ANALYSIS OF A CROSSLINKED PEPTIDE FROM CALF BONE COLLAGEN: EVIDENCE THAT HYDROXYLYSYL GLYCOSIDE PARTICIPATES IN THE CROSSLINK

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SUMMARY A crosslinked, double-chained peptide has been isolated from calf bone collagen after digestion with crude bacterial collagenase. Initially, the ^{3}H-labelled peptide was isolated from collagen that had been treated with [^{3}H]-$NaBH_4$, but an almost identical peptide was also isolated from collagen without prior reduction. After periodate oxidation of the reduced peptide the two component chains were resolved by further chromatography. Amino acid compositions showed that the peptide probably derived from an intermolecular crosslink between a carboxyterminal sequence of the collagen molecule and a sequence near the aminoterminus that previously has been shown to be the site of a glycosylated hydroxylysine residue. The crosslinking compound in the reduced peptide, hydroxylysinohydroxynorleucine, appeared to have derived mainly by reduction with borohydride of hydroxylysinooxonorleucine, the keto-amine rearranged form of the dehydro crosslink. The remaining hydroxyl group of the crosslink, the one not derived by reduction of the keto group, appeared to be glycosylated.

The major crosslinking compounds that have been detected in bone collagen are the aldimines, dehydro-hydroxylysinohydroxynorleucine (dehydro-HylOHNle) and dehydro-hydroxylysinonorleucine (dehydro-HylNle)(1,2). These compounds appear to be stabilized *in vivo* during tissue maturation by unknown reactions (3), although evidence has been presented that suggests that their reduction to the secondary amines, HylOHNle and HylNle, occurs to a significant extent in bone collagen (1).

Reduction of aldimines to secondary amines should stabilize these crosslinking bonds and, therefore, increase the strength of the collagen fibrils. However, the major reducible compound in bone, dehydro-HylOHNle, is unusually stable for an aldimine (2,4; Eyre and Glimcher, unpublished data), and it has been suggested by R. Fairweather that the initial aldimine may rearrange to a more stable keto-amine structure (5,6) by an Amadori type of rearrangement.

Data is not available on the precise location of intermolecular crosslinks in bone collagen. However, similar to the situation in soft tissue collagens, aldehydes that participate in crosslink formation are concentrated mainly at

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the ends of the α chains (7), and the absence of the carboxyterminal peptide, α1CB6B, from CNBr-digests of insoluble chicken bone collagen suggests that this region of the molecule is a major site of crosslinking (8). Recently a crosslinked peptide α1CB1-α1CB6 was isolated from collagen of rat tail tendon (9). This peptide derived from an intermolecular crosslink between the aminoterminus of an α1 chain and the carboxyterminus of another α1 chain. In fact, innumerable findings (10,11,12) support the concept that the ends of the collagen molecules are crucial sites of intermolecular crosslinks in most tissues, including bone.

The present paper reports the isolation and analysis of crosslinked peptides from bone collagen after digestion with bacterial collagenase. The results provide new knowledge on the structure, stability and site of the reducible intermolecular crosslinks in bone collagen.

MATERIALS AND METHODS

Demineralized, powdered bone collagen was prepared from foetal bovine tibiae and femora (5 to 6 months), by the method previously described (13). It was treated with [^{3}H]-$NaBH_4$ (1 Ci/M) as a suspension in 0.1M sodium phosphate, pH 7.4, using 3 mg $NaBH_4$ per 100 mg collagen (14), and digested with bacterial collagenase (Cl. histolyticum, Schwarz/Mann, crude) as previously described (13).

After concentrating the crosslinked peptides by molecular sieve chromatography on columns of Bio-Gel P-2 and P-10 (Figures 1 and 2), they were fractionated by column chromatography on phosphocellulose (Figure 3), and purified on Bio-Gel P-6 (150 cm x 1.5 cm) in either 0.05M NH_4HCO_3 or 0.1M acetic acid. For periodate treatment peptides were reacted in 0.02M acetic acid with 0.02M sodium metaperiodate at room temperature in the dark for 3 hours (15). Excess periodate was destroyed by addition of one drop of ethylene glycol, and reaction products were reduced at pH 7.4 in phosphate buffer by addition of sodium borohydride (4).

Amino acid analyses were performed on an automatic instrument equipped for stream splitting. Using the split-stream device, tritium activity could be continuously monitored by a scintillation counter with a specially constructed flow-coil (Intertechnique, model SL 20), and mixing the column effluent with scintillation fluid (Aquasol, New England Nuclear Corp., Boston, Mass.) (Eyre, to be published). The crosslinking compounds were identified on the basis of their elution positions as previously described (16).

