Solubilization of an α-(1⟶3)-D-mannosyltransferase from pancreas which utilizes synthetic dolichyl pyrophosphate trisaccharide β-Man-(1⟶4)-β-GlcNAc-(1⟶4)GlcNAc as substrate

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Calf pancreas microsomes were treated with 0.5–1% Triton X-100 and the resulting soluble enzyme preparation was incubated with GDP-D-[^{14}C]mannose. The addition of synthetic Dol-PP derivative of the trisaccharide β-Man-(1⟶4)-β-GlcNAc-(1⟶4)GlcNAc stimulated the synthesis of labeled lipid-bound tetrasaccharide 50–100-fold. The labeled tetrasaccharide thus formed was identified as α-Man-(1⟶3)-β-Man-(1⟶4)-β-GlcNAc-(1⟶4)GlcNAc by its chromatographic properties and by its sensitivity to α-mannosidase and to endo-β-*N*-acetylglucosaminidase D. The solubilized α-(1⟶3)mannosyltransferase did not require divalent cation and was active in the presence of 10 mM EDTA.

(1⟶3)-D-Mannosyltransferase *Dolichyl pyrophosphate* *Tetrasaccharide biosynthesis*

1. INTRODUCTION

The major pathway for the biosynthesis of *N*-linked oligosaccharides in glycoproteins involves the transfer of a $Glc_3Man_9GlcNAc_2$ oligosaccharide from dolichyl pyrophosphate to protein. A scheme for the biosynthesis of this oligosaccharide, based upon the characterization of dolichol-linked oligosaccharides labeled in intact cells, has been proposed [1]. However, detailed knowledge of the enzymology of this pathway is still incomplete. Using synthetic dolichol derivatives as substrates we have characterized the 3 enzymes involved in the biosynthesis of Dol-PP-trisaccharide containing a β-mannose linked to di-*N*-acetylchitobiose [2–4]. Here, we report the solubilization and partial characterization of the α-(1⟶3)-D-mannosyltransferase from pancreas responsible for the formation of Dol-PP-tetrasaccharide from synthetic Dol-PP-trisaccharide and GDP-D-[^{14}C]mannose.

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Abbreviations: 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-β-D-glucopyranosyl]pyrophosphate; Dol-PP-tetrasaccharide, P^1-dolichyl-*P*^{2}-[*O*-α-D-mannopyranosyl-(1⟶3)-*O*-β-D-mannopyranosyl-(1⟶4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1⟶4)-2-acetamido-2-deoxy-β-D-glucopyranosyl] pyrophosphate; Dol-P-Man, dolichyl β-D-mannopyranosyl phosphate; Dol-P, dolichyl phosphate; BSA, bovine serum albumin

2. MATERIALS AND METHODS

The source of chemicals has been described in [3]. The chemical synthesis of Dol-PP-trisaccharide and of the tetrasaccharide, α-D-

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Man-*p*-(1⟶3)-β-D-Man*p*-(1⟶4)-β-D-Glc*p*NAc-(1⟶4)-D-GlcNAc will be described separately (submitted). Endo-β-*N*-acetylglucosaminidase D was purchased from Miles (Elkhart IN). Jack bean α-mannosidase was obtained from Sigma (St Louis MO) and concanavalin A–Sepharose from Pharmacia (Piscataway NJ).

2.1. *Analytical methods*

Protein was estimated as in [5] using BSA as stansdard and carbohydrate was monitored with phenol/sulfuric acid [6]. 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.); and (B) chloroform/methanol/water (10:10:3, by vol.). Compounds were detected on TLC with the anisaldehyde reagent [7]. Gel filtration was performed on a column (1 × 110 cm) of Bio-Gel P-6 (200–400 mesh) in 0.1 M pyridine acetate buffer (pH 5.0) with bovine serum albumin to determine V_o and D-mannose for V_e. Fractions of 1 ml were collected and assayed for radioactivity and carbohydrate. High pressure liquid chromatography (HPLC) was done with a Varian model 5000 apparatus using a Hibar EC column (4 mm × 250 mm) packed with 5 μm Lichrosorb NH_2 (EM Reagents, Gibbstown NJ). Acetonitrile/water (60:40, v/v) pumped at 1 ml/min was used for elution, and 1-ml fractions were collected and assayed for radioactivity.

Radioactivity was located on TLC with Kodak X-Omat R film, and was determined with a Packard liquid scintillation spectrometer, model 3255 or an LKB Rackbeta, model 1218 using Hydrofluor (National Diagnostics, Somerville NJ), or Aquassure (New England Nuclear, Boston MA) for solutions, and Ready-Solv HP (Beckman, Fullerton CA) for TLC scrapings.

