RELATIVE STABILITIES OF THE INTERMEDIATE REDUCIBLE CROSSLINKS PRESENT IN COLLAGEN FIBRES

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1. Introduction

Three different reducible crosslinks have been shown to be present in the collagen fibres of skin, tendon, and bone obtained from young tissues. All three are based on the reactions of lysine- and hydroxylysine-derived aldehydes and, after reduction with borohydride, result in the formation of hydroxylysinonorleucine (I) [1], dihydroxylysinonorleucine (II) [2, 3] and histidinohydroxymerodesmosine (III) [4, 5].

During our early studies into the nature of these bonds we showed that in their non-reduced form two of them, I and III, were extremely labile, being readily cleaved by dilute acids, α-amino-β-thiols and thermal denaturation [6, 7] as might be expected from their aldimine bond structures. However, the crosslink present in the less soluble tissues such as bone and cartilage, designated syndesine [8], and later shown to be dehydro-dihydroxylysinonorleucine [3] was found to be relatively stable to the above reagents. In order to account for this unusual stability we suggested that migration of the double bond of this aldimine could occur to form an enaminol and thereby stabilize the crosslink [9, 10].

This paper presents evidence confirming the proposal of an enaminol-type structure for dehydro-dihydroxylysinonorleucine. In addition, the apparent lability of the non-reduced form of Fraction C or 'post-histidine' peak is shown to be inconsistent with the structure of the reduced compound, histidinohydroxymerodesmosine [4], suggesting that this proposed crosslink is an artefact of the borohydride reduction procedure.

2. Methods and results

2.1. *Degradative studies of di-OH-LNL*

De-calcified calf bone was reduced with NaB^2H_4 and the deuterated di-OH-LNL isolated from an acid hydrolysate by using the methods previously described [8, 10]. To a solution of the crosslink in 0.1 M citrate buffer (pH 5.5) was added an aqueous solution of $NaIO_4$ to give a final concn. of 0.001 M. After 5 min, the excess of periodate was destroyed by the addition of sodium arsenite, the pH then adjusted to 8.5 and the solution reduced with KBH_4 for 30 min. The products, predominantly proline but with smaller amounts of OH-norvaline, were isolated by using the Locarte analyser.

Fig. 1 shows the mass spectral traces of the trifluoroacetyl methyl ester derivatives of both authentic proline and the proline isolated from the degraded deutero-di-OH-LNL. In the latter, the molecular ion peaks at *m/e* 225 and 226 are due to the non-deuterated and mono-deuterated derivatives respectively, and measurements of the relative intensities showed that 46% of the proline was present as the deuterated derivative. On applying the periodate and reduction degradation to the tritium-labelled crosslink, 86% of the radioactivity was recovered as [^{3}H]proline and [^{3}H]OH-norvaline.

2.2. *Effect of acid phosphate buffer on the formation of Fraction C*

In fig. 2 the elution profile of an acid hydrolysate of rat tail tendon reduced with KB^3H_4 is compared with that of an alkaline hydrolysate of the same tissue

