Plant Physiol. (1978) 61, 248-251

Biosynthesis of Dolichyl Phosphate

CHARACTERIZATION AND SITE OF SYNTHESIS IN ALGAE[1]

Received for publication June 13, 1977 and in revised form September 9, 1977

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ABSTRACT

This is the first report not only on the presence of polyprenyl phosphates and their site of synthesis in algae, but also on the formation of their sugar derivatives in this system.

A glucose acceptor lipid was isolated from the nonphotosynthetic alga *Prototheca zopfii*. The lipid was acidic and resistant to mild acid and alkaline treatments. The glucosylated lipid was labile to mild acid hydrolysis and resistant to phenol treatment and catalytic hydrogenation, as dolichyl phosphate glucose is. These results are consistent with the properties of an α-saturated polyprenyl phosphate.

The polyprenylic nature of the lipid was confirmed by biosynthesis from radioactive mevalonate. The [^{14}C]lipid had the same chromatographic properties as dolichyl phosphate in DEAE-cellulose and Sephadex LH-20. Strong alkaline treatment and enzymic hydrolysis liberated free alcohols with chain lengths ranging from C_{90} to C_{105}, C_{95} and C_{100} being the most abundant molecular forms. The glucose acceptor activity of the biosynthesized polyprenyl phosphate was confirmed.

The ability of different subcellular fractions to synthesize dolichyl phosphate was studied. Mitochondria and the Golgi apparatus were the sites of dolichyl phosphate synthesis from mevalonate.

The role of polyprenyl phosphates as sugar carriers in the synthesis of bacterial wall polymers is now well established (10). In addition, considerable evidence has accumulated, indicating that this type of compound serves as an intermediate in the formation of glycoproteins in higher plants (9, 12) and yeast (2, 13).

The polyprenylic nature of the carrier lipids has not been clearly established in all systems. However, it seems quite clear that the presence of sugar acceptors with the properties of long chain α-saturated polyprenyl phosphates, as Dol-P,[5] may be a general feature of eukaryotic cells, since they have now been found in yeast (11), mammals (17), and plants (4, 19). On the other hand, the sugar acceptors in prokaryotic cells are short chain allylic deca- or undecaprenyl phosphates (10).

[1] This work was partially supported by a grant from the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina).

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[5] Abbreviations: Dol: dolichol; Dol-P: dolichyl phosphate: Fic: ficaprenol; Fic-P: ficaprenyl phosphate; AL-P: algal lipid phosphate; MVA: mevalonic acid.

This paper reports the identification of a glucose acceptor in the nonphotosynthetic alga *Prototheca zopfii* as Dol-P. The biosynthesis of algal Dol-P as well as its site at subcellular level have been studied.

MATERIALS AND METHODS

Chemicals. *RS*-[2-^{14}C]mevalonic acid lactose (13 Ci/mol) was obtained from Amersham/Searle Corp. UDP-[U-^{14}C]glucose (263 Ci/mol) was obtained from the Instituto de Investigaciones Bioquímicas "Foundación Compomar". Dol, Lot No. 65C-7202, from Sigma, is composed of five molecular species: C_{85} to C_{105}. The greatest concentration corresponds to C_{95}. Dol-P was obtained from Sigma. Ficaprenol, a mixture of C_{50} and C_{55} alcohols, was isolated and phosphorylated as described (20). Liver Dol-P-[^{14}C]glucose was a gift from Dr. R. Staneloni. All other chemicals were analytical grade.

Enzyme Preparation. *P. zopfii* strain PR-5, a colorless Chlorophyta, was obtained from the American Type Culture Collection (ATCC 16533). Cells were grown on 0.8% glucose and 0.2% peptone at 25 C. The cells were harvested from log phase cultures at 50% of maximum growth, washed three times with 50 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, and 1 mM EDTA. The same buffer was used for cell disruption by sonication (100 w, 30 min with 5 μm of glass powder in a cooling cell with circulating methanol at −30 C). The homogenate was centrifuged at 25,000*g* for 30 min. Aliquots of the resulting supernatant (S_{25}) were used as enzyme source for biosynthetic assays. Centrifugation of the S_{25} fraction at 100,000*g* for 120 min gives the pellet (P_{100}) used in the glucosylation reactions.

