BIOSYNTHESIS OF DOLICHYL PYROPHOSPHATE TRISACCHARIDE FROM SYNTHETIC DOLICHYL PYROPHOSPHATE DI-*N*-ACETYLCHITOBIOSE AND GDP-D-[^{14}C]MANNOSE IN CALF PANCREAS MICROSOMES

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1. Introduction

The biosynthesis of asparagine-linked oligosaccharides in glycoproteins begins with the transfer of an oligosaccharide from dolichyl pyrophosphate to protein [1,2]. The major oligosaccharide thus transferred has a composition of $Glc_3Man_9GlcNAc_2$ [3,4], and a scheme for its biosynthesis in vivo has been proposed [5]. However, details of the complete enzymic synthesis of the oligosaccharide moiety remain to be elucidated. We have shown that calf pancreas microsomes are very active in the synthesis of dolichyl pyrophosphate oligosaccharides [6,7]. Using synthetic dolichol derivatives as substrates we have characterized the first two enzymic reactions involved in the synthesis of the oligosaccharide moiety [8,9]:

Dol-P + UDP-GlcNAc → Dol-PP-GlcNAc + UMP

Dol-PP-GlcNAc + UDP-GlcNAc → Dol-PP-$(GlcNAc)_2$ + UDP

With this approach we now demonstrate the next enzymic reaction which is the formation of Dol-PP-trisaccharide from synthetic Dol-PP-$(GlcNAc)_2$ and GDP-D-[^{14}C]mannose.

Abbreviations: Dol-P, dolichyl phosphate; Dol-PP-GlcNAc, P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate; Dol-PP-$(GlcNAc)_2$, P^1-di-*N*-acetyl-α-chitobiosyl P^2-dolichyl pyrophosphate; Dol-P-Man, dolichyl β-D-mannosyl phosphate; Dol-PP-trisaccharide, P^1-dolichyl P^2-[*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] pyrophosphate

2. Materials and methods

The source of chemicals is described in [6,9]. GDP-D-[U-^{14}C]mannose (192 mCi/mmol) and Dol-P-[1-^{3}H]Man (0.91 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Dol-P, Dol-PP-GlcNAc and Dol-PP-$(GlcNAc)_2$ were synthesized chemically as in [10]. The synthetic trisaccharide, β-Man*p*-(1→4)-β-GlcNAc*p*-(1→4)-GlcNAc*p*, was prepared as in [11,12]. Jack bean α-mannosidase was purchased from Sigma, Louis, MO. β-Mannosidase from *Polyporus sulfureus* was a gift from Y. T. Li, Tulane University Medical Center, New Orleans, LA.

2.1. *Analytical methods*

Protein was estimated by the method in [13] with bovine serum albumin as standard.

Thin-layer-chromatography (TLC) was performed on Merck precoated plates of silica gel G (0.25 mm thick, Merck, Darmstadt) in solvents: (A) chloroform/methanol/water (60:35:6, by vol.); (B) chloroform/methanol/water (10:10:3, by vol.); (C) 2-propanol/ethyl acetate/water (3:3:2, by vol.); (D) *n*-butanol/acetic acid/water (3:3:2, by vol.). Compounds were detected with the anisaldehyde reagent [14].

Descending paper chromatography was performed on Whatman no. 1 paper in solvents: (E) 1-butanol/pyridine/water (6:4:3, by vol.); (F) 1-butanol/pyridine/water (4:3:4, by vol.); (G) ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Standard sugars were detected with the silver nitrate reagent [15].

Radioactivity was detected on TLC with Kodak

X-Omat R film, and on paper chromatograms with a Packard radiochromatogram scanner model 7201. Radioactivity was determined with a Packard liquid scintillation spectrometer, model 3255, using Aquasol (New England Nuclear, Boston, MA) or Hydrofluor (National Diagnostics, Somerville, NJ) for solutions, and Ready-Solv HP (Beckman, Fullerton, CA) for TLC scrapings.

2.2. *Standard incubation conditions*

Calf pancreas microsomes (total) were prepared by method 2 as in [7]. They were incubated (7–9 mg protein/ml) for 30 min at 37°C in 500 μl total vol. consisting of Tris-maleate buffer (40 mM) (pH 8.2), $MnCl_2$ (10 mM), sodium taurocholate (0.5%), Dol-PP-$(GlcNAc)_2$ (17 μg/ml) and GDP-D-[^{14}C]mannose (0.1 μCi/ml). The solution of Dol-PP-$(GlcNAc)_2$ in chloroform/methanol (2:1, v/v) was first dried under a stream of N_2 in the incubation tubes, and then the other components of the mixture were added.