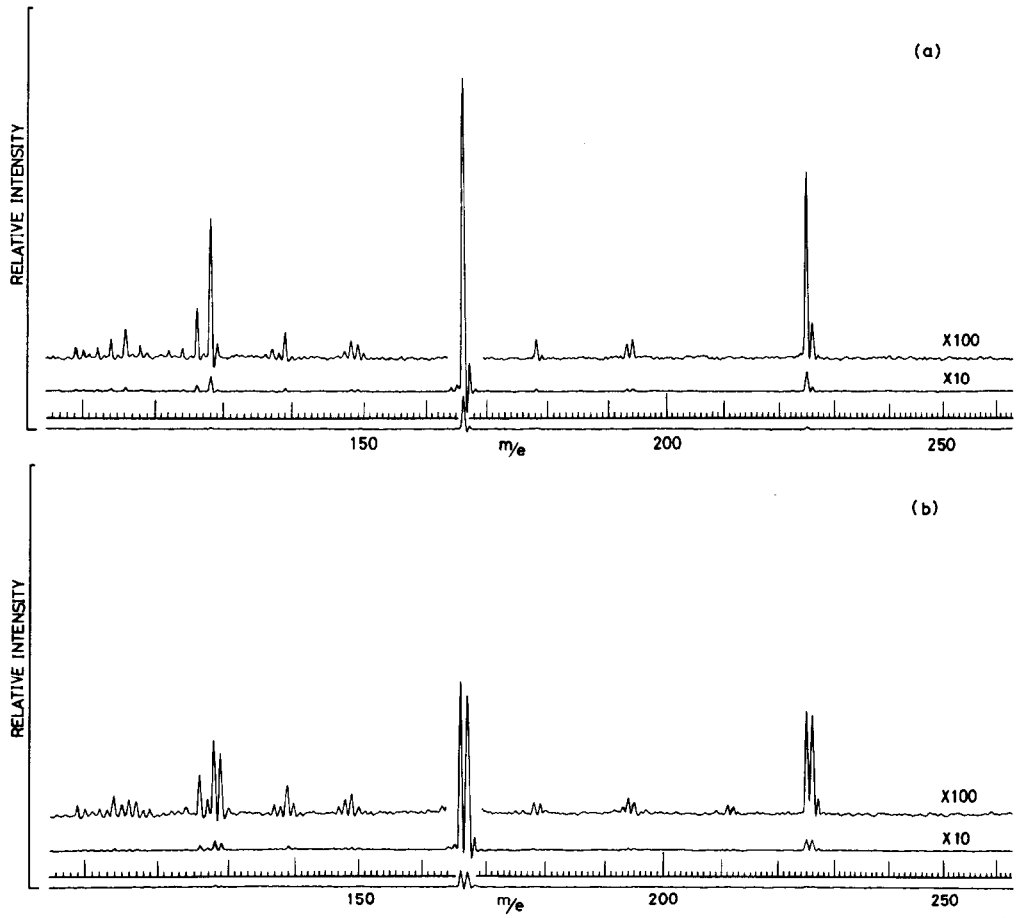


Fig. 1. The partial mass spectra of trifluoroacetyl methyl ester derivatives of (a) authentic proline and (b) proline isolated from degraded deutero-dihydroxylysinonorleucine.

treated for 2 hr at room temp. with 0.5 M NaH_2PO_4 (pH 4.3) before reduction. Whereas OH-LNL is stable to the acid buffer treatment, Fraction C is not present in the phosphate-treated sample, but is replaced by an approximately equivalent amount, in terms of ^3H-label, of material that elutes in the position of the reduced aldol condensation product (fig. 2). Although this compound is unstable to acid hydrolysis, alkaline hydrolysates of tendon reduced without phosphate treatment showed only trace amounts of aldol.

3. Discussion

The isolation and characterisation of proline, half of which was deuterium labelled, from deuterated dihydroxylysinonorleucine after the periodate and reduction degradation procedure, clearly indicates that reduction of the structure existing *in vivo* must have occurred at C-5.

On reduction of the aldimine bond form of the crosslink, the isotope would be retained only at C-6 and therefore migration of the double bond must have taken place to give the more stable enaminol structure. Borohydride reduction of the keto form of this tautomer would yield dihydroxylysinonorleucine.

These findings confirm our original proposal that the increased stability of the dihydroxy- compared to the monohydroxy-derivative of dehydro-lysinonorleucine is indeed due to the rearrangement of the structure to form an enaminol. The absence of the additional hydroxyl group in the case of dehydro-hy-