RESULTS

Initially, crosslinked peptides were concentrated by elution of the collagenase digest of 1 g bone collagen from the Bio-Gel P-2 column (Figure 1)

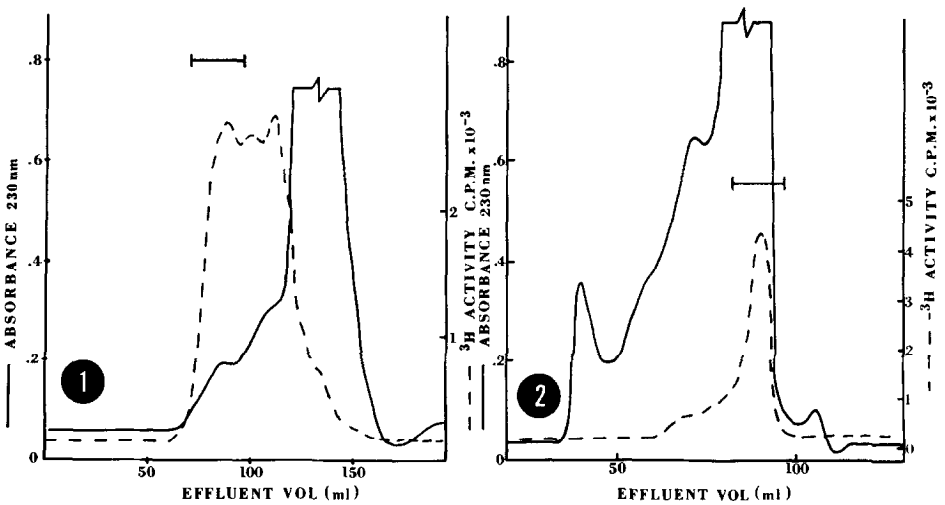


Figure 1 Gel filtration of a collagenase-digest of [^{3}H]-$NaBH_4$-treated calf bone collagen on Bio-Gel P-2, 100-200 mesh (2.5 cm x 30 cm) in 0.1M acetic acid.

Figure 2 Gel filtration of material from the Bio-Gel P-2 column (the region of effluent marked with a bar in Figure 1) on Bio-Gel P-10, 200-400 mesh (1.5 cm x 90 cm) in 0.1M acetic acid.

with further purification on a Bio-Gel P-10 column (Figure 2). By pooling the regions of effluent indicated, approximately one third of the total tritium activity that was introduced into the collagen by [^{3}H]-$NaBH_4$ was recovered in peptides representing 2% of the collagen. The crosslinked peptides were then fractionated by ion-exchange chromatography on a column of phosphocellulose (Figure 3). A major peak of tritium activity eluted towards the end of the salt gradient. The crosslinked peptide in this peak was purified by elution from Bio-Gel P-6 (Figure 4), and its elution position suggested a molecular size of about 2,000 to 3,000 daltons. Amino acid analysis established the size at 22 residues (Table I), with one residue of the reduced crosslink, HylOHNle, per molecule. All the tritium activity of the peptide eluted with the ninhydrin-positive peak of HylOHNle. After treatment of the peptide with periodate, and elution from the Bio-Gel P-6 column in 0.05M NH_4HCO_3, the tritium activity eluted a few fractions later than prior to oxidation (Figure 4). In two experiments, with separate preparations of peptide, 62% and 78% of the tritium activity in the peptide was recovered in the slightly retarded peak after periodate oxidation. Amino acid analysis of this peak (Table I) showed $(Gly)_2$, Met,His,Arg had been lost from the original peptide. Also, the crosslink was no longer detected, and the tritium activity was recovered as two peaks where α-amino δ-hydroxyl valeric acid (hydroxynorvaline) and its derivative on hydrolysis,

