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Further Characterization of Galactosylhydroxylysyl Glucosyltransferase from Chick Embryos

AMINO ACID COMPOSITION AND ACCEPTOR SPECIFICITY

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A modified purification procedure, consisting of affinity chromatographies on concanavalin A–agarose, collagen–agarose and UDP-glucose-derivative–agarose and one gel filtration, is reported for galactosylhydroxylysyl glucosyltransferase. The enzyme obtained is entirely pure when studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The enzyme protein was rich in glutamic acid + glutamine, aspartic acid + asparagine, glycine and alanine. The enzyme catalysed no significant glucose transfer to any of the glycoproteins tested, except for collagens. This included all the glycoproteins that have previously served as glucosyl acceptors for impure enzyme preparations, thus indicating a high degree of specificity of the enzyme for galactosylhydroxylysine. Galactosylsphingosine would act as a glucosyl acceptor, however. This compound has a close structural similarity to galactosylhydroxylysine in that they both have an unsubstituted amino group next to the hydroxy group to which the galactose is attached.

UDP-glucose–5-hydroxylysine-collagen glucosyltransferase (EC 2.4.1.66) has earlier been known as collagen glucosyltransferase, but, as discussed elsewhere (Kivirikko & Myllylä, 1978), this terminology is not strictly accurate, and hence the name galactosylhydroxylysyl glucosyltransferase (Kivirikko & Myllylä, 1978) is used here. The enzyme catalyses the synthesis of glucosylgalactosylhydroxylysine in collagen by transferring the monosaccharide unit from UDP-glucose to galactosylhydroxylysyl residues. The reaction probably occurs within the cisternae of the rough endoplasmic reticulum, and requires the presence of a bivalent cation, this requirement being fulfilled by Mn^{2+} (for a review, see Kivirikko & Myllylä, 1978). The enzyme has been isolated as a homogeneous protein from chick embryos (Myllylä *et al.*, 1977) and shown to be a glycoprotein (Myllylä *et al.*, 1976; Anttinen *et al.*, 1977), probably consisting of only one polypeptide chain with a mol.wt. of about 70000 (Myllylä *et al.*, 1977). The present paper reports an improved purification procedure for the enzyme and describes the amino acid composition of the protein.

Free galactosylhydroxylysine (Spiro & Spiro, 1971; Smith *et al.*, 1977) and galactosylhydroxylysine residues in denatured collagens and isolated collagen α-chains from various sources (see Kivirikko & Myllylä, 1978) serve as glucosyl acceptors for the enzyme. Some reports, however, have suggested that its specificity for these acceptors may not be absolute, as significant glucose transfer has been found to some other glycoproteins, in particular to *N*-acetylneuraminic acid-free thyroglobulin (Spiro & Spiro, 1971), transferrin (Barber & Jamieson, 1971) and serum α_1-glycoprotein (Henkel & Buddecke, 1975). In addition, galactosylsphingosine serves as the glucosyl acceptor for crude rat kidney-cortex enzyme preparations (Spiro & Spiro, 1971). All these studies have been carried out with highly impure enzyme preparations, and it is not known whether the transfer noted was due to the activity of a contaminating enzyme. The acceptor specificity of the enzyme was therefore reinvestigated with entirely pure enzyme preparations.

Experimental

Materials

Fertilized eggs of White Leghorn chickens were purchased from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and were incubated at 37°C in humidified incubators for 14 or 15 days. Calf skin gelatin substrate (Myllylä *et al.*, 1975*a*), diffusible collagen peptides (Myllylä *et al.*, 1977) and citrate-soluble rat skin collagen (Gallop & Seifter, 1963) were prepared. UDP-[^{14}C]glucose was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), and non-radioactive UDP-glucose from Sigma Chemical Co. (Kingston upon Thames, Surrey, U.K.). UDP-glucose-derivative–agarose (Anttinen & Kivirikko, 1976) and collagen–agarose (Risteli *et al.*, 1976) were prepared. Concanavalin A–Sepharose 4B (concanavalin A–agarose) was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden) and galactosylsphingosine from

K & K Laboratories (Hollywood, CA, U.S.A.). α_1-Glycoprotein was prepared from calf serum (Weimar *et al.*, 1950). The other glycoprotein acceptors were commercially available.