2.2. *Standard assay conditions*

Calf pancreas microsomes were prepared by method 2 as in [8] and kept frozen at −80°C until use. The solubilized enzyme source was prepared by suspending the microsomes in 50 mM Tris–maleate buffer (pH 6.8) containing 1% Triton X-100, using a hand-driven Potter-Elvejehm homogenizer with a Teflon pestle; after standing in ice for 1–2 h, the mixture was centrifuged for 1 h at 40000–50000 rev./min in a Beckman rotor 50 Ti. An aliquot (200 μl) of the supernatant was then immediately incubated at 37°C for 30 min in a total volume of 250 μl containing 0.05 μCi GDP-D-[^{14}C]mannose (spec. act., 269 mCi/mmol). Synthetic Dol-PP-trisaccharide was added to the incubation tube from a chloroform/methanol (2:1, v/v) solution, and the solvent was evaporated under a stream of N_2 before addition of the other components. At the end of the incubation, the products extracted with chloroform/methanol (2:1, by vol.) and with chloroform/methanol/water (10:10:3, by vol.) were isolated as in [8]. TLC of these lipid fractions was followed by radioautography and the radioactivity in Dol-PP-tetrasaccharide was estimated by scraping the appropriate area of silica gel.

2.3. *Preparation of labeled tetrasaccharide*

The labeled products in the lipid extracts were hydrolyzed with mild acid as in [4,8]. The hydrolysate was either chromatographed on a column of Bio-Gel P-6, or was passed through coupled columns (0.5 × 5 cm) of AG 50W-X8 (H^+-form, 200–400 mesh) and AG 1-X8 (formate form, 200–400 mesh), concentrated and then fractionated by HPLC as above.

2.4. *Treatment with endo-β-N-acetylglucosaminidase D*

Labeled tetrasaccharide obtained by gel filtration or by HPLC was mixed with 200 μg of synthetic tetrasaccharide and incubated for 18 h at 37°C in a total volume of 100 μl containing 0.05 M citrate/phosphate buffer (pH 6.0), 0.5 M NaCl, 0.05% BSA, 25 μg cycloheximide, 25 μg chloramphenicol and 10 munits of endo-β-*N*-acetylglucosaminidase D. At the end of the incubation the mixture was boiled for 3 min and the samples were chromatographed on Bio-Gel P-6. One-ml fractions were collected and assayed for radioactivity. Unlabeled carbohydrate was detected with the phenol/sulfuric acid reagent.

2.5. *Treatment with α-mannosidase*

Labeled tetrasaccharide was incubated for 18 h at 37°C in a total volume of 100 μl containing 0.01 M sodium acetate (pH 5.0), 0.025 M NaCl, 0.02 mM $ZnSO_4$, 5% bovine serum albumin, 25 μg cycloheximide, 25 μg chloramphenicol and 1.4 units of α-mannosidase. At the end of the incuba-

tion the mixture was boiled for 3 min and chromatographed on Bio-Gel P-6.

3. RESULTS AND DISCUSSION

When calf pancreas microsomes were incubated with GDP-D-[^{14}C]mannose, the addition of synthetic Dol-PP-trisaccharide greatly stimulated the incorporation of [^{14}C]mannose into several lipid-bound oligosaccharides extracted with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.). TLC of the chloroform/methanol extract in solvent A, and of the chloroform/methanol/water extract in solvent B showed that the labeling of several lipid-bound oligosaccharides was stimulated. The greatest stimulation was observed for the products with the fastest mobility on TLC, and the shortest oligosaccharides after mild acid hydrolysis. Addition of detergent (Triton X-100 or Nonidet P-40) greatly increased the extent of this stimulation; the maximum effect was observed at concentrations of 0.5–1.0%. Under these conditions, fewer products were formed, and about 85% of the enzyme activity observed in the presence of synthetic Dol-PP-trisaccharide was solubilized.

Incubation of the soluble enzyme preparation with GDP-D-[^{14}C]mannose followed by TLC of the chloroform/methanol extract showed that the addition of synthetic Dol-PP-trisaccharide stimulated by 50–100-fold the formation of a major product with $R_{\text{Dol-P-Man}}$ of about 0.2 (fig.1). In some experiments, but not in all cases, the formation of Dol-P-Man was also greatly stimulated by the addition of synthetic Dol-PP-trisaccharide, possibly because of degradation of the latter to Dol-P.