Isolation of Acceptor Lipid. Five hundred g of algal cells were extracted twice overnight with chloroform-methanol (2:1, v/v) and the extract filtered. The filtrate was washed by the method of Folch *et al.* (8) and poured into a DEAE-cellulose column (12 × 450 mm) in the acetate form (20). The column was washed with chloroform-methanol (2:1) and eluted with a gradient of 0 to 0.4 M ammonium acetate in the same solvent. Fractions of 4 ml were collected. Aliquots of each fraction were washed by the method of Folch and assayed for glucose acceptor activity. Active fractions were pooled and concentrated under N_2.

Lipid Glucosylation Assay. The sample to be assayed (Dol-P, Fic-P, or AL-P) was mixed with 0.5 μmol Mg-EDTA and evaporated to dryness under N_2. The components of the reaction mixture were added in a final volume of 50 μl: 0.6% Triton X-100, 2.5 μmol of Tris-HCl (pH 7.5), 0.5 μmol of β-mercaptoethanol, 0.4 nmol of UDP-[^{14}C]glucose (263 Ci/mol), and 100 to 300 μg of P_{100} protein. Standard incubations were carried out for 30 min at 25 C. The reaction was stopped by addition of 0.1 ml of butanol and extracted as described (20). Only fresh enzyme preparations exhibit glycosylating activity.

Biosynthesis of Endogenous Lipid. Standard incubations were

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carried out at 25 C for 90 min in a total volume of 50 μl of the following: 2.5 μmol of Tris-HCl (pH 7.5), 0.25 μmol of β-mercaptoethanol, 50 nmol of EDTA, 0.5 μmol of NaF, 0.5 μmol of ATP, 0.5 μmol of $MgCl_2$, 0.35 nmol of *RS*-[2-^{14}C]MVA (13 Ci/mol), and S_{25} enzyme or subcellular fractions containing 100 to 200 μg of protein. Free lipids and hydrocarbons were extracted three times with 0.1 ml of petroleum ether (b.p. 35–65 C). Prenyl phosphates were extraced with chloroform-methanol (2:1, v/v) and washed by the Folch method.

Enzymic Hydrolysis of Polyprenyl Phosphates. Polyprenyl phosphates resistant to mild acid hydrolysis were incubated with alkaline phosphatase from *Escherichia coli* (Sigma) at 37 C for 20 hr in the following medium: 2.5 μmol of Tris-HCl (pH 8.4), 0.5 μmol of $MgCl_2$, 0.1% Triton X-100, and 100 μg of alkaline phosphatase, in 50% methanol in a total volume of 50 μl. Another 100 μg of alkaline phosphatase was added after the first 10 hr of incubation. The resulting free alcohols were extracted with light petroleum ether (35–65 C ditillation range).

Subcellular Fractionation. For the preparation of subcellular fractions from algae, cells were disrupted with a French pressure cell (Aminco) model 4-3339, at the pressure of 1,550 kg/cm^2, in 10 mM HEPES buffer (pH 7.5) containing 0.25 M sucrose. Subcellular fractions were separated as described (7).

Marker enzymes were used for the determination of purity of the different fractions. Methods for measurement of enzyme activities were identical with those described by Morré (18).

Plasma membrane was checked by 5′-nucleotidase activity, glucose 6-phosphatase was used as a marker enzyme for ER, and thiamine pyrophosphatase as a marker for Golgi apparatus. Mitochondria were associated with ubiquinone content; detection of ubiquinone was made by its UV spectrum at 275 nm and the characteristic shift to 290 nm produced by reduction with sodium borohydride (14).

Estimation of Dol-P synthesis by different subcellular fractions was made by chromatography on LH-20 column of the acid-resistant chloroform extract. Radioactivity associated with the same elution volume as Dol-P marker was considered the biosynthesized Dol-P. Enzymic hydrolysis followed by reverse phase TLC of these fractions confirmed this assumption.

Chromatography and Electrophoresis. Paper chromatography was performed on Whatman No. 1 paper with the following solvents: solvent A: ammonium acetate 1 M (pH 7.6) - ethanol (3:7, v/v); solvent B: butanol-pyridine-water (6:4:3, v/v); solvent C: isopropyl alcohol-acetic acid-water (27:4:9, v/v); solvent D: butanol-water (4:1, v/v).