Purification of galactosylhydroxylysyl glucosyltransferase

All procedures were carried out at 0–4°C and all columns equilibrated with a solution consisting of 0.12M-NaCl/2mM-$MnCl_2$/50μM-dithiothreitol/1% (v/v) glycerol/50mM-Tris/HCl buffer, adjusted to pH7.4 at 4°C (Anttinen *et al.*, 1977; Myllylä *et al.*, 1977), termed enzyme buffer.

Preparation of $(NH_4)_2SO_4$-precipitated enzyme. The chick embryos were homogenized, and the 15000*g* supernatant of the homogenate was prepared as reported previously (Myllylä *et al.*, 1976). The $(NH_4)_2SO_4$ fraction (0–55% saturation) of the embryo extract was likewise prepared as described previously (Myllylä *et al.*, 1976) and the dialysis was carried out against the enzyme buffer. The enzyme was stored at −20°C.

Chromatography on a concanavalin A–agarose column. The $(NH_4)_2SO_4$ fraction of the enzyme was thawed and centrifuged at 15000*g* for 30min, and the supernatant (about 4000ml) was then passed, in a protein concentration of about 20mg/ml, through a column (4cm×8cm) of concanavalin A–agarose at a flow rate of 70–80ml/h. After the sample had passed through it, the column was washed with the enzyme buffer until A_{225} was about 0.1. The enzyme was eluted with 1500ml of a solution consisting of 1M-methyl α-D-glucoside and 20% (v/v) ethylene glycol in the enzyme buffer with a flow rate of about 80ml/h, and fractions (10ml) were collected. The fractions containing most of the enzyme activity were pooled and dialysed for 24h against 2×8 litres of the enzyme buffer.

Chromatography on a collagen–agarose column. The pooled enzyme from the previous step was passed through the collagen–agarose column (1.7cm×16cm) at a flow rate of 40ml/h. The column was then washed with the enzyme buffer until A_{225} was below 0.1, and the enzyme was then eluted either with 10g of diffusible collagen peptides in 10ml of the enzyme buffer or with 300ml of 50% (v/v) ethylene glycol in the enzyme buffer. Fractions (5ml) were collected and all fractions containing the elution peptides (detected by A_{280}) or ethylene glycol were pooled and concentrated to a volume of about 5ml in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA, U.S.A.) with a PM 30 membrane.

Sephadex G-150 gel filtration. The pooled enzyme was applied to a column (2.5cm×85cm) of Sephadex G-150 (Pharmacia). Fractions (5ml) were collected, and those containing most enzyme activity were pooled.

Chromatography on a UDP-glucose-derivative–agarose column. The pooled enzyme was passed through the UDP-glucose-derivative–agarose column (1cm×18cm) at a flow rate of 10ml/h and the column was then washed with 50ml of enzyme buffer. The enzyme was eluted from the column with 300ml of 0.5M-NaCl/2mM-$MnCl_2$/50μM-dithiothreitol/1% glycerol/50mM-Tris/HCl, adjusted to pH7.4 at 4°C. Fractions (5ml) were collected and those containing most enzyme activity were pooled and concentrated to a volume of about 1ml in an Amicon ultrafiltration cell with a PM 10 membrane. This constituted the final galactosylhydroxylysyl glucosyltransferase pool.

Assay of galactosylhydroxylysyl glucosyltransferase activity

The enzyme activity was assayed in a reaction volume of 100μl as described previously (Myllylä *et al.*, 1975*a*, 1976, 1977) with calf skin gelatin or citrate-soluble rat skin collagen as the substrate. One unit of enzyme activity was defined as the activity required to synthesize an amount of radioactive glucosylgalactosylhydroxylysine in d.p.m. corresponding to 1μmol in 1h at 37°C, as established previously (Myllylä *et al.*, 1976).