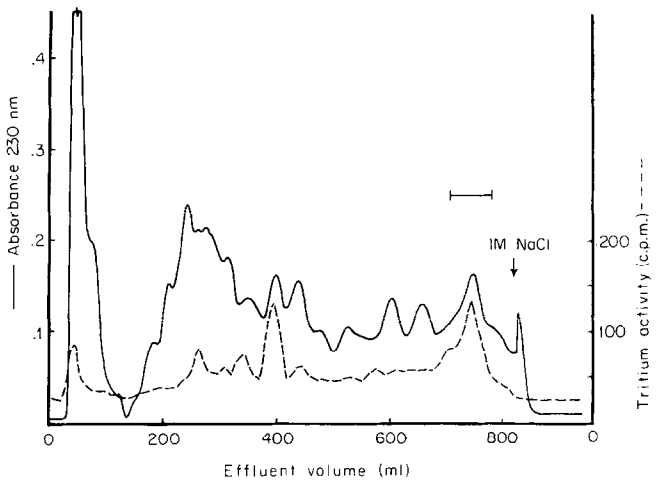


Figure 3 Chromatography of peptides recovered from the Bio-Gel P-10 column (Figure 2) on phosphocellulose (Whatman, P-11; 2.0 cm x 15 cm) at 42°. A linear gradient of 0 to 0.6M NaCl in 1mM Na acetate, pH 3.6, was applied in 800 ml total volume.

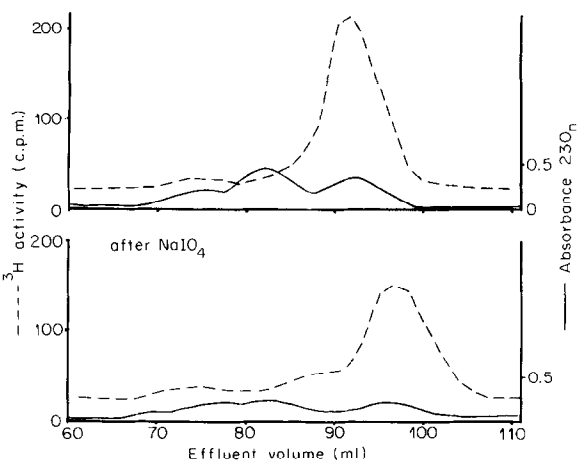


Figure 4 Molecular sieve chromatography on Bio-Gel P-6, 200-400 mesh (1.5 cm x 150 cm) in 0.05M NH_4HCO_3 of a sample of the crosslinked peptide indicated by a bar in the phosphocellulose chromatogram (Figure 3). The lower chromatogram is of a similar sample after treatment with $NaIO_4$ and $NaBH_4$.

chlornorvaline, elute (Figure 5). Although no other peaks of tritium activity, or UV-absorbing peptide, were detected in the P-6 chromatogram, amino acid analysis of fractions eluting later than the tritiated peptide revealed traces of the peptide $(Gly)_2$,Met,Hyl,His,Arg. In further experiments, periodate digests of the peptide were eluted from the Bio-Gel P-6 column in 0.1M acetic acid rather than in 0.05M NH_4HCO_3. The peak of radioactivity now eluted later, and

as a broader peak. Analysis of the main peak revealed the same peptide as before (Table I), but the trailing shoulder, and subsequent few fractions of this peak, now contained an equimolar amount of the missing peptide $(Gly)_2$,Met,Hyl,His,Arg. These results established that the two chains in the crosslinked peptide had the

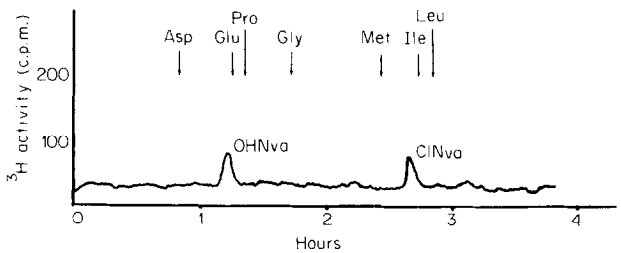


Figure 5 Elution profile of tritium activity on amino acid analysis after reduction with non-radioactive $NaBH_4$ and acid hydrolysis of the main peptide chain released from the reduced crosslinked peptide by $NaIO_4$ oxidation (Figure 4). OHNva is hydroxynorvaline and ClNva is chlornorvaline.

compositions shown in Table I.