TLC was done on Silica Gel G plates developed with the following solvents: solvent E: chloroform-methanol-water (60:25:4, v/v); solvent F: chloroform-isopropyl alcohol-ethanol-1 N acetic acid (5:6:6:5, v/v). Reverse phase TLC was carried out on Kieselguhr G plates, immersing the plate in 5% (v/v) solution of paraffin oil in light petroleum (4), and developed with acetone.

Column chromatography was performed on a Sephadex LH-20 column (10 × 200 mm) equilibrated with 0.1 M ammonium acetate in 99% methanol (6). Standards of Dol-P and Fic-P were used for calibration.

Paper electrophoresis was made with Whatman No. 1 paper at 1,000 v (20 v/cm) for 3 hr in pyridine acetate 1.2 M (pH 6.5). Proteins were determined by the method of Lowry *et al.* (15) and phosphate by the method of Taussky and Shorr (21). Reducing sugars were located with the silver nitrate reagent (22). Radioactivity measurements were made in a scintillation fluid containing 4 g of PPO in 1 liter of toluene, using a Beckman LS-233 scintillation spectrometer.

RESULTS

Isolation and Properties of Acceptor Lipid. Column chromatography on DEAE-cellulose of chloroform extracts from algae gave a single peak of glucose acceptor capacity, eluting at 0.12 M ammonium acetate (Fig. 1). A similar profile was obtained when liver Dol-P was chromatographed in the same way.

Algal acceptor lipid, Dol-P, and Fic-P were submitted to mild acid hydrolysis (pH 2, 100 C, 10 min) and then assayed for acceptor activity. Results shown in Table I indicate that the algal acceptor lipid and Dol-P were almost completely stable, while Fic-P was unstable under the conditions used. Alkaline treatment of the lipids showed little loss of their acceptor activities (Table I). Strong alkaline treatment (1) of the algal acceptor, followed by reverse phase TLC, showed the same chromatographic mobility as Dol carrier.

Isolation and Properties of Glucosylated Lipid. When P_{100} preparations were incubated with UDP-[^{14}C]glucose, radioactivity was incorporated into butanol-soluble products. Butanolic extracts were submitted to paper chromatography in solvent C. All of the radioactivity migrated with the solvent front (R_F:0.95) indicating that no traces of glucose, glucose-1-P, or UDP-glucose contaminated this fraction. Column chromatography on DEAE-cellulose showed a sharp peak of a polar glucolipid eluting at 0.125 M ammonium acetate. Mild acid treatment of the algal glucolipid rapidly hydrolyzed it and the radioactivity (76%) became water-soluble. Paper chromatography of the water phase in solvents A, B, and C indicated that glucose was the only radioactive product. TLC of the algal glucosylated lipid in solvents E and F, and paper chromatography in solvent D, consistently showed a similar mobility as liver Dol-P-glucose.

The algal glucolipid, Dol-P-[^{14}C]glucose, and Fic-P-[^{14}C]glucose were treated with 50% phenol for 3 hr at 68 to 70 C (19).

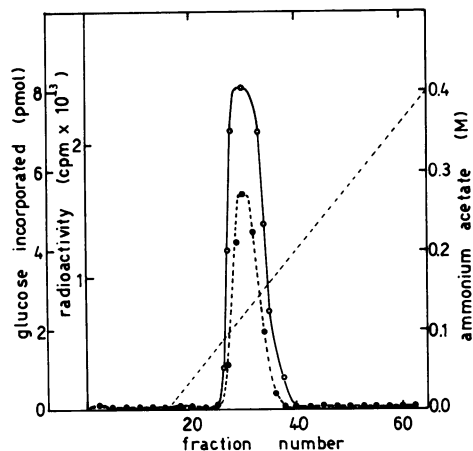

FIG. 1. Column chromatography on DEAE-cellulose of endogenous acceptor lipid. Each fraction was tested for glucose acceptor activity with P_{100} algal enzyme (○——○). These preparations have endogenous acceptor activity, and the figure represents only the activation produced by the added material. An aliquot of the [^{14}C]lipid (●- - -●) biosynthesized from mevalonate was chromatographed in the same column.

Table I

Acid and alkaline treatment of algal acceptor and polyprenyl phosphates

Samples were treated with 0.01 N HCl, 10 min at 100 C, and 0.1 N KOH at 37 C for 1 hr. The lipids were extracted with butanol and then assayed for their acceptor activity with P_{100} enzyme, as described in Materials and Methods. Figures in brackets represent the loss of acceptor capacity in per cent of the untreated sample.