When galactosylsphingosine was used as the acceptor, the transfer of glucose was determined, as described by Spiro & Spiro (1971), by extraction of the products into chloroform/methanol (2:1, v/v) and paper chromatography in butan-1-ol/acetic acid/water (4:1:5, by vol.). The dried paper strips were cut into 5mm fractions from the origin and their radioactivity was assayed (Myllylä *et al.*, 1975*a*). The position of galactosylsphingosine was detected by staining with ninhydrin (Spiro & Spiro, 1971).

The glucose transferred to various protein acceptors was assayed by precipitating the protein of the reaction mixture with 2ml of 1% phosphotungstic acid in 0.5M-HCl. The precipitate was collected by using a Millipore filter (0.45μm pore size, HAWP 01300) and washed with 3×2ml of 10% (w/v) trichloroacetic acid. The filters were dried and their radioactivity was measured in a toluene scintillant (Prockop & Ebert, 1963).

Other assays

The protein concentration was measured by peptide absorbance at 225nm, with bovine serum albumin as a standard, which gave an absorbance of $A_{225}^{1mg/ml} = 7.40$ with a 1cm-light-path cell.

Disc electrophoresis was performed, after reduction and denaturation of the polypeptide chains in the presence of sodium dodecyl sulphate, on 10% (w/v) polyacrylamide gels (Weber & Osborn, 1969). The gels were stained with Coomassie Brilliant Blue.

For amino acid analysis, the enzyme protein (0.1–0.2mg) was dialysed exhaustively against water,

Table 1. *Purification of galactosylhydroxylysyl glucosyltransferase from chick-embryo extract*

Enzyme fraction	Total protein (mg)	Total activity (munits)	Recovery (%)	Specific activity (munits/mg)	Purification over 15000*g* supernatant (-fold)
$(NH_4)_2SO_4$ (0–55% satn.)	80600	153000	100	1.9	2.2
Concanavalin A–agarose	514	75000	49	146	169
After collagen–agarose and gel filtration	11.3	23000	15	2030	2350
UDP-glucose-derivative–agarose	0.36	12300	8	34400	39800

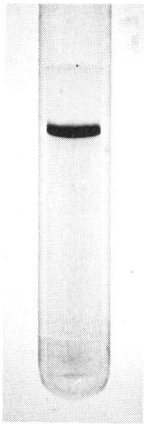

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of galactosylhydroxylysyl glucosyltransferase from chick embryos*

Table 2. *Amino acid composition of galactosylhydroxylysyl glucosyltransferase from chick embryos*

No correction is made for losses during hydrolysis. Cysteine, methionine and tryptophan were not determined, owing to the limited amount of enzyme sample available. The results are expressed as the mean ± S.D. of assays on four separate enzyme preparations.

Amino acid	Residues/1000 residues
Lysine	63 ± 2
Histidine	18 ± 2
Arginine	50 ± 3
Aspartic acid + asparagine	115 ± 4
Threonine	50 ± 2
Serine	71 ± 12
Glutamic acid + glutamine	133 ± 12
Proline	56 ± 2
Glycine	139 ± 18
Alanine	103 ± 3
Valine	60 ± 7
Isoleucine	34 ± 10
Leucine	67 ± 6
Tyrosine	10 ± 2
Phenylalanine	31 ± 6

and hydrolysed in 6M-HCl for 24h in tubes sealed under N_2. The sample was evaporated to dryness, and the analysis was carried out with a JEOL JLC-5AH amino acid analyser.