Upon mild acid treatment (0.1 M HCl at 90°C for 30 min) the labeled glycolipid (compound B in fig.1) yielded a labeled oligosaccharide which was not retained on concanavalin A–Sepharose and which had the same chromatographic mobility (R_f = 0.1) on TLC in solvent B, and the same elution profile from Bio-Gel P-6 (fig.2) as the synthetic tetrasaccharide, α-Man-(1⟶3)-β-Man-(1⟶4)-β-GlcNAc-(1⟶4)GlcNAc. Treatment of the labeled tetrasaccharide with α-mannosidase released all the labeled mannose, and incubation with endo-β-*N*-acetylglucosaminidase D resulted in the formation of a labeled product which was

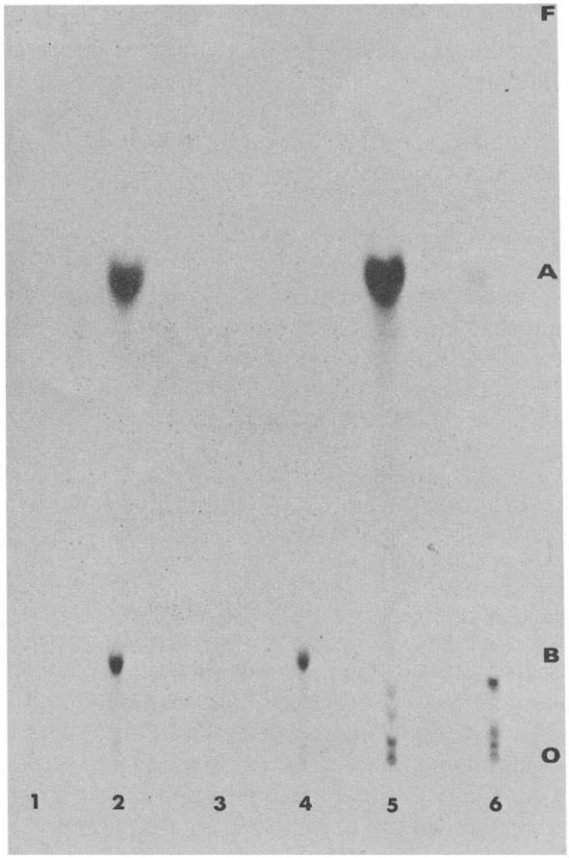

Fig.1. TLC of chloroform/methanol (2:1, v/v) extract in solvent A. Incubation of the solubilized preparation with GDP-D-[^{14}C]mannose was performed under standard conditions with: (1) no addition; (2) 40 μg/ml Dol-PP-trisaccharide; (3) 10 mM EDTA; (4) 40 μg/ml Dol-PP-trisaccharide and 10 mM EDTA; (5) 40 μg/ml Dol-PP-trisaccharide and 10 mM $MnCl_2$; (6) 10 mM $MnCl_2$. Radioautography of the TLC was obtained after 4 days of exposure: (O) origin; (F) solvent front; (A) Dol-P-Man; (B) Dol-PP-tetrasaccharide.

eluted with α-Man-(1⟶3)-β-Man-(1⟶4)-GlcNAc; the latter compound was obtained by endo-β-*N*-acetylglucosaminidase D treatment of the synthetic tetrasaccharide (fig.2). Since endo-β-*N*-acetylglucosaminidase D has a specific requirement for the presence of a terminal α-1,3-linked mannose residue attached to the β-linked mannose [9], these results demonstrate that the labeled tetrasaccharide has the structure, α-D-Man-*p*(1⟶3)-β-D-Man*p*-(1⟶4)-β-Glc*p*NAc-(1⟶4)-D-GlcNAc.

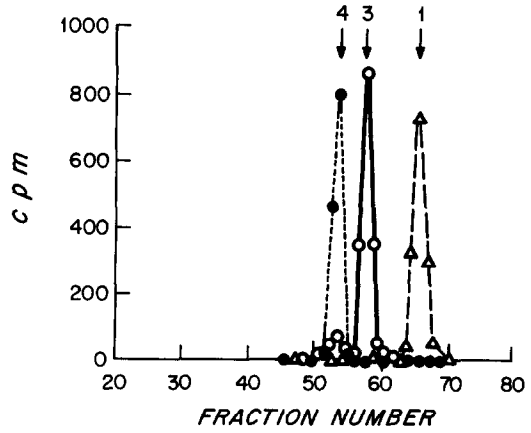

Fig.2. Effect of α-mannosidase and endo-β-*N*-acetylglucosaminidase D. Labeled tetrasaccharide mixed with synthetic tetrasaccharide was chromatographed on Bio-Gel P-6 before (●---●) and after treatment with α-mannosidase (Δ---Δ) or with endo-β-*N*-acetylglucosaminidase D (○—○) as in section 2. The positions of (1) Mannose, (3) α-Man-(1⟶3)-β-Man(1⟶4)-GlcNAc, (4) α-Man-(1⟶3)-β-Man(1⟶4)-β-GlcNAc, are indicated.