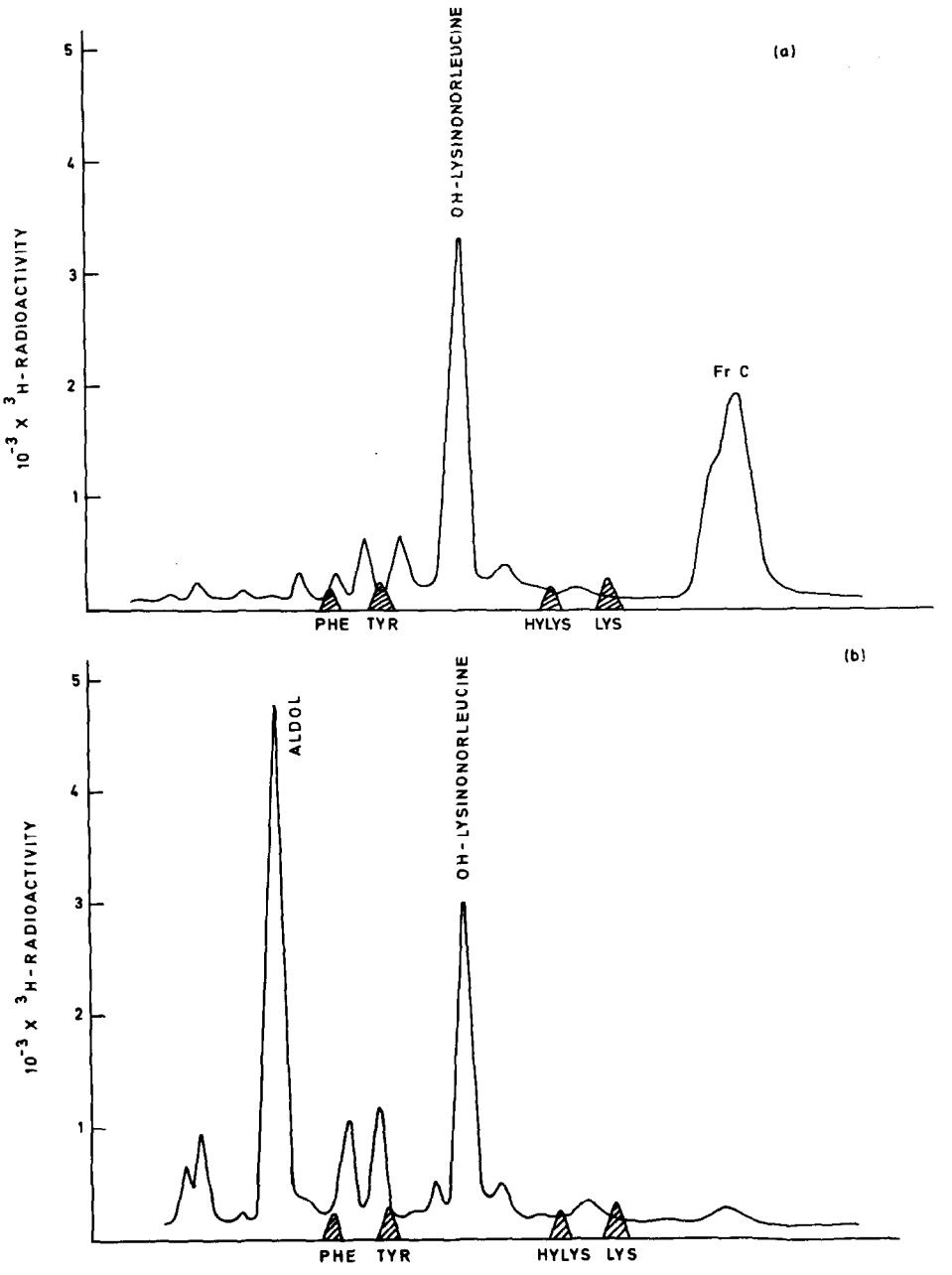
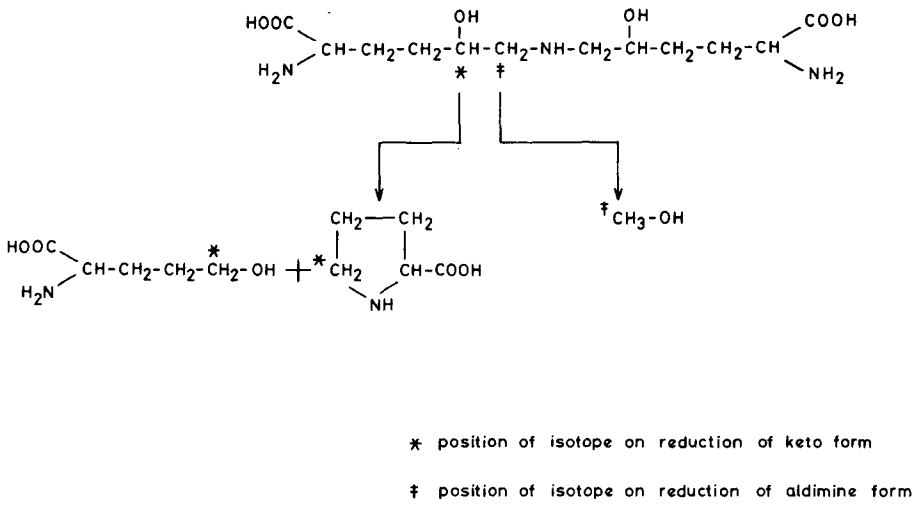


Fig. 2. The ^{3}H radioactivity elution profiles using pyridine–formate buffers of (a) an acid hydrolysate of reduced rat tail tendon and (b) an alkaline hydrolysate of the tendon treated with acid phosphate buffer before reduction.

droxylysinonorleucine prevents rearrangement of the structure occurring and the crosslink consequently has the lability expected of an aliphatic aldimine bond.

