Collagen Crosslinks: Direct Quantitative Determination of Stable Structural Crosslinks In Bone and Dentin Collagens

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Received August 5,1974

Summary: Direct determination of the stable keto-imine forms of [^{3}H]$NaBH_4$-reducible precursors of dihydroxylysinonorleucine [$(OH)_2$Lys-norLeu] and hydroxylysinonorleucine [(OH)Lys-norLeu] in bone and dentin collagen are described. Acidic thermal conditions were used to break labile imminium bonds and acetylate the resulting amino groups to prevent reformation of the original cross-links under the conditions used for $NaBH_4$-reduction.

The intractability of the hard tissue collagens may be in part related to the fact that the crosslinks dihydroxylysinonorleucine [$(OH)_2$Lys-norLeu] and hydroxylysinonorleucine (OHLys-norLeu) are partially reduced in vivo (1). In addition to in vivo reduction, the stable keto-imine forms of hydroxy-imminium compounds exist. Mechanic (4) has recently demonstrated direct evidence for 5-keto-5'-OHLys-norLeu and 5-keto-lys-norLeu in bone collagen. Previously the keto-imine structure was proposed by Eyre and Glimcher (2), on the basis of indirect evidence using periodate cleavage on a $(OH)_2$Lys-norLeu peptide isolated from $NaBH_4$-reduced bone collagen. They suggested that it arose via an Amadori rearrangement of the hydroxy-imminium compound, Δ^6-dehydro-5,5'-$(OH)_2$Lys-norLeu. Other indirect evidence has subsequently been reported for other collagens (3, 5, 6).

Robins and Bailey (5) have attempted to quantitatively determine, by indirect means, the amount of the keto-imine form of Δ^6deH-5,5'-$(OH)_2$Lys-norLeu in borodeuteride-reduced and borotritiide-reduced bone collagen. They analyzed the proline from the [^{1}H]KBH_4-reduced reaction products of periodate-cleaved, isolated $(OH)_2$Lys-norLeu, and reported that 46% of the proline was recovered as the deuterated

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derivative from [^{2}H]-$(OH)_2$Lys-norLeu and 86% of the radioactivity in [^{3}H]-$(OH)_2$Lys-norLeu was recovered as [^{3}H] proline and [^{3}H]hydroxynorvaline (5). These results indicate that the minimum amounts of 5-keto-5'-OHLys-norLeu are 92% and 86% respectively, because periodate cleavage of $(OH)_2$Lys-norLeu theoretically yields 2 equivalents of proline (6, 12, 13). In contrast, Davis (6), using the identical degradative methodology on [^{3}H]-$(OH)_2$Lys-norLeu from bovine bone and dentin collagen, reports that only 27-34% and 17-25% of the keto-imine form of Δ^6deH-5,5'-$(OH)_2$Lys-norLeu, exists in the two collagen tissues respectively.

In order to quantitate directly the amounts of 5-keto-5' (OH)Lys-norLeu and 5-keto-Lys-norLeu, we have used acidic thermal conditions to break labile imminium bonds and acetic anhydride to acetylate the resulting new amino groups to prevent reformation of Schiff base under the conditions used for $NaBH_4$ reduction of collagen (see Fig. 1). Isolation and quantitation of $(OH)_2$Lys-norLeu and OHLys-norLeu after reduction with standardized [^{3}H]$NaBH_4$ would yield the amounts of 5-keto-5' (OH)Lys-norLeu and 5-keto-Lys-norLeu. This procedure was deemed feasible because Brown and Beauregard (7) reported that quantitative acetylation of a protein occurs when it is treated for 3 hours at 80° in 16% (v/v) acetic anhydride in acetic acid solution. Although less stringent conditions are available for the quantitative acetylation of collagen (8) we chose the former to ensure both breakage of the labile imminium bond and subsequent acetylation of the resulting ε-amino group (See Fig. 1).

We report here the direct quantitation of significant amounts of the stable keto-imine forms of both Δ^6deH-5,5'-$(OH)_2$Lys-norLeu and Δ^6deH-5-OHLys-norLeu in bovine bone and dentin collagen.

Methods and Materials

The collagen used was bovine dentin and bone obtained from a 2.5 year old animal (Holstein, Japan) and was prepared as previously described (1, 15). The [^{3}H]$NaBH_4$ was standardized by the method of Paz, et al, (9) using p-nitrobenzoyl amidobutanal. The standardized [^{3}H]$NaBH_4$ had a specific activity of 4.2×10^7 CPM/micromole.

