GLYCOPROTEIN BIOSYNTHESIS: THE BIOSYNTHESIS OF THE HYDROXYLYSINE-GALACTOSE LINKAGE IN COLLAGEN*

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Collagen a glycoprotein predominating in connective tissue contains a small amount of carbohydrate existing primarily as glucosyl-galactose units linked O-glycosidically to hydroxylysine (Butler and Cunningham 1966, 1967). Recently we reported on the biosynthesis of the glucosyl-galactose linkage in collagen (Bosmann and Eylar, 1968a) by a collagen:glucosyl transferase. This enzyme showed a high receptor specificity; only collagen containing exposed galactose had significant receptor activity. This enzyme is found in the plasma membranes of HeLa cells (Hagopian et.al., in press) and its distribution in fibroblasts before and after transformation by oncogenic viruses has also been studied (Bosmann and Eylar, 1968b). This report deals with an enzyme, a collagen: galactosyl transferase, which is responsible for the synthesis of the carbohydrate-protein linkage of collagen represented by the galactosyl-O-hydroxylysine unit.

Citrate-soluble collagen was prepared as previously described (Bosmann and Eylar, 1968a). The main receptor used in this study was prepared by removal of the glucose and galactose from the collagen by two successive periodate oxidations and mild acid hydrolysis. Approximately 700 mg of guinea pig skin collagen was dispersed in 30 ml of water and heated at 100° for 1 hour. The sample was diluted and sodium periodate and sodium acetate added, each to a final concentration of 0.05 M (pH 4.5). The reaction mixture was stirred in the dark at 4° for 24 hours. Then 10 ml of 1 M ethylene glycol were added and

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the mixture was exhaustively dialyzed. The dialyzed material was dissolved in 60.0 ml of 0.1 M sodium borate buffer (pH 8.0), and 10.5 ml of 1.0 M $NaBH_4$ was added. This reaction was carried out for 24 hours at 4°C. The reaction mixture was adjusted to pH 5.0 with acetic acid, exhaustively dialyzed, and hydrolyzed in 0.05 N H_2SO_4 for 1 hour at 80°. The above procedure was then repeated on this material, except that the periodate oxidation was performed for 5 hours instead of 24 hours. The hydrolysate was neutralized, dialyzed, and lyophilized and made up to 4 ml with distilled water. The final concentration of protein (Lowry, et.al., 1951) was approximately 50 mg/ml. This material was used as the "receptor", and is designated as such in this report.

The enzyme was extracted from mid-term embryonic guinea pig skin and cartilaginous ends of limb bone rudiments as described previously (Bosmann and Eylar, 1968a). The tissue was homogenized in 0.1% Triton X-100 in a Virtis homogenizer at 5° and extracted for 16 hours in 20 volumes of the same solution with stirring at 5°. The suspension was centrifuged at 20,000 xg for 1 hour at 3°; the supernatant solution is referred to as the crude enzyme preparation. Crude collagen:galactose transferase prepared in this manner was further purified 160 fold, and separated from the collagen:glucosyl enzyme by the procedures outlined in Table I. Enzyme purification was carried out only with the embryonic guinea pig skin extract; however, extracts from embryonic guinea pig cartilage and HeLa cells were also highly active. The purified enzyme had an optimum pH of 6.0 and was activated by either of two divalent cations, Mn++ or Co++ (Table II). The complete assay mixture, which was incubated for 1 hour at 37°, required enzyme, receptor, and metal ion (Mn++). Triton X-100 was also routinely added to the incubation mixture. It should be noted that on longer incubation times (12-24 hrs.), using the same assay system, from 1-10% of the theoretical receptor sites (calculated from the galactose removed) could be reacted with galactose-^{14}C. The enzyme exhibited a K_m of 5.7 μ moles/liter with respect to UDP-galactose.