Lipid acceptor	Glucose acceptor capacity		
	Untreated cpm	Treatment	
		Acid cpm (%)	Alkaline cpm (%)
AL-P	3,415	3,041 (12)	2,994 (12)
Dol-P	5,322	4,837 (10)	4,793 (11)
Fic-P	4,007	510 (87)	3,107 (23)

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Under these conditions, Fic-P-[^{14}C]glucose prepared with algal enzyme was hydrolyzed and the label became water-soluble (Table II). Electrophoresis of the water phase showed that glucose phosphate was the product. In contrast, algal glucolipid and Dol-P-[^{14}C]glucose were not degraded under these conditions and radioactivity remained phenol-soluble. Similar results were obtained by catalytic reduction (19). Table II shows that neither Dol-P-[^{14}C]glucose nor the glucosylated algal lipid was hydrolyzed by this procedure, but about 90% of the label from Fic-P-[^{14}C]glucose became water-soluble.

Biosynthesis and Analysis of Algal Lipid. The results presented strongly support the hypothesis that the algal acceptor lipid is an α-saturated polyprenyl phosphate. In order to confirm this assumption, experiments were done to synthesize the endogenous lipid from mevalonate. Algal preparations (S_{25} enzyme) were incubated with *RS*-[2-^{14}C]MVA, and radioactive free alcohols and hydrocarbons were extracted by light petroleum. About 2% of the radioactivity from the active mevalonate isomer was recovered in the chloroform-methanol (2:1) fraction, indicating the presence of polyprenyl phosphates. The chloroform extract was submitted to mild acid hydrolysis (0.01 N HCl, 100 C, 10 min); 26% of the radioactivity became extractable by light petroleum, indicating that this treatment hydrolyzed part of the prenyl phosphates. The remaining 84% was acid-stable.

The acid-stable [^{14}C]polyprenyl phosphates were chromatographed together with Dol-P and Fic-P carriers on a Sephadex LH-20 column. The elution profile is shown in Figure 2. The radioactive peak co-chromatographed with the acid-stable phosphate in Dol-P (mol wt ~1400) and far from the labile one corresponding to Fic-P (mol wt ~700). Fractions 20 to 50 were pooled, desalted by the Folch method, and concentrated under N_2. About 65% of the radioactivity from the acid-stable [^{14}C]polyprenyl phosphates was recovered in this fraction. An aliquot of the Sephadex column eluate was chromatographed on a DEAE-cellulose column eluted with a linear gradient of ammonium acetate in 99% methanol. A single radioactive peak was eluted at 0.12 M ammonium acetate, like the glucose acceptor activity isolated from algae (Fig. 1) and liver Dol-P.

Another aliquot of the Sephadex column eluate was treated with alkaline phosphatase. The resulting free alcohols (35%) were extracted with petroleum ether. Bidimensional reverse phase TLC of this fraction was performed using liver Dol as carrier. After treatment of the plate with iodine vapors, spots were scraped from the plate and counted. Results indicated that radioactivity was associated with the following molecular species: C_{90} (0–3%), C_{95} (30–35%), C_{100} (43–47%), and C_{105} (19–22%). Some radioactivity was observed at the origin; after elution and paper chromatography in solvent D it was concluded that this activity came from Dol-P contaminating this fraction.

A third aliquot of the column was treated for 30 min at 70 C under N_2 in 60% aqueous KOH-absolute ethanol (1:5, v/v) (1).

Table II

Phenol treatment and catalytic reduction of glucosylated lipids.

Samples were treated with 50% phenol at 68-70 C for 3 hr and the water and phenol phases were separated as described (19). Reduction was carried out with H_2 and Pt catalyst (19) in water saturated butanol for 4 hr. Radioactivity was measured in a scintillator after butanol-water partition.

Treatment	Glucolipids	Organic phase cpm	Water phase cpm	Hydrolysis %
Phenol	AL-P-(^{14}C)glucose	2,375	146	6.8
	Dol-P-(^{14}C)glucose	1,336	65	5.6
	Fic-P-(^{14}C)glucose	213	1,956	90.2
Catalytic reduction	AL-P-(^{14}C)glucose	8,362	779	9.5
	Dol-P-(^{14}C)glucose	3,616	775	17.7
	Fic-P-(^{14}C)glucose	691	5,436	89.7

The resulting free alcohols (75%) were extracted by addition of water and light petroleum. Reverse phase TLC of the organic phase was done and the results are in agreement with the conclusion concerning the molecular species obtained by enzymic hydrolysis.

