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Note

Detection of uridine 5′-diphosphate glucose–collagen glucosyltransferase activity by high-performance liquid chromatography and possible participation of a novel intermediate as a glucose donor in collagen biosynthesis

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Glucosyl galactosyl hydroxylysine (GGH) is a well known glycopeptide derived from collagen by alkaline treatment [1]. It is distributed widely in vertebrates and invertebrates [2–4] and can be also found in the complement, C1q [5].

We have reported previously the use of the dansyl derivative of GGH (GGH-Dns) for the detection of collagen-specific glucosidase activity in the crude enzyme preparation. In that report, GGH-Dns was treated with the enzyme, and the product thus obtained, by means of high-performance liquid chromatography (HPLC), dansylated galactosyl hydroxylysine (GH-Dns), was analyzed and quantified following its separation from the substrate (GGH-Dns) [6]. Thus, in the present study, it was considered that detection of the reverse reaction, i.e. glucosyltransferase activity, would be possible by the same procedure. Collagen-specific glucosyltransferase (EC 2.4.1.66) activity was successfully detected using the dansylated acceptor and HPLC.

Until now, collagen-specific glucosyltransferase activity was detected using denatured collagen as an acceptor and radiolabelled uridine 5′-diphosphate glucose (UDPG) as a glucose donor [7]. Although the transfer of the sugar to the collagen molecule occurs within the Golgi complex [8], the soluble fraction or the affinity-purified enzyme obtained from it is generally used as the enzyme source [9]. Detection of enzyme activity by the conventional method was possible without too much difficulty, but it was vague in some cases owing to the presence of a heterogeneous acceptor, such as undefined denatured collagen.

In the present work, glycopeptide mapping developed for the fractionation of

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N-glycosidic-type glycopeptides [10,11] was extended to the analysis of enzymes involved in collagen metabolism. Our objectives were to devise an easy and rapid method for the detection of collagen-specific glucosyltransferase activity and to determine the control mechanism for the biosynthesis of the collagen carbohydrate chain.

EXPERIMENTAL

Materials

The following compounds and materials were obtained commercially: UDPG, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS), maltose, gentamycin sulphate and UDP from Sigma (St. Louis, MO, U.S.A.); 1-deoxynojirimycin from Seikagaku Kogyo (Tokyo, Japan); N-ethylmaleimide and retinol (vitamin A alcohol) from Nakarai Chemicals (Tokyo, Japan); acetonitrile from Wako (Osaka, Japan); Inertsil ODS column from Gasukuro Kogyo (Tokyo, Japan). Glycogen was prepared from rat liver by trichloroacetic acid extraction and ethanol fractionation.

All solvents for HPLC were filtered through a Toyo Roshi filter (pore size 0.45 μm). All other reagents used were of the highest grade available and used without further purification.

Preparation of the dansylated acceptor

Dansyl derivatives were prepared as reported previously [6]. Purified GGH-Dns components a, b and c corresponded to the α-amino dansylated molecule, its diastereoisomer and the ϵ-amino dansylated molecule, respectively [6]. Each GH-Dns as an acceptor was prepared by treatment of the GGH-Dns component with mouse liver crude enzyme, which possessed strong glucosidase activity. For preparation of GH-Dns from component c, galactono lactone was added to the reaction mixture to inhibit galactosidase activity.

Glucosyltransferase assay

Unless otherwise stated, incubation was carried out in all cases in standard buffer containing 20 m*M* Tris–HCl (pH 7.2), 10 m*M* manganese chloride, 0.5 m*M* dithiothreitol, 0.1% Triton X-100, 10 μ*M* 1-deoxynojirimycin and 1.0 m*M* UDPG. A 40-μl volume of enzyme (microsomal fraction) and ca. 1 nmol of dansylated acceptor were added to the standard buffer to bring the final volume to 0.1 ml. 1-Deoxynojirimycin was a potent inhibitor of collagen-specific glucosidase (unpublished data). The reaction mixtures was incubated at 37°C for 16 h, followed by centrifugation at 8500 *g* for 10 min to remove insoluble material. A 50-μl portion of the supernatant was then analysed by HPLC.