TABLE I

PROCEDURE FOR PURIFICATION OF THE
COLLAGEN:GALACTOSYL TRANSFERASE FROM EMBRYONIC GUINEA PIG SKIN

Treatment	CPM/mg Protein	Recovery %
Triton X-100 extract	110	100
Centrifugation at 350,000 xg, 4 hours, supernatant	420	80
Sephadex G-200 chromatography	1,690	64
Centrifugation at 350,000 xg, 4 hours, supernatant	8,100	51
Sephadex G-100 column	17,600	33

TABLE II

THE ASSAY SYSTEM FOR THE COLLAGEN:GALACTOSYL TRANSFERASE
FROM EMBRYONIC GUINEA PIG SKIN

		CPM
I.	Complete System*	17,900
	-$MnCl_2$	745
II.	Complete System +EDTA (7.5×10^{-7} mole)	78
	+EDTA + Na+	89
	" + K+	31
	" + Cu++	39
	" + Pb++	47
	" + Cd++	24
	" + Co++	15,350
	" + Ca++	980
	" + Mg++	785
	" + Mn++	17,800
III.	Complete System (pH 6.0)	18,080
	pH 2.8	648
	3.5	742
	5.5	11,670
	6.5	10,070
	8.5	987
	11.1	802

* Complete system, in 0.08 ml, contained: 20 µl purified enzyme preparation (approximately 400 µg protein) (Lowry et.al., 1951), 20 µl of receptor preparation (approximately 900 µg protein), 10 µl of 0.1% Triton X-100, 10 µl of 0.1 M $MnCl_2$, 10 µl of UDP-galactose-^{14}C (approximately 40,000 cpm; 1.4×10^{-10} mole), and 10 µl of 0.1 M Tris buffer, pH 7.2. Ions were tested by adding 10 µl of 0.1 M solutions. After 1 hour of incubation, the protein of the reaction mixture was precipitated with 1% phosphotungstic acid in 0.5 N HCl and the radioactivity was determined as given elsewhere (Bosmann and Eylar, 1968a).

In order to evaluate the receptor and nucleotide sugar specificity of the collagen:galactosyl transferase, a variety of glycoprotein materials and labelled nucleotide diphosphate sugars were examined as shown in Table III.

None of the glycoprotein receptors showed significant activity, except for the α_1-glycoprotein (-sialic acid and galactose); this result suggests that a small amount of a glycoprotein:galactosyl transferase (Eylar et.al., 1967) may be present in the purified enzyme preparation. It should be noted, however, that the number of theoretical sites on the α_1-glycoprotein receptor on a weight basis far exceeds that present in the collagen receptor. The system was relatively inactive when nucleotide sugars other than UDP-galactose-^{14}C were employed.

TABLE III

SPECIFICITY OF THE COLLAGEN:GALACTOSYL TRANSFERASE

		CPM
I.	Complete system*	17,900
II.	Complete system (-receptor)	43
	+ Native guinea pig collagen	53
	+ Guinea pig skin collagen (-glucose)+	94
	+ PSM (procine submaxillary glycoprotein)	72
	+ PSM (-sialic acid)	23
	+ PSM (-sialic acid, fucose)	119
	+ Fetuin	72
	+ Fetuin (-sialic acid)	584
	+ Fetuin (-sialic acid, galactose)	238
	+α_1 glycoprotein	86
	+α_1 glycoprotein (-sialic acid)	89
	+α_1 glycoprotein (-sialic acid, -galactose)	900
	+ BSM (bovine submaxillary glycoprotein)	160
	+ BSM (-sialic acid)	135
	+ BSM (-sialic acid, N-acetylgalactosamine)	109
III.	Complete system (-enzyme)	128
	+ 80°C treated enzyme	100
	+ Embryonic guinea pig cartilage extract (approximately 2 mg protein)	8,111
IV.	Complete system (-UDP-galactose-^{14}C)	0
	+ UDP-glucose-^{14}C	205
	+ UDP-N-acetylgalactosamine-^{14}C	198
	+ UDP-xylose-^{14}C	0
	+ GDP-mannose-^{14}C	0
	+ GDP-fucose-^{14}C	0

* The complete system is given in Table II. The nucleotide sugars were tested at the same level of cpm as the UDP-galactose.

+ Guinea Pig skin collagen (-glucose) refers to guinea pig skin collagen in which the glucose has been removed by acid or enzymatic degradation leaving an exposed galactose residue (Bosmann and Eylar, 1968a); similarly for the other receptors as described in detail elsewhere (Hagopian et.al., in press). All receptors were present at 1 mg/ml protein.