The recovery of hydroxylysine in one chain of the crosslinked peptide after periodate oxidation and acid hydrolysis suggested that this hydroxylysine residue, derived by periodate oxidation of the crosslink, was derivatized through its hydroxyl group, thereby preventing attack by periodate. This idea was supported by the failure to detect HylOHNle on amino acid analysis of the peptide after hydrolysis in 2M KOH, conditions under which glycosidic attachments are stable. Instead, a peak of equivalent color yield and tritium activity to the HylOHNle now eluted earlier, just before ammonia; in some preparations of peptide two new peaks were observed, the one eluting before ammonia plus a smaller one coinciding with tyrosine. Peaks of tritium activity in similar positions were also observed on analyses of base hydrolysates of reduced bone collagen. Acid hydrolysis after the base hydrolysis eliminated both these peaks and HylOHNle was fully recovered. Even relatively mild conditions (4 hours in 2N HCl at 105°C) were sufficient for this conversion. Analysis for hexoses by t.l.c. on cellulose detected both glucose and galactose in a mild acid hydrolysate of the crosslinked peptide. These findings indicated that the HylOHNle in the peptide was probably glycosylated.

DISCUSSION

The composition of the component chains in the crosslinked peptide from bone collagen are highly characteristic and can be allocated to particular regions of the collagen molecule. Thus, $(Gly)_2$,Met,Hyl,His,Arg almost certainly is the

TABLE 1

AMINO ACID COMPOSITIONS OF THE CROSSLINKED PEPTIDE AND ITS COMPONENT CHAINS

	Crosslinked Peptide	Chains Released by Periodate Oxidation			Carboxyterminal Sequence of α1 Chain from Calf Skin Collagen	
		Chain A*	Chain A†	Chain B†	Last 20 Residues (Ref.20)	Last 18 Residues (Ref.21)
Asp	1 (1.3)	1 (1.3)	1 (1.3)	-	2	1
Ser	-	-	-	-	1	-
OHNva[Ψ]	-	1 (0.7)	1 (0.7)	-	-	-
Glu	3 (3.2)	3 (3.0)	3 (3.5)	-	3	4
Pro	3 (2.8)	3 (2.7)	3 (3.3)	-	3	3
Gly	5 (5.2)	3 (3.0)	3 (3.2)	2 (2.3)	3	2
Ala	1 (1.3)	1 (1.1)	1 (1.3)	-	1	1
Met	1 (0.8)	-	-	1 (1.0)	-	-
Leu	1 (1.1)	1 (0.9)	1 (0.9)	-	2	1
Tyr	0.4§	0.3§	0.2§	-	1	2
Phe	1 (1.1)	1 (0.8)	1 (0.7)	-	1	1
HylOHNleζ	2 (2.2)	-	-	-	-	-
Hyl	-	-	-	1 (0.9)	-	-
Lys	-	-	-	-	1φ	1φ
His	2 (1.9)	1 (1.0)	1 (1.0)	1 (0.9)	1	1
Arg	2 (2.2)	1 (1.2)	1 (1.3)	1 (1.0)	1	1
TOTAL	22	16	16	6	20	18

* Isolated by elution from Bio-Gel P-6 in 0.05M NH_4HCO_3.

† Isolated by elution from Bio-Gel P-6 in 0.1M acetic acid. The two compositions were corrected for about 25% cross-contamination.

Ψ Includes chlornorvaline.

§ The values for tyrosine ranged from 0.2-1.6 res/molecule in various preparations of the peptides.

ζ Leucine equivalents.

φ Present mainly as a lysine aldehyde.

sequence Gly-Met-Hyl-Gly-His-Arg, with the hydroxylysine participating in the crosslink. This hexapeptide had previously been isolated from collagenase and trypsin digests of collagen from guinea pig skin, carp swim bladder and human skin (17,18), where it was a major source of the hydroxylysyl glycoside content of the collagen. More recently, this glycosylated peptide was found in the α1 chain of collagen from rat skin (19) at the junction of the peptides α1CB4 and α1CB5. In a quarter-stagger model for the packing of collagen molecules in a fibril, this region of the α1 chain would be exactly adjacent to the carboxy-terminus of a neighboring molecule (11). Interestingly, the amino acid composition of the other chain of the crosslinked peptide isolated in the present study is remarkably similar to that of the extra-helical carboxyterminal sequence of the α1 chain of calf skin collagen, which includes a major site of lysine aldehyde (20,21). This sequence is included in the following hypothetical structure for the crosslinked peptide, with hydroxylysine replacing lysine.