Enzyme preparation

Preparation of the microsomal fraction from rat liver was carried out by differential ultracentrifugation as reported by Lahav et al. [12]. Approximately 6 ml of this enzyme fraction were prepared from 19.2 *g* of rat liver tissue.

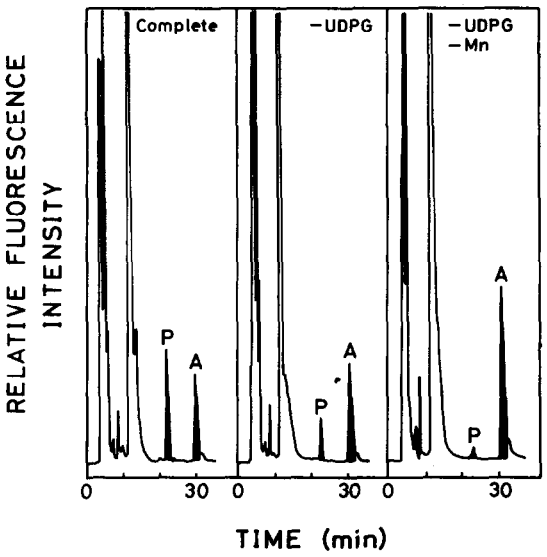

Fig. 1. Assay of glucosyltransferase activity by reversed-phase HPLC. A reaction mixture was analysed as described in the text. Shaded peaks A and P indicate the acceptor (GH-Dns) and product (GGH-Dns). Glucosyltransferase activity was calculated based on the heights of peaks, H_a and H_p. Glucosyltransferase activity expressed as product percent in Fig. 2 was calculated from $100 \times H_p/(H_a+H_p)$. Left panel, standard reaction mixture: middle panel, standard reaction mixture minus UDPG; right panel, standard mixture minus UDPG and manganese. All reaction mixtures were incubated at 37°C for 16 h.

High-performance liquid chromatography

The HPLC apparatus used in this study consisted of a Tri Rotar SR2 (Japan Spectroscopic, Tokyo, Japan) solvent delivery system, an Inertsil ODS column (5 μm; 25 cm×4.6 mm I.D.) protected by a Brownlee Labs. MPLC guard column (30 mm×4.5 mm I.D.) (Santa Clara, CA, U.S.A.), a Shimadzu fluorescence monitor RF-530 (Shimadzu, Kyoto, Japan) and a Unicorder U-228 recorder. For the enzyme assay, the incubation mixture containing 0.5 nmol of the dansyl derivatives was applied onto the column, which was then eluted at a flow-rate of 0.5 ml/min with 20% acetonitrile (v/v) in 25 m*M* sodium borate buffer (pH 7.0). For detection of dansylated derivatives, the effluent was monitored fluorimetrically (excitation, 325 nm; emission, 530 nm).

RESULTS AND DISCUSSION

Glucosyltransferase activity toward GH-Dns was analyzed under the standard conditions described in the experimental procedure. Surprisingly, a fair amount of GGH-Dns was produced from GH-Dns without UDPG in the reaction mixture (Fig. 1). This unexpected result indicated the glucose residue to be transferred via an unknown endogenous activated glucose intermediate in the microsomal fraction. When manganese ions were removed at the same time as UDPG from the reaction mixture, the transferase activity underwent a remarkable decrease (Fig. 1). The formation of an enzyme complex containing manganese and UDPG