Results

Modified purification procedure and amino acid composition of the enzyme

The original purification procedure for galactosylhydroxylysyl glucosyltransferase enabled enzyme preparations to be obtained that were homogeneous when studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but which, as reported previously, contained in some instances protein contaminants representing up to about 5% of the total protein (Myllylä *et al.*, 1977). Subsequent studies indicated that some preparations also contained trace amounts of the elution peptides, and thus were not ideal for experiments on the properties of the enzyme protein, such as amino acid analysis.

The modified purification procedure described above consists of affinity chromatographies on concanavalin A–agarose, collagen–agarose and UDP-glucose-derivative–agarose and one gel filtration between the second and third affinity-chromatography steps to separate the enzyme from the elution peptides or the ethylene glycol used as the alternative eluting agent (Table 1). The UDP-glucose-derivative–agarose column, which constitutes the last affinity-column step, can be eluted in this procedure by increasing the NaCl concentration to 0.5M, although such a solution does not elute the enzyme if the same column is used at an earlier stage in the purification (Anttinen & Kivirikko, 1976; Myllylä *et al.*, 1977).

The final enzyme is entirely pure when studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). The specific activity of the preparation shown in Table 1 is about 1.3 times that reported previously, although owing to partial inactivation during the purification, the specific activities of

Table 3. *Acceptor specificity of galactosylhydroxylysyl glucosyltransferase*

The glucose transfer to protein acceptors was assayed by precipitating the protein of the reaction mixture and determining the radioactivity of the washed precipitate as described in the Experimental section. The treatment with H_2SO_4 (25mM at 80°C for 1h) was carried out to remove *N*-acetylneuraminic acid. The product of the reaction with galactosylsphingosine was assayed by paper chromatography (see the Experimental section). The value obtained with 3.0mg of citrate-soluble rat skin collagen/ml was taken as 100%.

	Acceptor	Concentration (mg/ml)	Glucose transferred (d.p.m.)	Glucose transferred (%)
Expt. I	None		0	0
	Citrate-soluble rat skin collagen	0.3	1020	21
	Citrate-soluble rat skin collagen	3.0	4860	100
	Ovalbumin	3.0	0	0
	Ovalbumin; H_2SO_4-treated	0.3	0	0
	Casein	0.3	0	0
	Casein; H_2SO_4-treated	0.3	0	0
	Chondroitin sulphate	0.3	0	0
	Ovomucoid	3.0	0	0
	Ovomucoid; H_2SO_4-treated	3.0	0	0
	Transferrin	3.0	40	<1
	Transferrin; H_2SO_4-treated	3.0	30	<1
Expt. II	None		0	0
	Citrate-soluble rat skin collagen	3.0	3870	100
	α_1-Glycoprotein	3.0	0	0
	α_1-Glycoprotein; H_2SO_4-treated	3.0	0	0
	Fetuin	3.0	10	<1
	Fetuin; H_2SO_4-treated	3.0	20	<1
	α-Lactalbumin	3.0	0	0
	Thyroglobulin	3.0	0	0
	Thyroglobulin; H_2SO_4-treated	0.3	0	0
Expt. III	None		0	0
	Citrate-soluble rat skin collagen	3.0	8320	100
	Galactosylsphingosine	2.8	7640	92

entirely pure enzyme preparations tend to show considerable variation, ranging between about 20 and 35 units/mg.

The amino acid analysis of the enzyme, expressed as the means ± S.D. of four separate preparations, is shown in Table 2. The protein was rich in glutamic acid+glutamine, aspartic acid+asparagine, glycine and alanine.

Acceptor specificity

A number of glycoproteins was tested as possible glucosyl acceptors in the galactosylhydroxylysyl glucosyltransferase reaction (Table 3), and most were also examined after heating with 25mM-H_2SO_4 at 80°C for 1h to remove *N*-acetylneuraminic acid. No significant transfer of glucose was found to any of these acceptors with the pure enzyme (Table 3).

