FORMATION OF LIPID-LINKED SUGAR COMPOUNDS IN *VOLVOX CARTERI* F. *NAGARIENSIS* IYENGAR

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1. Introduction

During the last decade evidence has accumulated that polyisoprenyl phosphate intermediates have an important function as 'carriers' of activated glycosyl groups in the glycosylation processes in many mammalian tissues [1] as well as in plants [2–4] and yeasts [5,6], although there are still large gaps in our knowledge of the complete reaction sequence and the nature of polypeptides that are glycosylated.

Little information is available on the formation and significance of polyisoprenyl-linked sugars in algae. The synthesis of dolichyl-P-glucose by membrane fractions of the non-photosynthetic alga *Prototheca zopffi* was demonstrated [7]. This alga was also shown to be equipped with an enzyme system capable of synthesizing polyisoprenyl lipids, which appear to be identical with dolichol compounds from other sources.

Here we report on both dolichyl phosphate- and dolichyl pyrophosphate-activated sugar intermediates, which are synthesized by membrane bound glycosyltransferases from the alga *Volvox carteri* f. *nagariensis*.

2. Materials and methods

2.1. *Materials*

GDP-[^{14}C]mannose (spec. act. 166 Ci/mol), UDP-[^{14}C]galactose (347 Ci/mol) and UDP-*N*-acetyl-[^{14}C]glucosamine (323 Ci/mol) were obtained from Radiochemical Centre, Amersham. Unlabeled nucleoside diphospho-sugars and dolichyl phosphate were purchased from Sigma. DEAE-cellulose was from Serva. Dol-P-P-*N*-acetyl-[^{14}C]glucosamine and Dol-P-P-di-*N*-acetyl-[^{14}C]chitobiose were synthesized according to [8]. All other chemicals and solvents used were of reagent grade.

Abbreviations: Dol-P, Dol-P-P, dolichyl mono(di)phosphate; GlcNAc, *N*-acetylglucosamine

2.2. *Preparation and assay of particulate membrane*

HK 10 asexual strains of *Volvox carteri* f. *nagariensis* were grown as in [9] and harvested after 4–6 days. Cells were separated from the culture medium by filtration and washed 3 times with 100 mM Tris/HCl (pH 7.4) containing 5 mM mercaptoethanol and 1 mM EDTA. The same buffer was used for cell disruption with the Bio-X-press. After thawing, the crude homogenate was centrifuged at 8000 × *g* for 20 min. Centrifugation of the supernatant at 100 000 × *g* for 2 h yielded the particulate fraction, which was used as the enzyme source.

Standard incubation mixtures for the synthesis of the various $CHCl_3/CH_3OH$ (2/1) soluble products contained the following components in 100 μl final vol.: 0.8% Triton X-100, 10 mM $MgCl_2$, 4 mM mercaptoethanol, 0.8 mM EDTA, 80 mM Tris/HCl (pH 7.4) nucleoside diphospho-sugars (5×10^4 to 5×10^5 cpm) and 100–300 μg membrane protein. Incubations were run at room temperature and stopped by adding 1 ml $CHCl_3/CH_3OH$ (2/1) at chosen times. Lipids were extracted by the procedure in [10]. When Dol-P (5–50 μg in benzene) was added, the solvent was first evaporated and then continued as described above.

2.3. *Analytical procedures*

Lipids were fractionated by DEAE-cellulose chro-

matography as in [11]. Mild acid and alkaline hydolysis of dolichol-linked saccharides was according to [12]. Protein was estimated by the Lowry method [13], using bovine serum albumin as a standard. Transfer of Dol-P-P-activated di-*N*-acetyl-[^{14}C]chitobiose to exogenous peptide substrates was carried out as in [14]. Thin-layer chromatography of lipid-linked and free sugars was done on Silica gel plates (Merck, Darmstadt) using the following solvent systems: (A) $CHCl_3$/CH_3OH/1 M NH_4OH (65/25/4, v/v/v); (B) *n*-butanol/acetone/H_2O (4/5/1, v/v/v); (C) *n*-butanol/acetic acid/H_2O (4/1/5, v/v/v). Radioactive material was detected either by radioautography with Sakura QH X-Ray Film or measured in a liquid scintillation counter (Delta 300, Searle Analytic Inc.).