The experimental collagens were treated as described (7). After removal of the acetylating solution the collagens as well as the controls were reduced with

the standardized [^{3}H]$NaBH_4$ (1, 15) and hydrolyzed with 3N tosyl acid (10). Aliquots were chromatographed for separation and quantitative isolation of $(OH)_2$Lys-norLeu and OHLys-norLeu (1). The appropriate peaks were pooled and the total amount of radioactivity in each was assessed. Hydroxyproline was determined on aliquots of the hydrolyzate to determine the amount of collagen used for the separation of the cross-links. An automatic amino acid analyzer (Jeol JLC-6AH) was used.

To evaluate the procedures, a pure sample of [^{3}H]$(OH)_2$Lys-norLeu was hydrolyzed and chromatographed in an identical manner, as was [^{3}H] $(OH)_2$Lys-norLeu not subjected to hydrolysis. The recoveries were complete.

Results

It was assumed that bone and dentin contain 14% hydroxyproline and this was reconfirmed by the amino acid analyses (Table 1). A molecular weight of 290,000 was

TABLE I

DIRECT DETERMINATION OF KETO-IMINES IN BONE AND DENTIN COLLAGEN

	Dentin (Control)	Dentin (Acetylated)	Bone (Control)	Bone (Acetylated)
Hypro on Crosslink Column				
mg	1.24	1.15	1.37	0.81
micromoles	9.44	8.78	10.43	6.22
Collagen on Column				
mg	8.86	8.21	9.79	5.79
micromoles	0.031	0.028	0.034	0.020
Total $(OH)_2$Lys-norLeu (CPM)	365,000	226,000	246,000	119,000
micromoles	0.0087	0.0054	0.0059	0.0028
Moles of $(OH)_2$Lys-norLeu / Mole of Collagen	0.28	0.19	0.17	0.14
Moles of OHLys-norLeu / Mole of Collagen	0.061	0.054	0.024	0.026
% Keto-imine $(OH)_2$Lys-norLeu	67.9		82.4	
% Keto-imine OHLys-norLeu	88.5		100	

$(OH)_2$Lys-norLeu - dihyroxylysinonorleucine
OHLys-norLeu - hydroxylysinonorleucine
Keto-imine $(OH)_2$Lys-norLeu - 5-keto-hydroxylysinonorleucine
Keto-imine OHLys-norLeu - 5-keto-lysinonorleucine
Specific Activity of Standardized sodium borohydride= 4.2×10^7 CPM/micromole

assumed for the collagen molecule in order to calculate molar quantities. See Table 1 for the results. It is interesting to note that all of the reducible precursor of OHLys-norLeu in bone is stable to thermal acidic conditions.

Discussion

Davis (6) has reported that only 27-34% and 17-25% of 5-keto-5'-$(OH)_2$Lys-norLeu exists in bone and dentin respectively after using a correction factor of 1.33. This correction factor was determined from earlier experiments by Davis and Bailey (12, 13). The discrepencies between the results reported here and those of Davis (6) cannot be easily accounted for. Perhaps tissues of different age were used. The direct method described in this paper may be more valid than, the indirect periodate cleavage degradative methods (2, 3, 5, 6, 12,13). It is well known that periodate may destroy amino compounds and other organic substances (14).

In the introduction it was concluded, from the values reported by Robins and Bailey (5), that the minimum amount of 5-keto-5'-OHLys-norLeu present in borodeuteride-reduced bone collagen is 92%. However they did not employ the correction factor of 1.33, determined by Davis and Bailey (12, 13), although they use identical procedures, Davis (6) points out that this correction factor is necessary. Application of the correction factor to the data of Robins and Bailey (5) yields the impossible value of 123% for 5-keto-5'-OHLys-norLeu.

Table 1 indicates that much of the OHLys-norLeu is present as a stable $NaBH_4$-reducible precursor. Direct evidence for the existence of 5-keto-Lys-norLeu in bovine bone collagen had been reported earlier (4). It has been noted that much, if not all, of the [^{3}H]$NaBH_4$-reducible precursor of OHLys-norLeu is labile to acid or heat (11, 16) in some soft tissue collagens. The same phenomena has also been noted with the [^{3}H]$NaBH_4$-reducible precursor of $(OH)_2$Lys-norLeu using chromatographic techniques that separate aldol-histidine (17) from $(OH)_2$Lys-norLeu. Davis (6) has suggested (See ref. 6, Fig. 1) that Δ^6deH-5,5'-$(OH)_2$Lys-norLeu is the only imminium compound that is capable of forming the keto-imine, because it has a hydroxyl bond conjugated with an imminium double bond, but he has overlooked the possible reaction shown in Fig. 2 to yield a compound with a hydroxyl bond conjugated with the imminium

