ISOLATION AND CHARACTERIZATION OF GLYCOSYL DERIVATIVES OF THE REDUCIBLE CROSS-LINKS IN COLLAGENS

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1. Introduction

The structural importance of hydroxylysine residues in collagen has become apparent in at least two respects. They have been shown to be involved in the linkage of carbohydrate to the polypeptide chain and to participate in the formation of the intermolecular bonds.

Butler and Cunningham [1] isolated a hydroxylysine residue linked to glucose and galactose by an alkali-stable *O*-glycosidic bond and Spiro [2] demonstrated that the structure was glucosylgalactosylhydroxylysine. Further studies showed that in addition to the disaccharide derivative, galactosylhydroxylysine is also present in a number of collageneous tissues [3, 4].

The formation of the reducible aldimine cross-links in collagen involves reaction of the ϵ-NH_2 group of hydroxylysine residues in the helical part of the molecule with aldehydes derived from lysine or hydroxylysine residues located in the terminal non-helical regions [5–7]. Several authors have speculated as to the role that the hydroxylysine-linked carbohydrate may play in either promoting or inhibiting cross-link formation. Eyre and Glimcher [8] have isolated from reduced bone collagen a peptide containing a cross-link that appeared to be glycosylated. This paper describes the isolation and characterization of reducible cross-link glycosides from alkaline hydrolysates of several borohydride-reduced tissues.

2. Materials and methods

Skin, achilles tendons, bone and articular cartilages from 1-year-old calves and the skin and tail tendons from 5-month-old rats were prepared and reduced with KB^3H_4 as described by Bailey et al. [9]. The samples were dialysed against several changes of distilled water (1 litre) and freeze-dried. Alkaline hydrolyses in 2 M NaOH were performed in polypropylene bottles heated at 108°C for 24 hr. The hydrolysates were diluted 10-fold with water and desalted on a column ($1\ cm^2 \times 5$ cm) of Zeo-Karb 225 (H^+ form).

The hydrolysates were initially fractionated by using the Technicon analyser with pyridine-formate buffers [9]. The relevant ^3H-containing fractions were chromatographed on a column ($2\ cm^2 \times 140$ cm) of Bio-Gel P-2 at a flow-rate of 30 ml/hr with 0.1% acetic acid as eluent. Final purification of the ^3H-labelled glycosyl derivatives was effected by chromatography on the $0.8\ cm^2 \times 26$ cm column of the Locarte analyser using a stepwise 0.2 M-sodium citrate buffer system from pH 2.90 to pH 4.25. The components were desalted on Zeo-Karb 225 (H^+ form).

3. Results

The ^3H radioactivity profiles of alkaline hydrolysates of reduced bovine bone and articular cartilage and rat skin are shown in fig. 1. Each tissue contained a major

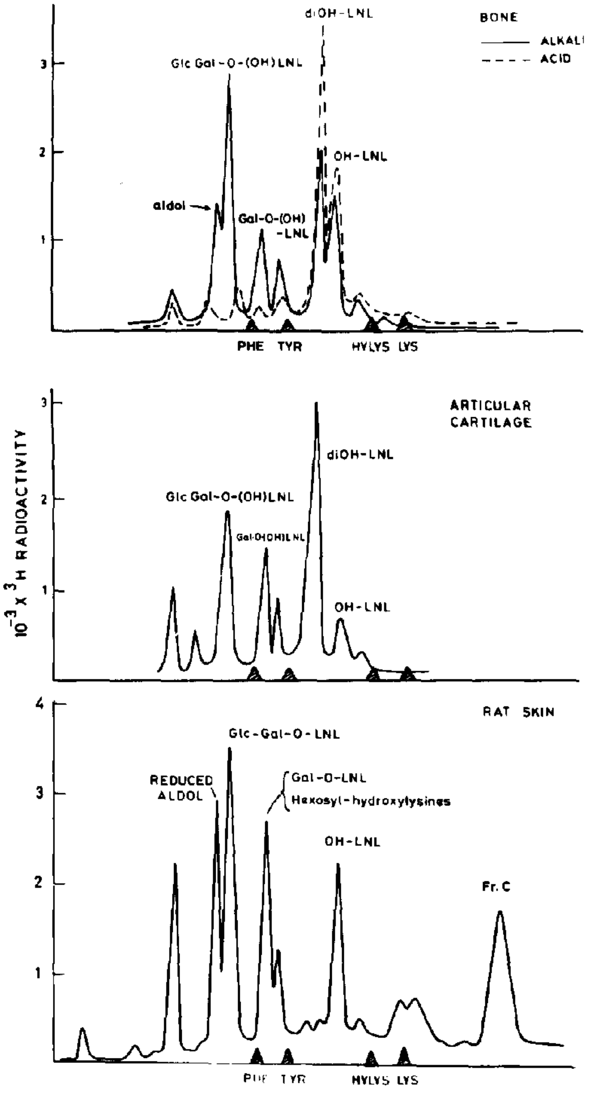

Fig. 1. ^{3}H radioactivity elution profiles hydrolysates of collagen reduced with KB^{3}H$_4$; (Upper) alkaline (——) and acid (– – –) hydrolysate of reduced bovine bone: (Middle) alkaline hydrolysate of bovine articular cartilage: (Lower) alkaline hydrolysate of reduced rat skin. The position of glycosylated cross-links are indicated; Glc-Gal-O(OH)LNL, glucosylgalactosyl-dihydroxylysinonorleucine; Gal-O(OH)LNL, galactosyl-dihydroxylysononorleucine; Glc-Gal-O-LNL, gluxosylgalactosyl-hydroxylysinonorleucine; Gal-O-LNL, galactosyl-hydroxylysinonorleucine. For experimental details see the Materials and methods section.

