dansyl-Cl or phenyl isothiocyanate.

there is only one sequence of "proline-hydroxylysine" which potentially forms one side of the cross-linked tripeptide. The location of this sequence is almost in the middle of α_1-CB7 of calf skin collagen (Fietzek et al., 1973) and is the 129 and 130th residue from the amino terminus of this cyanogen bromide peptide (Figure 7). This location is somewhat unusual because a number of investigations of cross-link location in collagen have suggested that it is each end of the α chains which are primarily involved in the formation of intermolecular cross-links (Gallop et al., 1972). Moreover, the other side of the cross-linked peptide, "hydroxylysine-valine", does not appear in the α1 sequence. Conceivably, it may be in the α2 chain or may be a sequence unique to bone α1 and α2 chains. We have provisionally postulated that the cross-link joins the interior of α1-CB7 with an interior section of the α2 chain (Figure 7). Further evidence on this point will necessitate the isolation of larger cross-linked peptides.

Acknowledgments

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