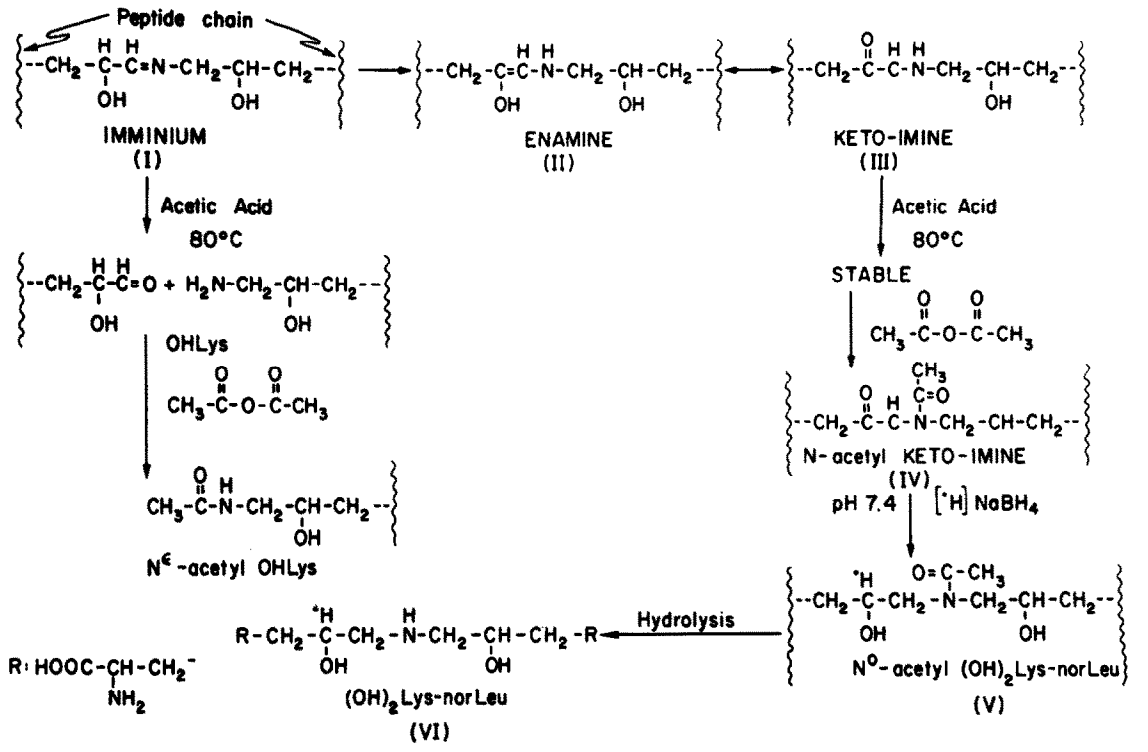

FIGURE 1

Figure 1: Compounds (I) and (III) are both $NaBH_4$-reducible precursors of dihydroxylysinonorleucine. The imminium compound (I) forms from α-amino-δ-hydroxyadipic-δ-semialdehyde and hydroxylysine providing a structure with a hydroxyl bond conjugated with an imminium double bond. This can then form the enamine (II) which readily tautermerizes to the keto-imine (III). When (I) and (III) are treated with 16% (v/v) acetic anhydride in acetic acid at 80° for 3 hours, (I) will break yielding its precursors while (III) will be stable. The hydroxylysine from (I) will be acetylated preventing reformation of (I) while (III) will be acetylated giving (IV). Upon reduction with $[^*H]NaBH_4$, (IV) will yield N^O-acetyl $(OH)_2$Lys-norLeu (V) which on hydrolysis yields $[^*H](OH)_2$Lys-norLeu (VI).

double bone. This reaction is quite feasible because of the larger amounts of α-amino-δ-hydroxy-adipic-δ-semialdehyde than α-aminoadipic-δ-semialdehyde in hard tissues (1). The results reported here indicate that the formation of the major portion of Δ^6-deH-5-OHLys-norLeu in dentin collagen and its total formation in bone collagen occurs via condensation of α-amino-δ-hydroxy-adipic-δ-semialdehyde and lysine (Fig. 2).

The crosslink chemistry of the various collagenous tissues in the body have been shown to differ, leading to the suggestion that each physiologically differing support tissue is under its own fine biochemical control (20) and may be altered in

