DIRECT EVIDENCE THAT D-GALACTOSAMINE INCORPORATION INTO GLYCOGEN OCCURS VIA UDP-GLUCOSAMINE

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1. Introduction

Previous studies by Maley et al. [1–3] and Keppler et al. [4] demonstrated that D-galactosamine is metabolized in rat liver by the same pathway as D-galactose. In addition to the formation of a variety of acid-soluble products (e.g., galactosamine 1-phosphate, UDP-GlcN, UDP-GalN and UDP-GlcNAc), radioactivity from [1-^{14}C]galactosamine was associated with glycogen in the form of glycosidically linked [1-^{14}C]glucosamine. Since galactosamine is enzymically converted to glucosamine by epimerization at the uridine nucleotide level [5], the presence of glucosamine in glycogen could be explained by the substitution of UDP-GlcN for UDP-Glc in the glycogen synthetase reaction, as proposed earlier [1]. However, because the UDP-GlcN employed had been isolated in small amounts from rat liver and was contaminated with UDP-GalN, unequivocal proof could not be presented. This report provides direct confirmation for the incorporation of glucosamine into glycogen by utilizing chemically synthesized UDP-GlcN in the glycogen synthase (EC 2.4.1.11) reaction. These findings also suggest that another reason for the hepatotoxicity of galactosamine, in addition to those already proposed [6], may be the substitution of UDP-GlcN and UDP-GalN for their corresponding UDP-hexoses in reactions concerned with membrane and glycoprotein synthesis [7].

2. Materials and methods

2.1. *Preparation of rat liver glycogen synthase*

A procedure similar to that described by Leloir and Goldenberg [8] was used. The final pellet was dissolved in 15 ml of buffer (0.05 M glycylglycine, pH 8.0; 5 mM EDTA) to yield a bluish-opalescent solution containing 20 mg/ml protein and 2.75 mg/ml glycogen.

2.2. *Glycogen synthase assay*

The complete system contained glycylglycine buffer (pH 8.0), 10 μmol; EDTA, 1 μmol; rabbit muscle glycogen, 3 mg; glucose 6-phosphate, 1.5 μmol; enzyme extract, 0.2 mg protein; and 0.1 μmol of either UDP-[1-^{14}C]Glc (2.4×10^6 cpm/μmol) or UDP-[1-^{14}C]GlcN (2.6×10^6 cpm/μmol), in a final volume of 0.1 ml. The tubes were incubated at 37°C for various times and the reactions terminated by applying 0.05-ml aliquots to a 1-cm band on a sheet of Whatman 3 MM paper. Chromatograms were developed descendingly, in solvent A for 4 h.

After thorough drying, the origin containing the glycogen was excised, placed in a counting vial with 10 ml of toluene fluor, and analyzed for radioactivity in a liquid scintillation counter.

2.3. *Analytical methods*

The *N*-acetylated β-amylase product was hydrolyzed at 110°C for 2 h with 2 N HCl in evacuated, nitrogen-

flushed tubes. After the removal of HCl by repeated rotary evaporation in vacuo, a portion of the sample was analyzed for glucosamine with a Jeolco Model 5 AH amino acid analyzer. The remainder of the sample was assayed for glucose by the spectrophotometric-hexokinase procedure [8]. A sample of the β-amylase product, equal in radioactivity to that used for acid hydrolysis of the corresponding *N*-acetylated dissaccharide, was subjected to nitrous acid deamination [2]. Aliquots of the reaction were analyzed for 2,5-anhydromannose [9] and for glucose as described above. *N*-Acetylation [10] and borotritide reduction were conducted as described previously [11].

Protein was determined by the Lowry method [12] and glycogen by the iodine method of Van der Vies [13].

2.4. *Chromatography*

Descending paper chromatography was carried out with Whatman 3MM paper in the following solvent systems: (A) 95% ethanol-1 M ammonium acetate, pH 7.5, (5:2); (B) ethyl acetate/pyridine/water (10:4:3); (C) 1-butanol/pyridine/water (6:4:3). Reducing sugars were detected on chromatograms with the alkaline $AgNO_3$ reagent [14] and radioactive compounds with a Nuclear Chicago 4π windowless strip scanner (Actigraph III).

2.5. *Synthesis of UDP-[1-^{14}C]GlcN*

The radioactive compound was prepared by a small-scale adaptation of our previously described method [15], which was improved by employing a prolonged room temperature incubation for the synthesis of compound I.