The formation of the labeled Dol-PP-tetrasaccharide was proportional to the concentration of synthetic Dol-PP-trisaccharide in the medium (fig.3), and was time-dependent for about 30 min incubation at 32 μg Dol-PP-trisaccharide/ml (fig.4). The addition of Mn^{2+} inhibited the synthesis of the tetrasaccharide–lipid, presumably because the formation of Dol-P-Man and of other lipid-bound oligosaccharides was enhanced in the presence of divalent cation (fig.1). Furthermore, the synthesis of Dol-PP-tetrasaccharide was observed in the presence of 10 mM EDTA; under these conditions Dol-P-Man synthesis was completely inhibited (fig.1). These results indicate that the α-(1⟶3)-D-mannosyltransferase from pancreas has no requirement for divalent cation, in direct contrast to the α-(1⟶6)-D-mannosyltransferase from the same tissue (submitted). It is also different from the liver α-(1⟶3)-D-mannosyltransferase described in [10]. The latter enzyme required divalent cation for optimum activity and was greatly inhibited by as low as 0.1% detergent. The reasons for the different properties of the liver and pancreas α-(1⟶3)-D-mannosyltransferases are not clear, but it should be pointed out that the

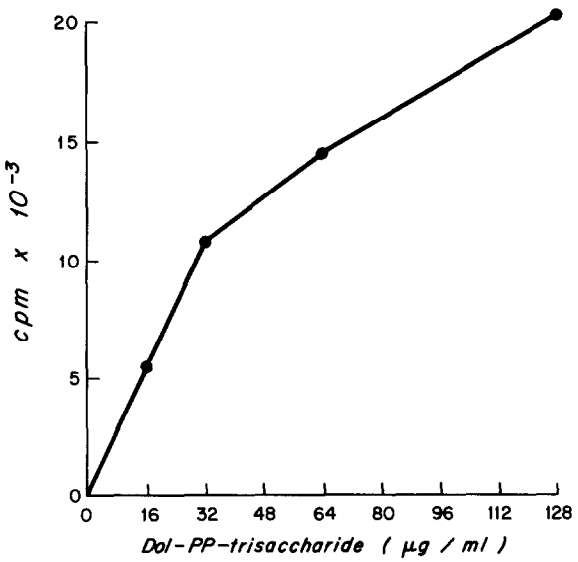

Fig.3. Effect of synthetic Dol-PP-trisaccharide concentration on labeling of Dol-PP-tetrasaccharide from GDP-D-[^{14}C]mannose. The soluble enzyme preparation was incubated with GDP-D-[^{14}C]mannose under standard conditions, but with different concentrations of synthetic Dol-PP-trisaccharide as indicated. At the end of the incubation, the chloroform/methanol extract was chromatographed in solvent A and the radioactivity corresponding to Dol-PP-tetrasaccharide was located by radioautography and estimated by scraping the appropriate area.

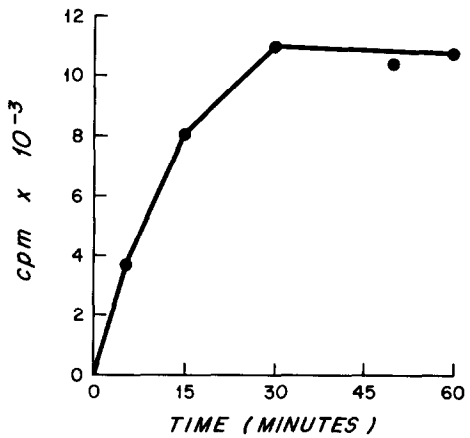

Fig.4. Time course of Dol-PP-tetrasaccharide synthesis. The soluble enzyme preparation was incubated for different periods of time under standard conditions with 32 μg/ml synthetic Dol-PP-trisaccharide. The incorporation of radioactivity from GDP-D-[^{14}C]mannose into Dol-PP-tetrasaccharide was determined after TLC as described in fig.3.

substrate used for the characterization of the liver enzyme was a mixture of tetrasaccharide–lipids with both α-(1⟶3)- and α-(1⟶6)-mannose residues attached to the β-D-mannose residues, and although the liver mannosyltransferase activity was partially purified, the preparation still contained both α-(1⟶3)- and α-(1⟶6)-D-mannosyltransferase activities. The α-(1⟶3)-mannosyltransferase described here is likely to be the enzyme responsible for the biosynthesis of dolichyl pyrophosphate tetrasaccharide according to the scheme proposed in [1].

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