In order to demonstrate the site of galactose attachment, 6 samples were incubated, precipitated and washed in the manner described above. The precipitates were pooled and hydrolyzed for 6 hours at 105°C in 3 N NaOH and the resulting peptides separated as previously described for the isolation of the glucosyl-galactosyl-hydroxylysine compound from an alkaline hydrolysate of guinea pig skin collagen (Bosmann and Eylar, 1968a). The radioactive fraction (approximately 65% of the original radioactivity) was hydrolyzed in 1 N HCl for 11 hours and chromatographed in butanol:acetic acid:water (4:1:5) and subjected to high voltage electrophoresis at pH 4.7; only galactose and hydroxylysine were present and all of the radioactivity resided with galactose. Thus a hydroxylysine-galactosyl linkage was synthesized by the enzyme.

When the collagen:glucosyl transferase (Bosmann and Eylar, 1968a) in contrast to the collagen:galactosyl transferase, was incubated as shown in Table II with receptor and UDP-glucose-^{14}C, only 215 cpm of glucose-^{14}C were incorporated. This small degree of incorporation is probably due to a small number of galactose residues yet remaining on the receptor. However, when both the collagen:glucosyl and collagen:galactosyl enzymes were incubated together along with UDP-gal and UDP-glc-^{14}C in the assay system shown in Table IV, 3270 cpm of glucose-^{14}C were incorporated. Thus, the inactive receptor is converted by addition of galactose into a structural form now recognized by the collagen:glucosyl transferase.

In addition to testing the glycoprotein receptors listed in Table II, galactose, glucose, glucosamine and N-acetylgalactosamine were substituted for the collagen receptor in the assay procedure to see if a disaccharide might be formed. The supernatant fluid from each reaction mixture, after precipitation with ethanol, was tested chromatographically in butanol:pyridine:0.1 NHCl (5:3:2) for 40 hours. No evidence of a disaccharide was found; in each instance all the radioactivity continued to be associated with UDP-galactose or galactose-1-phosphate. Similarly, hydroxylysine, lysine, hydroxyproline, threonine and serine were substituted for the collagen receptor and the supernatant fluid was

TABLE IV

THE INCORPORATION OF GLUCOSE USING THE COLLAGEN:GLUCOSYL TRANSFERASE

	Condition[1]	CPM
A.	Receptor alone	215
B.	Receptor + 50 μ moles UDP-galactose	3,270

[1]Assay system as described in Table II (with the substitution of UDP-glucose-^{14}C for UDP-galactose-^{14}C) using the collagen receptor from which both galactose and glucose had been removed. Collagen:glucosyl transferase was presented in each instance. In Condition B, both the collagen:glucosyl and collagen: galactosyl were present with an additional 50 μ moles of UDP-galactose.

examined by high voltage electrophoresis at pH 4.7 for 1 hour. Only in the case of hydroxylysine was the radioactivity associated with a compound other than UDP-galactose or galactose 1-phosphate. This compound had the same mobility as that of hydroxylysine-galactose (prepared by hydrolysis of the glucosyl-galactosyl-hydroxylysine compound described previously, Bosmann and Eylar, 1968a).

The finding of the collagen:galactosyl transferase, the subject of this report, along with the collagen:glucosyl transferase (Bosmann and Eylar, 1968a) together account for the synthesis of the carbohydrate unit of collagen as given by the following reactions:

UDP-gal + hydroxylysine collagen $\xrightarrow{Mn^{++}}$ gal-O-lysine-collagen + UDP

UDP-glc + gal-O-lysine-collagen $\xrightarrow{Mn^{++} \text{ or } Co^{++}}$ glc-gal-O-lysine-collagen + UDP.

One of the aspects of most interest in this study is the high specificity of the two collagen:glycosyl transferases for appropriate receptors. The galactosyl transferase recognizes the hydroxylysine residue alone; however, a special amino acid sequence or tertiary structure containing the galactosyl-hydroxylysine unit is required by the glucosyl transferase. Since hydroxylysine occurs only in collagen, the specificity of the collagen:galactosyl transferase for collagen is thus assured.

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