2.3. *Extraction of radioactively-labeled products*

At the end of the incubation, the tubes were cooled to 4°C and 5 vol. chloroform/methanol (2:1, v/v) were added. The suspension was mixed with a Vortex mixer, kept at room temperature for 10–20 min, mixed again and centrifuged. The lower chloroform/methanol extract was removed with a Pasteur pipette. An aliquot of this extract was subjected to TLC in solvent (A), and then to radioautography. Radioactive areas corresponding to Dol-P-Man and to Dol-PP-trisaccharide were scraped and counted.

2.4. *Preparation of [^{14}C]mannose-labeled Dol-PP-trisaccharide*

For preparative purposes the chloroform/methanol extract obtained from several incubation mixtures scaled up to 3 ml total vol. each was subjected to preparative TLC in solvent A. After radioautography overnight, the area containing the Dol-PP-trisaccharide was scraped and the labeled lipid was eluted by stirring the silica gel in several changes of chloroform/methanol/water (10:10:3, by vol.) at room temperature. The suspension was filtered and the extract was dried under vacuum and redissolved in the same solvent. It was then passed through a column of LH-20 (1 cm × 12 cm) and eluted in chloroform/methanol/water (10:10:3, by vol.). The fractions containing the labeled Dol-PP-trisaccharide were pooled.

2.5. *Treatment with α- and β-mannosidase*

A sample of [^{14}C]mannose-labeled Dol-PP-trisaccharide purified by preparative TLC was dried under N_2 and hydrolyzed in 1 ml 0.01 M HCl for 45 min at 90°C. After neutralization with NaOH the sample was chromatographed on a column of Bio-Gel P-6 (1 cm × 114 cm) in 0.1 M pyridine acetate, (pH 5). Over 90% of the radioactivity was eluted with a V_e/V_o = 2.2. Aliquots of the [^{14}C]trisaccharide (3000 cpm) were then incubated at 37°C for 18 h in either 100 μl 0.05 M glycine–HCl buffer (pH 3.0) containing 2.5 μg cycloheximide, 2.5 μg chloramphenicol and 2.5 munits β-mannosidase, or in 100 μl 25 mM sodium acetate buffer (pH 5) containing 50 mM NaCl, 0.1 mM $ZnSO_4$, 2.5 μg cycloheximide, 2.5 μg chloramphenicol and 1.1 unit α-mannosidase. Control incubations without enzyme were done under the same conditions in each of the buffers. For each enzyme, one unit hydrolyzes 1 μmol of the corresponding anomer of *p*-nitrophenyl-D-mannopyranoside per minute under the conditions of the assay.

3. Results and discussion

The chloroform/methanol extract obtained from calf pancreas rough microsomes incubated with GDP-D-[^{14}C]mannose contained only dolichyl [^{14}C]mannosyl phosphate [16]. When total microsomes were used, small amounts of several ^{14}C-labeled products with a mobility $R_{\text{Dol-P-Man}}$ of 0–0.35 in solvent A were also extracted with chloroform/methanol (2:1, v/v). These labeled compounds are lipid-linked oligosaccharides varying in the size of the oligosaccharide moiety, but shorter than those extracted with chloroform/methanol/water (10:10:3, by vol.). The addition of synthetic Dol-PP-$(GlcNAc)_2$ to the incubation medium stimulated the labeling of several of these compounds. The greatest stimulation was observed for the lipid-bound oligosaccharide with the fastest mobility ($R_{\text{Dol-P-Man}}$ = 0.35). This labeled lipid was eluted and characterized. Its synthesis was proportional to the concentration of Dol-PP-$(GlcNAc)_2$ added to the medium (fig.1) and was not affected by the addition of similar amounts of Dol-P or Dol-PP-GlcNAc. The labeled lipid was adsorbed on DEAE-cellulose and subsequently eluted with 10–50 mM ammonium acetate under conditions for dolichyl pyrophosphoryl derivatives [6,7]. Treatment with mild acid yielded a ^{14}C-labeled product

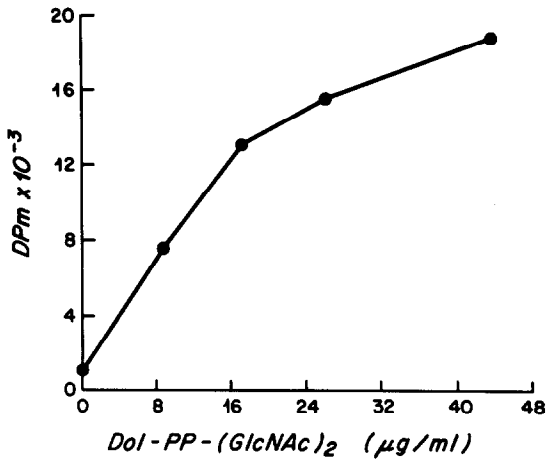

Fig.1. Effect of Dol-PP-$(GlcNAc)_2$ on the incorporation of radioactivity from GDP-D-[^{14}C]mannose into Dol-PP-trisaccharide. Standard conditions of incubation were used for 60 min with various concentrations of Dol-PP-$(GlcNAc)_2$.