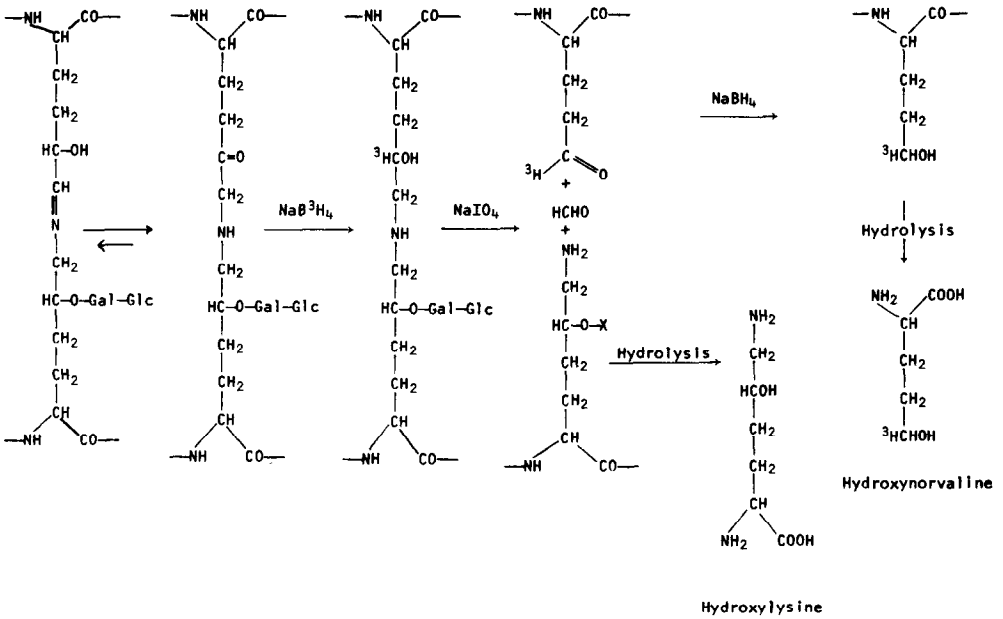
α1 • • —Phe-Leu-Pro-GlN-Pro-Pro-GlN-GlN-Glx-Hyl-Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr
| Intermolecular Crosslink
α1 • • —Gly-Met-Hyl-Gly-His-Arg— • •

Furthermore, in the present study, different preparations of the crosslinked peptide had variable contents of tyrosine, that ranged from 0.2 to 1.6 residues per peptide. This could be expected if the peptide was derived from the carboxy-terminus of the α1 chain, which apparently ends in -Tyr-Tyr-COOH in both calf skin (21) and chicken bone collagens (22). The ease with which these terminal tyrosine residues can be lost by proteolysis in non-denaturing solvents during preparation of the collagens has been established (21).

The data suggests therefore that the crosslinked peptide derives from linkage of an hydroxyallysine aldehyde near the carboxyterminus of an α1 chain to the ε-amino group of an hydroxylysine in a sequence at the junction of peptides α1CB4 and α1CB5 in another α1 chain. Although the data is not conclusive that this is the site of origin, the amino acid compositions of the peptide chains are so characteristic, being abundant in several of the amino acids that are rare constituents of collagen, such as histidine, methionine, tyrosine, phenylalanine and hydroxylysine, that they cannot be assigned to any other sites on the known structures of α1 chains from cow skin collagen. It is possible that they derive from regions of an α2 chain, for which sequence data is not available. Indeed the α2 chain may contain homologous sequences in similar locations to those in the α1 chain.

Analyses of the peptide chains after cleavage of the crosslink with periodate indicate that most of the crosslink HylOHNle in this particular peptide was produced in the demineralized tissue by borohydride reduction of hydroxy-lysinooxonorleucine, the keto-amine form of dehydro-HylOHNle (see Scheme). Such a rearranged form should be a more stable crosslink than the aldimine, and would explain the unusual thermal and acid stability of this reducible crosslink as previously proposed by R. Fairweather (5, 6). Indeed, in the present study the crosslinked peptides could be isolated in similar yields without prior reduction of the collagen. After isolation, the peptides could be treated with $NaBH_4$ and the reduced crosslink, HylOHNle, was recovered as before. A significant amount of naturally reduced crosslink was not detected (Eyre, in preparation).

The recovery of hydroxylysine from the crosslink after periodate oxidation and hydrolysis, indicates that the remaining hydroxyl group in the crosslink was derivatized. The results of analysis after base hydrolysis, the detection of glucose and galactose in the peptide, and the apparent origin of one of the peptide chains at a major site of hydroxylysyl glycoside in collagen, lead us to conclude



that a glycosylated hydroxylysine residue had participated directly in the biosynthesis of this crosslink. Previously, it had been suggested that glycosylated hydroxylysine residues might be important in collagen crosslinking (17), although there was a tendency to think of them as inhibitors rather than as direct participants (4,23).

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