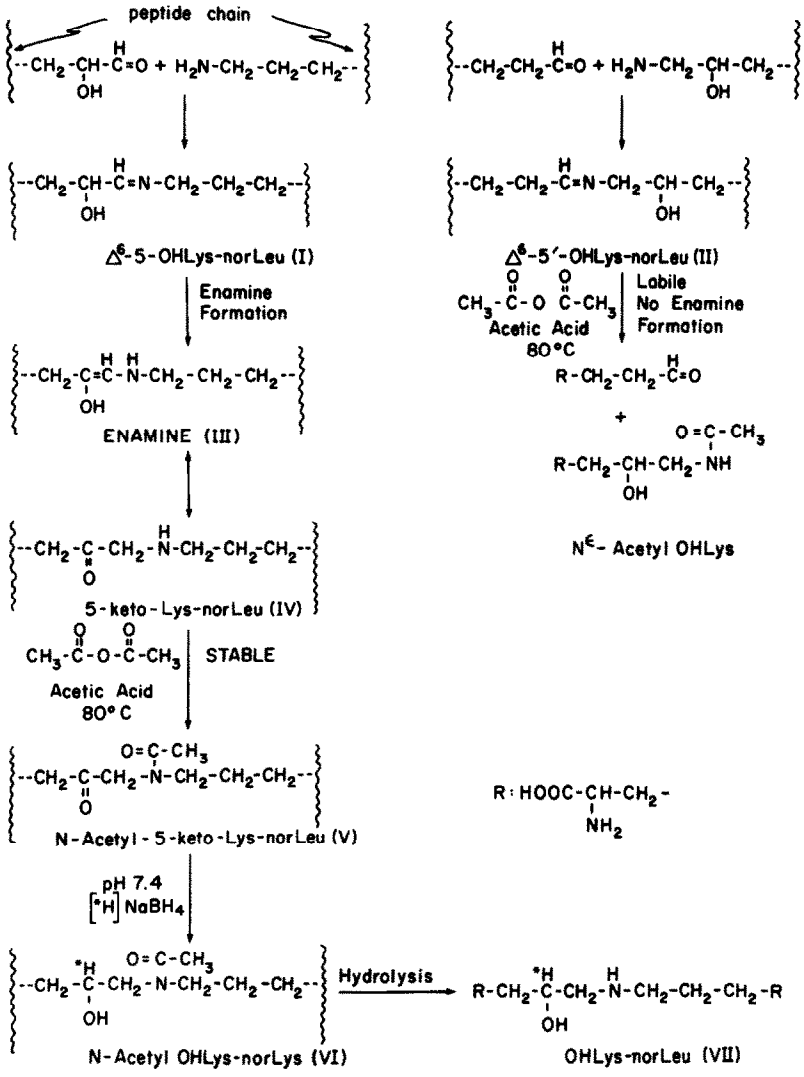

FIGURE 2

Figure 2: Two alternative methods exist for the formation of $NaBH_4$-reducible precursors of hydroxylysinonorleucine. On the left is the formation of Δ^6-dehydro-5-OHLys-norLeu (I) from α-amino-δ-hydroxyadipic-δ-semialdehyde and lysine yielding a compound with a hydroxyl bond conjugated with an imminium double bond. This compound can form the enamine (III) which can tautermerize to the keto-imine (IV). On the right is the formation of Δ^6-dehydro-5'-OHLys-norLeu (II) from α-amino-adipic-δ-semialdehyde and hydroxylysine. The latter does not contain a hydroxyl bond conjugated with an imminium double bond and therefore cannot form the enamine (III). (I) and (II) are labile to acidic thermal conditons while (IV) is not. If any imminum compound exists it will break by the treatment (see legend figure 1) and the resulting amino groups will be acetylated preventing any Schiff base reformation. Compound (IV) will yield (V) which will be reduced to (VI) and on hydrolysis will yield (VII).

pathological conditions (21). This biochemical control is probably due to extracellular post-translational phenomena. In addition it has been clearly demonstrated that although [^{3}H]$NaBH_4$-reduced, mature bovine tendon and dentin collagen, contain $(OH)_2$Lys-norLeu as their most abundant crosslink, its distributions in the two are different (19), although both consist of $[\alpha 1(I)]_2\alpha 2$ type collagens. Davis (6) has concluded that formation of the enamine and tautermerization to the keto-imine cannot explain the stability of the imminium compound, although it contains the necessary configuration, (hydroxyl bond conjugated with imminium double bond), because 95% of the $NaBH_4$-reducible precursor of $(OH)_2$Lys-norLeu exists in calf tendon collagen as Δ^6-deH-5,5'-$(OH)_2$Lys-norLeu. We do not agree with his hypothetical cyclic stable intermediate which is dependent upon hydrogen bonding, this would be disrupted very readily under thermal conditions. We suggest that among the post-translational phenomena influencing organization, packing and matrix structure of collagen there is an enzyme present in hard tissues that can catalyze the transformation of imminium to enamine so that ready tautermerization to the lower energy form, the keto-imine, can occur thereby forming a stable bond. It is suggested that this enzyme be called hydroxyenaminase and that it is important to the crosslink structure-function relationships of hard tissue collagens.

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Acknowledgements: This work was supported in part by NIH Grant #DE-02668 and Grant #RR-05333.