To 95 mg of α-D-glucosamine–HCl were added 15.9 mg of [1-^{14}C]glucosamine–HCl (10 mCi/mmol, New England Nuclear Corp.) and 0.2 ml of acetyl bromide, This mixture was shaken overnight at room temperature in a Teflon-capped tube by means of a vortex mixer. Excess acetyl bromide and HBr were removed in vacio over soda lime, and the residue was extracted with 5 to 7 ml of $CHCl_3$ from which the α-1-bromo-3,4,6-tri-*O*-acetyl–α-D-glucosamine–HBr (I) crystallized on the addition of ether. Dry benzene (3 ml) was added to 186 mg of (I) with 54 μl of triethylamine and stirred, followed by 1 ml of dry benzene containing 95 mg of diphenylphosphoric acid and 54 μl triethylamine. After 3 h the suspension was filtered to remove triethylamine–HBr and the filtrate concentrated in vacuo to a syrup. On addition of 1.4 ml of cold 2.0 N HCl, a precipitate formed which was kept at 4°C overnight. After filtering and washing with ether, the residue (1-diphenylphosphoro-3,4,6-tri-*O*-acetyl–α-D-glucosamine–HCl, II) was dried in vacuo over soda-lime and silica gel. Compound II was recrystallized from methanol-ether, 148 mg of which was dissolved in 4 ml of dry methanol and hydrogenated at room temperature and pressure with 30 mg PtO_2. The filtered cooled solution was treated with 0.89 ml of 0.58 N potassium methoxide, and on standing in the cold, 40 mg of monopotassium crystalline α-D-[1-^{14}C]glucosamine-1-phosphate was obtained. If crystallization did not occur, incomplete removal of the phenyl groups on hydrogenation is indicated, as evidenced by chromatography on Whatman No. 1 paper with ethanol–1 M ammonium acetate, pH 7.2 (5:2 v/v). Two radioactive compounds were obtained, with GlcN-1-P migrating slower than the second compound. Rereduction of the latter converted it to GlcN-1-P.

To prepare UDP-[1-^{14}C]GlcN, 100 μmol of the [1-^{14}C]GlcN-1-P (3.13×10^8 dpm) were converted to the pyridinium salt by passage through a column of Dowex-50 in the pyridinium form and concentrated with 50 μl of tri-*n*-octylamine to dryness in vacuo. The compound was further dried by concentration several times in vacuo with anhydrous pyridine and then taken up in 2 ml of dry dimethylsulfoxide. To this solution was added 45 mg (about 60 μmol) of UMP-morpholidate (Sigma Chemical Co.), which was incubated at room temperature for 5 days. About 5 ml of H_2O was then added and the solution passed through a column (0.9 × 8 cm) of AG 1-X8 formate, 200–400 mesh. The column was first washed with water until the radioactivity eluting was considerably reduced (1.25×10^8 dpm eluted), then with 0.1 N formic acid, which removed another 1.20×10^8 cpm. Finally, 1 N formic acid was used to elute 87×10^6 dpm of UDP-[1-^{14}C]GlcN (about 26 μmol). The eluted compound was lyophilized and taken up in H_2O. It possessed an $A_{280/260}$ ratio of 0.44 and a specific activity of 3.14×10^6 dpm/μmol.

3. Results and discussion

When UDP-GlcN was substituted for UDP-Glc,

Table 1
Conditions for the in vitro incorporation of UDP-[1-^{14}C]GlcN into glycogen

Assay conditions	cpm at origin
Complete system[a]	32 600
Complete system[a] + UDP-Glc(0.1 μmol)	120
Complete system[a] – glycogen	11 600
Complete system[a] – Glc-6-P	848
Complete system[a] – Glc-6-P + GlcN-6-P(1.2 μmol)	26 000

[a] As presented in Materials and methods.

the natural substrate for glycogen synthase, radioactivity was incorporated into a product which remained at the origin on paper chromatography in all of the solvent systems tested (table 1). The formation of this radioactive product was prevented by adding unlabeled UDP-Glc, suggesting that the two nucleotides compete for the same site on glycogen synthase. This conclusion is further supported by the finding that the incorporation of both UDP-GlcN and UDP-Glc is dependent on the presence of glucose 6-phosphate and is stimulated by exogenous glycogen. Glucosamine 6-phosphate substituted for glucose 6-phosphate as a cofactor, with either UDP-Glc or UDP-GlcN as the nucleotide sugar donor.

A comparison of the initial rates of incorporation at saturating levels of both substrates revealed that UDP-Glc is incorporated into glycogen 12 times faster than UDP-GlcN (insert, fig.1). However, with extended periods of incubation, 100% of the UDP-GlcN could be incorporated into the glycogen. No incorporation of radioactivity from UDP-[1-^{14}C]GlcNAc into glycogen was observed.