Galactosylsphingosine, which was also tested (Table 3), did prove to act as an acceptor. The position of the labelled product corresponded to that reported for glucosylgalactosylsphingosine (Spiro & Spiro, 1971), the mobility being about 0.9 compared with that of galactosylsphingosine (Fig. 2). The K_m of galactosylsphingosine was about 2.5mM (Fig. 3) and its $V_{max.}$ about twice that obtained with citrate-soluble rat skin collagen in the same experiment (results not shown).

Discussion

The enzyme preparations obtained by the purification procedure reported here were entirely pure when studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The turnover number of the enzyme, calculated from the highest specific activity and on the basis of a mol.wt. of about 70000 (Myllylä *et al.*, 1977), is about 0.7mol of glucosylgalactosylhydroxylysine formed/s per mol of enzyme. Turnover numbers that have been reported for several other glycosyltransferases (Powell & Brew, 1976; Paulson *et al.*, 1977; Schwyzer & Hill, 1977) are some 30–70 times higher, and that for proline hydroxylase (Tuderman *et al.*, 1975), another intracellular enzyme of collagen biosynthesis, is about 10 times higher, than the present value. It should be noted, however, that enzyme units are expressed with citrate-soluble rat skin collagen as substrate. Since some of the galactosylhydroxylysyl residues in this collagen had already been glucosylated *in vivo* (see Kivirikko & Myllylä, 1978), it seems possible that the value would be higher when studied for the glucosylation of the first

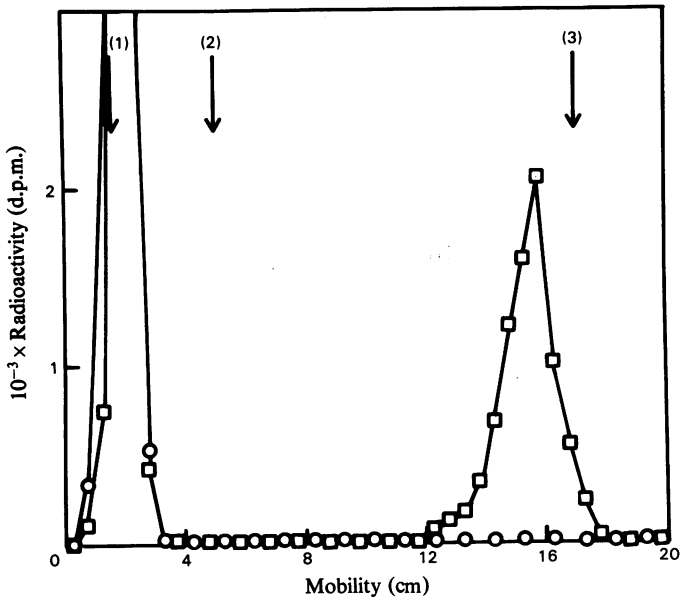

Fig. 2. *Assay by paper chromatography of the product formed in the reaction of galactosylsphingosine with galactosylhydroxylysyl glucosyltransferase*

The product was chromatographed and assayed as described in the Experimental section. The arrows indicate the mobilities of UDP-glucose (1), glucose (2) and galactosylsphingosine (3). The reaction was carried out both in the absence (○) and in the presence (□) of the enzyme.

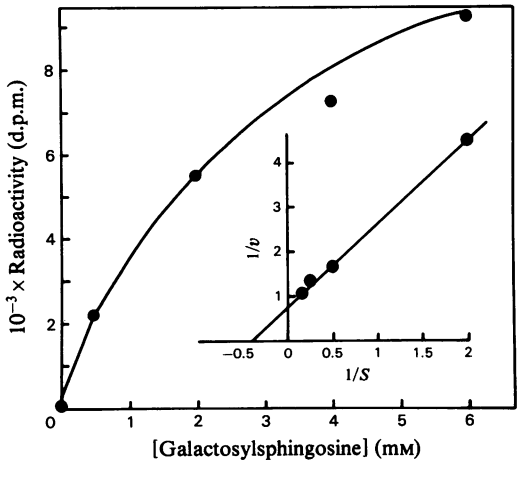

Fig. 3. *Effect of galactosylsphingosine concentration on the formation of glucosylgalactosylsphingosine in the reaction with galactosylhydroxylysyl glucosyltransferase*