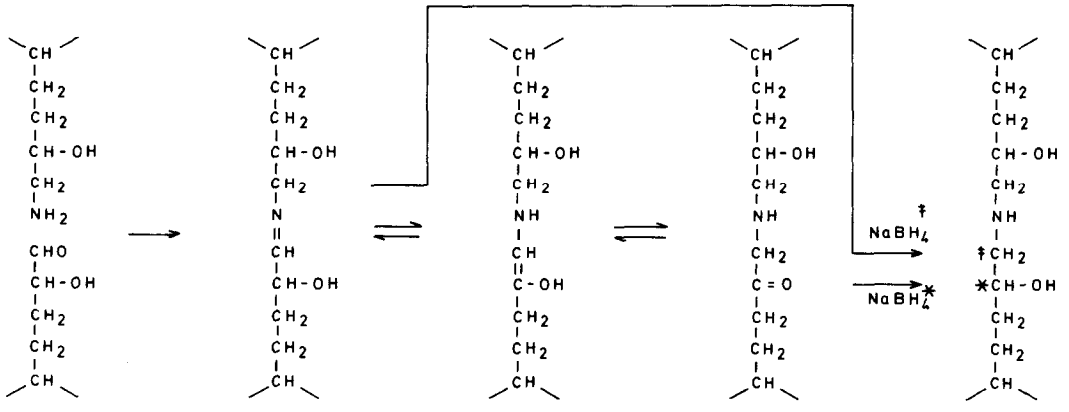
It is probable that the location of these crosslinks is identical and two of the possible sites, the quarter stagger and overlap regions contain a similar sequence in which the hydroxylysine may be glycosylated [11]. Preliminary studies indicate that the crosslink is sometimes glycosylated but it is unlikely that the presence of hexose units will affect the stability of the crosslink.



Scheme 1. Labelled products expected after periodate degradation and reduction of tritium- or deuterium-labelled dihydroxylysinonorleucine. Isotope at C-5 would produce labelled proline and hydroxynorvaline whilst at C-6 would result in the formation of labelled methanol.

The rearrangement of dihydroxylysinonorleucine presumably takes place soon after the biosynthesis of the crosslink and this stabilization is, therefore, independent of the subsequent changes with maturation that result in the formation of a non-reducible crosslink [10].

Our earlier studies of the third reducible component, previously referred to as Fraction C or 'post-histidine' peak indicated that it was extremely labile. This is confirmed by the present finding that the component appears to be cleaved by mild acid treatment of the tissue before reduction to yield the aldol con-



Scheme 2. Biosynthesis of dehydro-dihydroxylysinonorleucine and subsequent stabilization by the formation of an enaminol structure.

densation product. The identification of the reduced compound as histidino-hydroxymerodesmosine has recently been reported [4]. It was proposed that biosynthesis of the non-reduced form of this presumed crosslinking component proceeds through the Michael addition of a histidine residue to the aldol condensation product and Schiff base formation of the aldehyde function with a hydroxylysine residue. The precise mode of attachment of the histidine residue proposed by Tanzer et al. [4] was confirmed by ^{13}C NMR studies of Fraction C [5].

The presence of the aldimine bond in the proposed non-reduced form existing *in vivo* would readily account for the labile nature referred to above. However, although cleavage of the aldimine bond under mild acid conditions is conceivable, rupture of the Michael addition product involving histidine would not be expected; based on the structure proposed, aldolhistidine should have been obtained after acid treatment. A more detailed study of the effects of acid pH suggested that inhibition of the formation of this compound on reduction is due to protonation of the imidazole ring [12], thus indicating that the histidine residue cannot be involved in covalent aldol linkage in the native fibre. We conclude, therefore, that histidinohydroxymerodesmosine is an artefact produced during the reduction of the collagen fibres, the borohydride promoting a base-catalysed Michael reaction. The non-reduced form of this structure consequently does not exist as an intermolecular crosslink *in vivo*.

Acknowledgements

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