3. Results

3.1. *Formation and characterization of* N-*acetyl-[^{14}C]glucosamine-labeled lipids*

Particulate membrane fractions of *Volvox carteri* f. *nagariensis* catalyze the transfer of *N*-acetyl-glucosamine (GlcNAc) from UDP-GlcNAc into $CHCl_3$/CH_3OH soluble products. Figure 1 shows the time course of incorporation into the lipid fraction. Figure 2 demonstrates that this transfer is strongly stimulated by exogenous Dol-P and requires both the presence of divalent cations and detergent. The app.

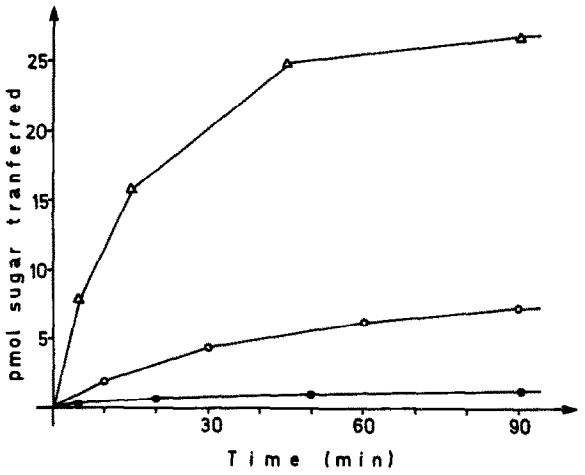

Fig.1. Time course of galactose (–△–△–), mannose (–●–●–) and *N*-acetyl-glucosamine (–○–○–) imcorporation into the $CHCl_3$/CH_3OH (2/1) soluble fraction. Incubation conditions see section 2. Nucleotide sugar was 0.7 μM.

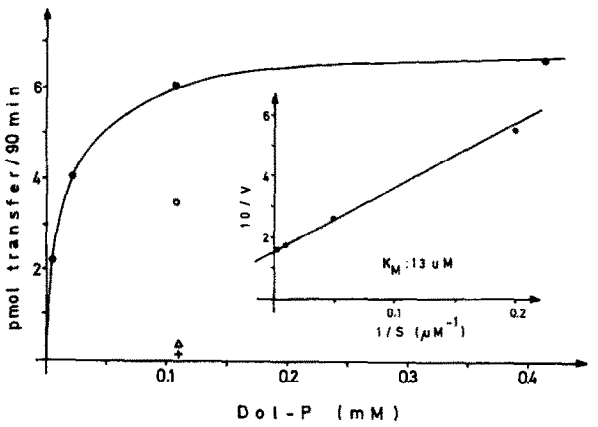

Fig.2. Transfer of *N*-acetyl-[^{14}C]glucosamine to $CHCl_3$/CH_3OH (2/1) soluble material as a function of Dol-P concentration. Standard incubations as in section 2 in the absence of (△) Me^{2+}, (+) Triton X-100 and in the presence of (○) 10 mM Mn^{2+} or (●) 10 mM Mg^{2+}.

K_m for Dol-P is ~13 μM. Optimal incorporation was observed at 0.8–0.9% Triton X-100 conc.