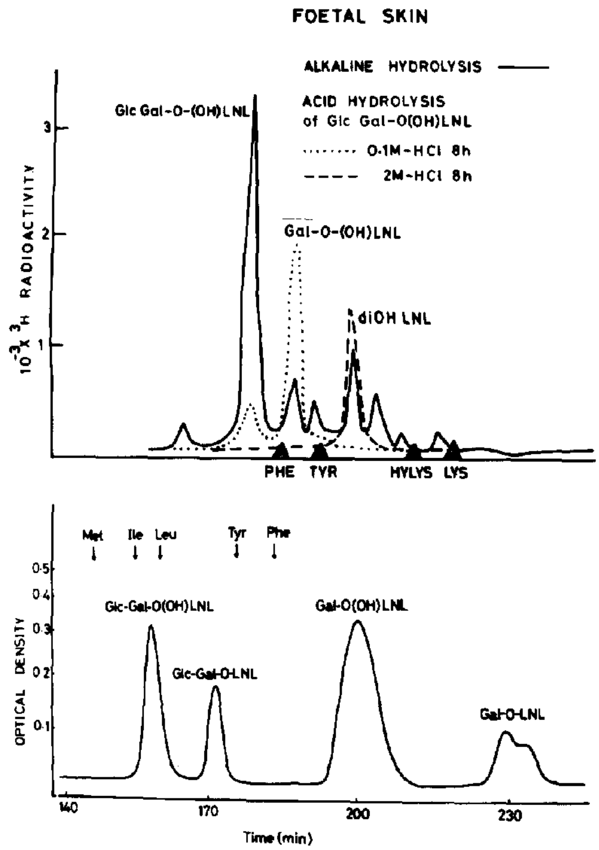

Fig. 2. Chromatographic behaviour of glycosylated cross-links. (Upper) alkaline hydrolysate (——) of reduced foetal calf skin and the products of partial (....) and complete (– – –) acid hydrolysis of the disaccharide cross-link. Analyses were on the Technicon analyser with pyridine–formate buffers. (Lower) elution positions relative to amino acids of the glycosylated cross-links on the Locarte analyzer. For notation see legend to fig. 1.

component not present in the acid hydrolysates that eluted close to the reduced aldol and which, after purification, was shown to comprise the disaccharide cross-link components as described below. The monosaccharide cross-links co-chromatographed in this system with the hexosyl-hydroxylysines. By contrasts, in alkaline hydrolysates of rat tail tendons, none of the carbohydrate-cross-link components could be detected.

In foetal calf skin the major radioactive component was the disaccharide cross-link (fig. 2). Mild acid hydrolysis in boiling 0.1 M-HCl of this compound released the monosaccharide derivative, galactosyl-dihydroxylysinonorleucine, and complete acid hydrolysis in boiling 2 M-HCl resulted in the formation of the free cross-link (fig. 2), the identity of the latter being confirmed by using several chromatographic systems [6, 9].

The elution positions of the glycosyl cross-link derivatives relative to other amino acids are shown in fig. 2. The mono- and disaccharide derivatives of dihydroxylysinonorleucine were isolated from bone collagen and those of hydroxylysononorleucine were from rat skin. The carbohydrate compositions of each of the four compounds were determined by g.l.c. after methanolysis and trimethylsilyl derivatization. For the disaccharide compounds, glucose and galactose were present in equimolar amounts whereas only galactose was detected in the monosaccharide derivatives. These findings together with the results of partial acid hydrolysis confirmed that the disaccharide components were present as glucosylgalactosyl derivatives.

4. Discussion

The present finding of glycosylated cross-link derivatives in indicates that hydroxylysine glycosides take part in aldimine bond formation with aldehydes from the terminal non-helical regions. Since the aldimine form of the dihydroxylated cross-link undergoes a spontaneous and complete rearrangement to a keto form [10], only one of the hydroxyl groups of the isolated compounds can be glycosylated, the second hydroxyl function being present only after the reduction with borohydride.

According to the quarter-stagger and overlap hypothesis [11], the C-terminal aldehyde would be adjacent to the hydroxylysine residue in the α1CB5 peptide which is known in some tissues to be a major site of attachment of the disaccharide [12]. Eyre and Glimcher [8] have reported the isolation of a glycosylated cross-linked peptide from bone collagen that appeared to be derived from this region of the molecule. Further, the N-terminal aldehyde would be adjacent to the α1CB6 peptide which in calf skin is known to contain a monosaccharide residue [13]. Thus, tissue differences in the relative amounts of mono- and disaccharide cross-links might indicate differences in their location. although further information on the location of carbohydrate in the α2 chain and in other tissues is clearly necessary.

In addition to the variations in the amounts of mono- and disaccharide cross-link derivatives, other tissues specific differences are manifest. There are large differences in the total extent of glycosylation ranging from about 80% for foetal calf skin to undetectable amounts in rat tail tendons. Since the latter is known to contain hydroxylysine glycosides [14], this result again might indicate differences in cross-link location. Although both mono- and dihydroxylysinonorleucine were shown to be glycosylated, there is evidence that in tissues that contain both these cross-links the dihydroxy derivative is glycosylated to a greater extent. For example, comparison of the relative amounts of the free cross-links in acid and alkaline hydrolysates of reduced bone (fig. 1) shows that their ratio is not constant as would be expected if the two compounds were equally glycosylated. Further studies are being carried out in an attempt to elucidate the role of the carbohydrate attachments in collagen cross-linking.

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