COLLAGEN CROSSLINKS: DIRECT EVIDENCE OF A REDUCIBLE STABLE FORM OF THE SCHIFF BASE Δ^6DEHYDRO-5,5'-DIHYDROXYLYSINONORLEUCINE AS 5-KETO-5'-HYDROXYLYSINONORLEUCINE IN BONE COLLAGEN.

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Received December 13,1973

Summary

Direct evidence is presented for the existence of 5-keto-5' hydroxylysinonorleucine and 5-keto-lysinonorleucine in bone collagen. The latter two are the keto-imino forms of the Schiff bases Δ^6-dehydro-5,5'-dihydroxylysinonorleucine and Δ^6-dehydro-5-hydroxylysinonorleucine respectively. The keto-imino forms of the crosslinks were reduced in acid solution with the selective reducing agent NaB^3H_3CN to dihydroxylysinonorleucine and hydroxylysinonorleucine.

The integrity and stability of the fibrillar macromolecular matrix of collagenous tissues depend on covalent crosslinks between collagen molecules. It has been demonstrated that an important crosslink in $NaBH_4$-reduced collagens from various tissues is dihydroxylysinonorleucine. It probably exists in unreduced collagen fibrils as Δ^6-dehydro 5,5' dihydroxylysinonorleucine, a Schiff base involving the aldehyde group of α-amino-δ-hydroxy-adipic-δ-semialdehyde and the ε-amino group of hydroxylysine (1). The crosslink is the most abundant NaB^3H_4-reducible compound in mature bovine tendon (1) embryonic skin (2) foetal bovine tendon (3) foetal bovine dentine, mature dentine and foetal bovine bone (4).

It has been demonstrated that, in some cases, much of the Schiff base Δ^6-dehydro 5,5'-dihydroxylysinonorleucine in mature tendon and scar collagen is stable to heat and mild acid conditions while much larger amounts of the Schiff base Δ^6-dehydro-5-hydroxylysinonorleucine are labile under the same conditions (5). The Schiff bases leading to

lysinonorleucine and histidinohydroxymerodesmosine are completely labile (5). The thermal stability of the Δ^6-dehydro-5,5'-dihydroxylysinonorleucine in tendon has also been noted by others (6).

Recently indirect degradative evidence has suggested that Δ^6-dehydro-5,5'-dihydroxylysinonorleucine may exist as its keto-imino form 5-keto-5'-hydroxylysinonorleucine in bone (7) and in cartilage (8).

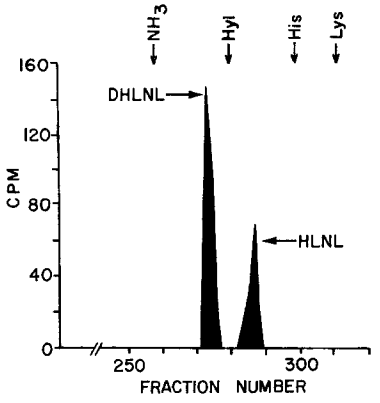
This laboratory in seeking a means of determining directly the existence of 5-keto-5'-hydroxylysinonorleucine in collagens has successfully established is presence using the selective reducing reagent sodium cyanoborohydride (9). It was found that the reduction of aldehydes and ketones would occur at around pH 3 to 4 and reduction of the imminium (>C=N+HR) moiety was rapid at pH 6-7, where its formation is favored, without reducing aldehydes or ketones (9). Since the Schiff bases in collagen are acid labile and the keto-imino form of Δ^6 dehydro 5,5' dihydroxylysinonorleucine would be stable, it was thought that treatment with the cyanohidridoborate anion at pH 3 would reduce the ketone, 5-keto-5'-hydroxylysinonorleucine to dihydroxylysinonorleucine and thereby establish a direct confirmation of its existence in collagen.

Methods and Materials

Bone collagen was prepared by demineralization of bovine cortical bone by exhaustive decalcification with 0.5M EDTA 0.05M Tris pH 7.4 in the cold, washing with 0.05M NH_4HCO_3 and water. The calcium free collagen was then lyophilized.

Tritium labeled NaB^3H_3CN was prepared as previously described (9) without isolation since the final pH of the preparation was at 3.0.

The bone collagen (23 mg) was suspended in 0.5 ml of citrate buffer (0.05M) at pH3.1 at 25°C and allowed to equilibrate overnight. To this was added a 10 fold molar excess of NaB^3H_3CN and allowed to stand 48 hrs at room temperature. The bone collagen suspension was diluted with water and dialyzed exhaustively against 0.05% acetic acid in the cold.



Legend to Figure 1

Elution profile of the crosslink portion of NaB^3H_3CN-reduced mature bone collagen at pH 3.1. DHLNL is dihydroxylysinonorleucine and HLNL is hydroxylysinonorleucine. The DHLNL and HLNL represent the reduced keto-imino forms of the Schiff bases. Δ^6-dehydro-5,5'-dihydroxylysinonorleucine and Δ^6-dehydro-5-hydroxylysinonorleucine respectively.

The collagen was lyophillyzed, hydrolyzed in vacuo with 3N Tosyl Acid, and analyzed for crosslinks (10).

Results

The radioactive elution profile of the hydrolyzate of the bone collagen reduced with NaB^3H_3CN at pH 3.1 is shown in figure 1. It can be readily seen that the crosslinks dihydroxylysinonorleucine and hydroxylysinonorleucine were the only two crosslinks observed. Mature bovine bone also contains some lysinonorleucine when reduced with NaB^3H_4 (11).

Discussion

The results reported here indicate that keto-imino forms of the Schiff bases Δ^6-dehydro-5,5'-dihydroxylysinonorleucine and Δ^6-dehydro-5-hydroxylysinonorleucine exist in bone collagen. These compounds are 5-keto-5'-hydroxylysinonorleucine and 5-keto-lysinorleucine respectively. The direct evidence for their existence was obtained by reduction at pH 3.1 with the selective reducing reagent NaB^3H_3CN to obtain dihydroxylysinonorleucine and hydroxylysinonorleucine.

The most abundant NaB^3H_4-reduced crosslink in mature tendon and scar collagen has been reported to be dihydroxylysinonorleucine (1,12). The Schiff base precursor of this crosslink was found to be one of the heat stable reducible compounds in mature tendon and scar collagen (5). Some of the Schiff base precursor of hydroxylysinonorleucine was also stable to heat at pH 7.4 (5).

These heat and acid stable crosslinks probably account for the gross insolublity of tendon and scar collagen (5). Despite the stability of these crosslinks, tendon swells enormously in acid while bone collagen does not. It was found earlier that as much as 25-50% of the crosslinks dihydroxylysinonorleucine and hydroxylysinonorleucine in dentin and bone resulted because of in vivo reduction of their respective Schiff bases (4). It is probably as a result of in vivo reduced crosslinks, in addition to reducible keto-imino forms of the Schiff bases described in this report, that may account for the extreme insolubility of bone and dentin collagen and why the latter two do not swell in mild acid solution.

Acknowledgement

The author wishes to thank Miss O. M. Perry and Mrs. Betty Hillard for excellent technical assistance. This investigation was supported by NIH research grant number DE 02668 from the National Institute of Dental Research and by NIH grant number RR 05333 from the Division of Research Facilities and Resources.

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