DEAE-cellulose chromatography of the [^{14}C]glycolipids yielded a product (fig.3, peak B), which eluted as a single peak at ~0.15 M NH_4-acetate in a gradient of 0–0.2 M NH_4acetate (pH 6.0) in $CHCl_3$/CH_3OH/H_2O (10/10/3, v/v/v), a position at which glycosyl polyisoprenyl pyrophosphates elute. Dol-P-[^{14}C]mannose, isolated from rat liver, elutes as monophospho-derivative at a corresponding lower salt concentration (fig.3, peak A). Thin-layer chromatography of peak B

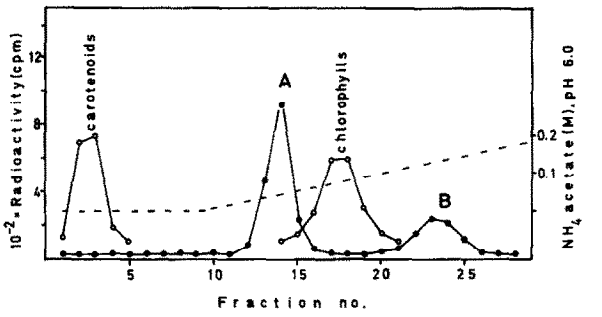

Fig.3. DEAE-cellulose chromatography of glycolipids obtained from incubations of (A) GDP-[^{14}C]mannose or UDP-[^{14}C]galactose and (B) UDP-*N*-acetyl-[^{14}C]glucosamine with particulate enzyme. The glycolipids were poured onto a DEAE-cellulose column (1 × 12 cm) in $CHCl_3$/CH_3OH/H_2O (10/10/3, v/v/v) and eluted with a linear gradient of NH_4-acetate (pH 6.0). Fractions of 4 ml were collected.

on silica gel revealed the existence of two ^{14}C-labeled compounds, the slower moving one increasing with longer incubation times. Both compounds co-chromatographed with the reference substances Dol-P-P-GlcNAc and Dol-P-P-GlcNAc$_2$, which were isolated from rat liver [8]. The total yield of both glycolipids was higher with Mg^{2+} as the stimulating cation than with Mn^{2+} (see fig.2). The relative amount of disaccharide, however, increased, when Mn^{2+} was used. Mild acid hydrolysis of the purified Dol-P-P-monosaccharide resulted in a radioactive product, which co-chromatographed with *N*-acetyl-glucosamine on silica gel in solvent C.

Recent studies have demonstrated that Dol-P-P-di-*N*-acetyl-chitobiose functions as glycosyl donor in the *N*-glycosylation of exogenous peptides [14,15]. When the Dol-P-P-disaccharide, synthesized in the *Volvox* system, is incubated with rat liver microsomes in the presence of the hexapeptide Tyr–Asn–Leu–Thr–Ser–Val, the carbohydrate residue is transferred to the hexapeptide in similar yield as was observed for the same glycolipid isolated from rat liver, indicating that a dolichyl pyrophosphate-linked disaccharide has been formed.

Incubation of membrane fraction with UDP-[^{14}C]-GlcNAc in the presence of GDP-mannose leads to the formation of an additional labeled compound with a lower R_F-value relative to Dol-P-P-GlcNAc$_2$, probably originating from a transfer of mannose to the latter glycolipid to give a polyisoprenyl pyrophosphate-bound trisaccharide. With unlabeled UDP-GlcNAc and GDP-[^{14}C]mannose the formation of this same [^{14}C]glycolipid was observed, which was not obtained, when UDP-GlcNAc was omitted.

3.2. *Transfer of sugars from GDP-[^{14}C]mannose and UDP-[^{14}C]galactose to dolichyl phosphate*

Figure 1 shows that both sugars are transferred to $CHCl_3/CH_3OH$ (2/1) soluble compounds. As with UDP-GlcNAc, ^{14}C incorporation into the lipid fraction is also stimulated by exogenous Dol-P and is dependent on divalent cations (table 1). The stimulation of the transfer by exogenous Dol-P suggests that both sugar lipids are dolichyl derivatives. Chromatography on DEAE-cellulose gave elution characteristics identical to Dol-P-mannose from rat liver, indicating that both compounds may be dolichyl monophospho-saccharides (fig.3A). Both glycolipids are stable to mild alkaline treatment known to cause cleavage of fatty acid esters [16], whereas mild acid hydrolysis released the free monosaccharides. Analysis of the water soluble fraction, obtained after acid hydrolysis, by chromatography on silica gel in solvent B yielded [^{14}C]glucose as the only radioactive product, when incubations were run with UDP-[^{14}C]galactose as the glycosyl donor.