In order to demonstrate that the glucosamine moiety of UDP-GlcN was actually incorporated into the glycogen and glycosidically linked to terminal glucosyl residues, a large-scale reaction containing 6 μmol of UDP-[1-^{14}C]GlcN (3.42×10^5 cpm/μmol) was conducted, and the radioactive glycogen was purified as follows: ethanol precipitation (3 times), exhaustive dialysis against water, passage through a mixed-bed ion-exchange column, and finally reprecipitation (95% recovery). Acid hydrolysis of the purified glycogen yielded a radioactive product which absorbed completely to AG 50W-X8 (H^+) and was verified as D-glucosamine on the amino acid analyzer.

Incubation of the radioactive glycogen for 4 h with 0.1 mg of β-amylase released 95% of the label as ethanol-soluble material. This compound was purified by absorption and elution from AG 50W-X8 (H^+) and migrated as a single spot on paper chromatography in solvents B and C. A comparison of the mobility of the *N*-acetylated β-amylase product with various markers suggested it to be a disaccharide. This conclusion is

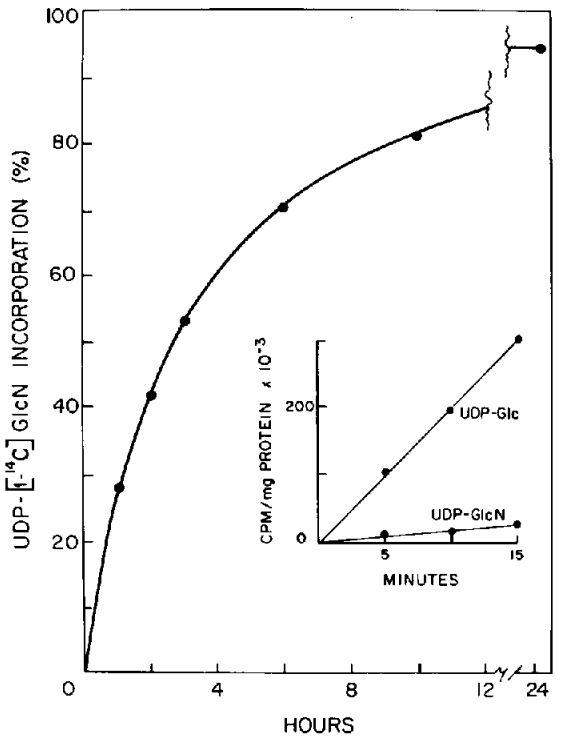

Fig.1. Rate of incorporation of UDP-[1-^{14}C]GlcN into glycogen. A comparison with the rate of incorporation of UDP-[U-^{14}C]Glc into glycogen is presented in the insert. The assay procedure is given in Materials and methods.

Table 2
Composition of the β-amylase disaccharide

Compound	Treatment[a]	Products	μmol
N-acetylated disaccharide	Acid hydrolysis	glucosamine	1.16
		glucose	0.90
Unacetylated disaccharide	Nitrous acid	2,5-anhydromannose	1.03
		glucose	0.92

[a] Analyses were performed as described in Materials and methods.

supported by the finding that on acid hydrolysis equimolar amounts of glucosamine and glucose were obtained (table 2).

To determine the sugar on the reducing end of the *N*-acetylated disaccharide, the compound was reduced with sodium borotritide and subjected to acid hydrolysis. The tritium-labeled product which passed through AG 50W-X8 (H^+) cochromatographed with glucitol in both solvent systems, while the ^{14}C-labeled product obtained on elution of the AG 50W-X8 (H^+) column, comigrated with glucosamine, 2-Amino-2-deoxy[1-^{3}H]glucitol was not detected in this fraction, and both amino sugars were easily separated in the solvent systems used. These results indicate that glucose is on the reducing end of the β-amylase disaccharide. This inference was confirmed by nitrous acid treatment of the unacetylated disaccharide, a procedure known to cleave hexosaminidic linkages [2]. As indicated in table 2, equimolar amounts of 2,5-anhydromannose and glucose were obtained. No evidence for a disaccharide containing 2 mol of glucosamine was obtained, as was found when 2-deoxyglucose was incorporated into glycogen [16].

The foregoing data demonstrate that UDP-GlcN is utilized as a substrate by glycogen synthase and that the glucosamine moiety is transferred to terminal glucosyl residues on the glycogen molecule. It is assumed that the glycosidic linkage of the β-amylase product is α by virtue of the nature of the synthase reaction and because a highly purified jack bean meal β-N-acetylhexosaminidase did not release *N*-acetyl-glucosamine from the *N*-acetylated β-amylase product. The capacity of glycogen synthase to utilize UDP-GlcN in vitro appears to explain the formation of glucosamine-labeled glycogen that is produced in vivo when rats are injected with D-galactosamine [1,2].

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