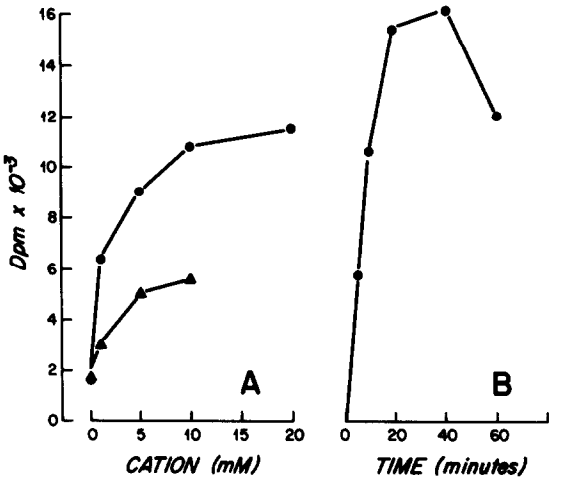

Fig.3. (A) Effect of divalent cations on the formation of Dol-PP-trisaccharide. Various concentrations of either $MnCl_2$ (•) or $MgCl_2$ (▲). (B) Time course of synthesis of Dol-PP-trisaccharide from Dol-PP-$(GlcNAc)_2$ and GDP-D-[^{14}C]mannose.

which migrated with synthetic β-Man*p*-(1→4)β-GlcNAc*p*-(1→4)-GlcNAc*p* on TLC in solvents B,C and D and on paper chromatograms in solvents E (fig.2), F, and G. Treatment of the labeled trisaccharide with β-mannosidase released [^{14}C]mannose, whereas incubation with α-mannosidase had no effect (fig.2). These results indicate that the labeled lipid formed from Dol-PP-$(GlcNAc)_2$ and GDP-D-[^{14}C]-mannose is Dol-PP-trisaccharide with a β-D-mannosyl residue linked to di-*N*-acetylchitobiose.

No labeled Dol-PP-trisaccharide was formed from synthetic Dol-PP-$(GlcNAc)_2$ when GDP-D-[^{14}C]mannose was replaced with Dol-P-[^{3}H]Man under similar conditions. These results show that the β-linked mannose residue comes directly from GDP-D-mannose as shown in other systems [2,17--21]. The optimum pH for synthesis of the Dol-PP-trisaccharide was ~8.2 in Tris-maleate buffer. The formation of Dol-PP-trisaccharide was stimulated by the addition of divalent cations, Mn^{2+} being more effective than Mg^{2+} (fig.3A), and was linear for ~20 min (fig.3B). The synthesis of the Dol-PP-trisaccharide was greatly stimulated (10–30-fold) by the addition of detergent (table 1). However, this stimulation was greater with sodium taurocholate than with Triton X-100, probably because sodium taurocholate depressed the synthesis of Dol-

Fig.2. Effect of α- and β-mannosidase on [^{14}C]trisaccharide. [^{14}C]Mannose-labeled trisaccharide obtained by mild acid treatment of Dol-PP-trisaccharide was treated with either α-mannosidase (– – –) or β-mannosidase (——). Control (. . .) incubations without enzyme showed a similar profile for each buffer used. The samples were chromatographed in solvent E with standards of D-mannose and synthetic trisaccharide. (O) origin.

Table 1
Effect of detergent on the incorporation of radioactivity from GDP-D-[^{14}C]mannose into lipids

Detergent	Dol-PP-$(GlcNAc)_2$ (17 μg/ml)	Radioactivity (dpm)	
		Dol-PP-trisaccharide	Dol-P-Man
None	+	370	8,860
Taurocholate (0.5%)	–	550	230
Taurocholate (0.5%)	+	10,650	900
Triton X-100 (0.1%)	+	3,320	11,170

Standard conditions were used with different detergents, and omitting Dol-PP-$(GlcNAc)_2$, as indicated

P-[^{14}C]Man from GDP-D-[^{14}C]mannose and endogenous Dol-P by ~90% (table 1). It appears that there is a competition for the available GDP-D-[^{14}C]mannose between the enzymes involved in the synthesis of Dol-P-Man and Dol-PP-trisaccharide, so that the synthesis of the latter is enhanced when Dol-P-Man formation is depressed. Under these conditions ~80% of the labeled products in the chloroform/methanol extract was recovered in Dol-PP-trisaccharide. These results demonstrate the third enzymic reaction in the synthesis of Dol-PP-oligosaccharide, using a synthetic substrate:

$$\text{Dol-PP-(GlcNAc)}_2 + \text{GDP-mannose} \rightarrow$$

$$\text{Dol-PP-(GlcNAc)}_2\text{Man} + \text{GDP}$$

The conditions described provide a useful source of labeled substrate to study subsequent elongation of the oligosaccharide moiety.

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