4. Discussion

In this study a particulate membrane fraction of *Volvox carteri* f. *nagariensis* was examined for the ability to transfer sugars from the nucleotide stage to lipid-extractable products. In all experiments a stimulation of incorporation into lipids by exogenous Dol-P was observed, strongly suggesting that dolichyl phospho-sugars have been synthesized (table 1). This assumption is supported and confirmed by a number of analytical criteria, such as mild acid and alkaline

Table 1
Transfer of sugars from nucleoside diphospho-sugars to lipid

Substrate	Transfer into lipid fraction		
	No Dol-P added 10 mM Mg^{2+}	Dol-P (10 μg) added no Mg^{2+}	10 mM Mg^{2+}
GDP-[^{14}C]mannose	1 pmol	1 pmol	7.7 pmol
UDP-[^{14}C]galactose	4.2 pmol	3.4 pmol	150 pmol
UDP-[^{14}C]GlcNAc	4.2 pmol	1.5 pmol	40 pmol

Assay for incorporation into lipid was done as in section 2. Sugar substrate was 5 μM

hydrolysis, elution characteristics on DEAE-cellulose chromatography and thin-layer chromatography with corresponding reference substances.

The use of UDP-GlcNAc as glycosyl donor gives rise to the formation of Dol-P-P bound mono- and disaccharides. When incubations are carried out in the presence of both UDP-GlcNAc and GDP-mannose, an additional product is observed, which seems to be a Dol-P-P-linked trisaccharide. The exact nature of this compound is under investigation. Nevertheless synthesis of these sugar-lipid derivatives represents primer reactions in a presumably general, but still tentative reaction scheme [1], which leads to the formation of lipid-bound oligosaccharides and finally results in the *N*-glycosylation of peptides and proteins, respectively. In addition, the general significance of polyisoprenyl intermediates for glycosylation in membranes is emphasized by the following observations:

(i) Dol-P, isolated from pig liver, functions as glycosyl acceptor in *Volvox*

(ii) Dol-P-P derivatives, synthesized in *Volvox*, act as glycosyl donors for peptide glycosylation with rat liver microsomes.

In a variety of tissues it has been demonstrated that the Dol-P-P-linked trisaccharide is elongated via Dol-P-mannose as glycosyl donor, before the completed oligosaccharide unit is transferred to protein [1]. It is not surprising therefore that Dol-P-mannose synthesizing enzyme activities are normally high. In this aspect it is remarkable that the amount of Dol-P-mannose, synthesized by *Volvox* membranes is extremely low in comparison to other membrane systems. One reason may be lack or inhibition of the corresponding glycosyltransferases. A more plausible explanation may be that GDP-mannose instead of Dol-P-mannose is the immediate donor for the chain elongation to oligosaccharides.

Yields of glycolipid synthesis are ~20-times higher in comparison to the sugar transfer from GDP-mannose, when UDP-galactose functions as glycosyl donor (fig.1, table 1). However, analysis of the lipid-soluble compound revealed that pure Dol-P-glucose has been formed instead of Dol-P-galactose. This unexpected result may be interpreted in terms of an epimerase present in the enzyme preparation. Kinetic studies and analysis of lipid and water-soluble products showed that the epimerisation occurs only at the nucleoside diphosphate stage. At present no reason can be given for the surprisingly high rate of synthesis of Dol-P-glucose, just as the significance of this compound in metabolism is obscure. Nevertheless one is tempted to speculate that Dol-P-glucose may function as an intermediate in the formation of those glucoproteins, which might be involved as precursors in the biosynthesis of polysaccharides, as is indicated in studies [17] on plant polysaccharide synthesis. This would agree with the finding that large quantities of matrix material, probably a polysaccharide, are synthesized during growth cycles of *Volvox* colonies. Finally it may be noted that the *Volvox carteri* inducer glycoprotein [9] contains a remarkably high proportion of glucose.

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