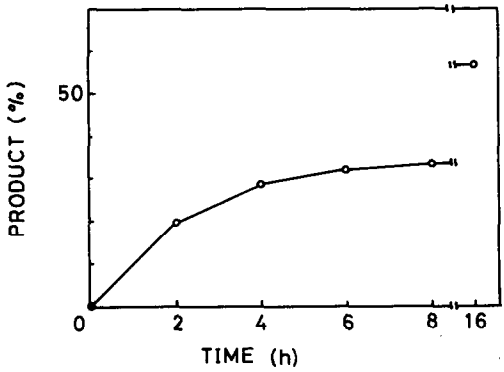

Fig. 2. Time course of glucosyltransferase activity toward the dansylated acceptor (GH-Dns) in the rat liver microsomal fraction. The assay was carried under the standard conditions as described in the text. GH-Dns prepared from GGH-Dns, component a, was used as the acceptor. The product percentage, i.e. glucosyltransferase activity, was calculated as shown in Fig. 1.

has already been reported [13], but in the present case the production of the enzyme complex in the reaction mixture probably required the presence of a large amount of UDPG in the organella. The time course of the reaction showed a biphasic change, suggesting the rapid consumption of the endogenous intermediate and slow transfer of glucose from UDPG (Fig. 2).

A previous paper reported how various monodansyl derivatives were obtained from GGH [6]. These components were used to examine the acceptor specificity of transferase.

ϵ-Amino dansylated GH from component c manifested a lower acceptor activity (Table I); however, the diastereoisomer of α-amino dansylated GH from component b was as good an acceptor as that from a native molecule, component a. The enzyme requirement for a free ϵ-amino group of hydroxylysine indicated that the action of the enzyme is probably specific toward the collagen molecule, as was also found to be the case for collagen-specific glucosidase [14].

The enzyme differed from glycogen synthetase since the addition of glycogen and maltose as competitive inhibitors did not interfere with enzyme action toward GH-Dns. The effects of other inhibitors, such as DIDS, UDP and N-ethylmaleimide were also examined. DIDS and UDP are known to inhibit the formation of dolichol phosphoryl glucose [15] from UDPG and of UDPG [16] from glucose 1-phosphate, respectively, and N-ethylmaleimide is known to inhibit the activity of collagen glucosyltransferase in the soluble fraction [17]. None of these three compounds was capable of inhibiting the activity of the glucosyltransferase examined in this study. Gentamycin, which possesses renal toxic activity [18], was found to activate the enzyme somewhat.

In this work, it was possible to detect specific glucosyltransferase activity toward the collagen carbohydrate chain without the use of radiolabelled materials. The transfer of the glucose residue from the endogenous glucose donor (maybe novel intermediate) to the dansyl-labelled acceptor was unexpectedly observed, and a preliminary experiment to characterize the intermediate was carried out. Since UDPG is a key material participating in various metabolic processes, such as

TABLE I

EFFECTS OF VARIOUS COMPOUNDS TOWARD GLUCOSYLTRANSFERASE ACTIVITY

Reaction mixture was incubated at 37°C for 16 h.

Reaction mixture	Final concentration	Relative activity
Complete*	–	100
–UDPG	–	49.1**
–UDPG, –manganese	–	8.7
GH-Dns (c)***	–	2.3
GH-Dns (b)***	–	70.4
DIDS	2 m*M*	107
Vitamin A§	5 mg/ml	96.7
N-Ethylmaleimide§§	10 m*M*	95.8
Glycogen	5 mg/ml	116
Maltose	10 mg/ml	115
Gentamycin	1 m*M*	124
UDP	2 m*M*	107

*Standard reaction mixture.
**Mean value from three determinations.
***GH-Dns (a) as the acceptor was replaced by each dansyl component.
§Retinol dissolved in a small amount of ethanol was added to the standard reaction mixture.
§§Dithiothreitol in the standard reaction mixture was substituted by N-ethylmaleimide.

glycogen synthesis, lipid intermediate formation in the synthesis of the N-glycosidic-type carbohydrates chain, and energy production via glycolysis, a specific intermediate may possibly perform an important role in regulating the biosynthetic processes of the collagen carbohydrate chain. Attempts are presently being made to separate and purify the endogenous glucose donor detected in the present study from the rat liver microsomal fraction.

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