In a double-reciprocal plot (inset) the values shown on the abscissa are reciprocal values of the galactosylsphingosine concentration and the values shown on the ordinate are reciprocal values of the velocity in 10^{-4} × radioactivity of the product formed.

galactosylhydroxylysyl residues. In accordance with this suggestion, the $V_{max.}$ value observed in the present study for the glucosylation of galactosylsphingosine was about twice that for the glucosylation of citrate-soluble rat skin collagen. Another factor that may lead to an artificially low turnover number is the tendency for the enzyme protein to lose activity during purification, and most of the losses in the total activity in Table 1 were due to inactivation rather than omission of the activity during pooling of the enzyme fractions.

An amino acid analysis of galactosylhydroxylysyl glucosyltransferase was obtained here for the first time. Several similarities were found to the corresponding analysis for chick-embryo prolyl hydroxylase (Berg & Prockop, 1973; Tuderman *et al.*, 1975), another intracellular enzyme of collagen biosynthesis. These include relatively high glutamic acid+glutamine, aspartic acid+asparagine and alanine contents and relatively low histidine and tyrosine contents. Distinct differences were also observed, however, such as higher glycine and serine contents in the glucosyltransferase, and lower isoleucine, leucine and phenylalanine contents. Fewer similarities in amino acid composition were found between the glucosyltransferase and the galactosyltransferase, which catalyses lactose synthesis (Powell & Brew, 1974).

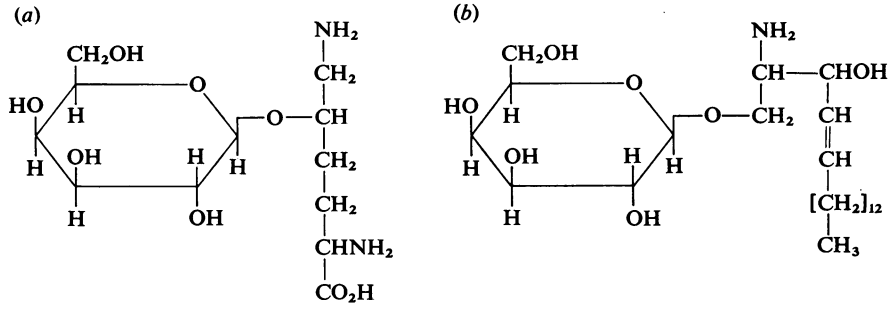

Fig. 4. *Galactosylhydroxylysine (a) and galactosylsphingosine (b)*

Re-examination of the acceptor specificity of galactosylhydroxylysyl glucosyltransferase showed no significant glucose transfer to any of the glycoproteins tested, except for collagens. This included all the glycoproteins that have previously served as glucosyl acceptors for impure enzyme preparations (Barber & Jamieson, 1971; Spiro & Spiro, 1971; Henkel & Buddecke, 1975), thus indicating a high degree of specificity of the enzyme for galactosylhydroxylysine. Galactosylsphingosine was found to act as a glucosyl acceptor, however. The $V_{max.}$ was even higher than that for citrate-soluble rat skin collagen, but the K_m was about 500–1000 times the K_m value previously reported for citrate-soluble rat skin collagen, expressed as the concentration of the galactosylhydroxylysyl acceptor sites (Myllylä *et al.*, 1975*b*). Galactosylsphingosine bears a close structural similarity to galactosylhydroxylysine in that both compounds have an unsubstituted amino group next to the hydroxy group to which the galactose is attached (Fig. 4). The importance of the free ε-amino group is also apparent from data indicating that *N*-acetylation (Spiro & Spiro, 1971) or deamination (Barber & Jamieson, 1971) of the ε-amino group in galactosylhydroxylysine or galactosylhydroxylysyl residues completely inhibits glucose transfer.

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