Acceptor Activity of Biosynthesized Lipid. Aliquots of Sephadex LH-20 eluate were incubated with nonradioactive UDP-glucose and P_{100} enzyme and the lipids were extracted with butanol. The butanolic extract was chromatographed with solvent D. A radioactive peak with identical mobility as Dol-P-[^{14}C]glucose from liver and AL-P-[^{14}C]glucose (Fig. 3B) appeared in the incubated extract. This peak was not present in the nonincubated lipid (Fig. 3A).

Biosynthesis of Algal Dolichyl Phosphate by Subcellular Fractions. In order to investigate the specific site of biosynthesis of algal Dol-P, incubations were made with *RS*-[^{14}C]MVA and different subcellular fractions from algae. Table III shows the specific activity of the marker enzymes in different fractions. Only two fractions, mitochondria and Golgi, showed ability to synthesize Dol-P; this fact made it unnecessary to analyze the purity of the other fractions. From the results of thiamine pyrophosphatase activity it is clear that only 4% of dyctiosome proteins was contaminating the mitochondrial fraction. On the

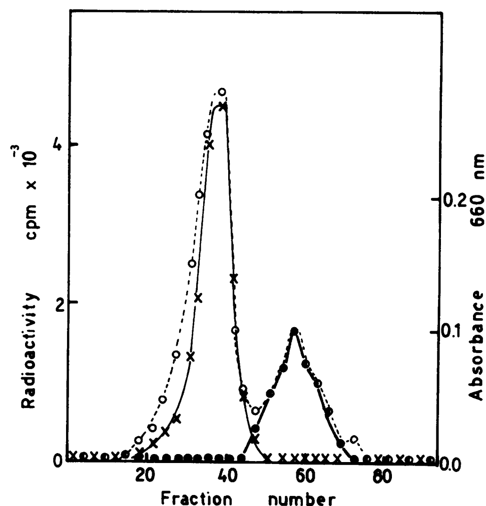

FIG. 2. Column chromatography on Sephadex LH-20. Acid-stable [^{14}C]polyprenyl phosphates were chromatographed together with Dol-P and Fic-P standards. Dol-P was detected by total phosphorus (O- - -O), Fic-P was detected by acid-labile phosphorus (●——●), and biosynthesized polyprenyl phosphate by radioactivity (×——×).

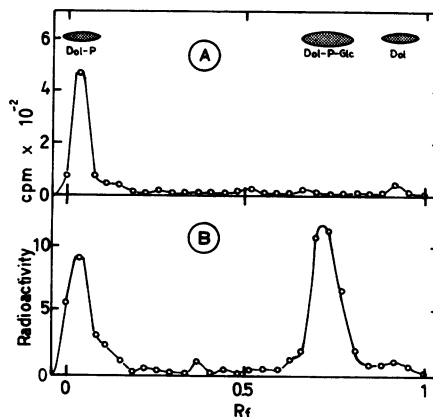

FIG. 3. Paper chromatography on solvent D of the [^{14}C]lipid. A: Unincubated lipid coming from the Sephadex LH-20 column; B: lipids extracted after incubation with UDP-glucose and P_{100} enzyme.

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Table III

Specific activities of marker enzymes in different subcellular fractions and synthesis of Dol-P

Fractions were separated by differential and discontinuous sucrose-gradient centrifugation. Enzyme assays were made with 0.1-ml samples of different fractions; biosynthesis assay of Dol-P was made with 30-µl samples. Ubiquinone was detected by UV absorbance at 275 nm.

Fraction	Ubiquinone	5'-Nucleotidase		Glc-6-Pase		Thiamine pyrophosphatase		Dol-P pmol/mg
	nmol	nmol/min·mg	RSA[1]	nmol/min·mg	RSA	nmol/min·mg	RSA	90 min
Mitochondria	407	10.7	0.12	10.7	0.03	8.6	0.11	267
Endoplasmic reticulum	16	20.6	0.24	929.0	3.12	68.8	0.91	ND
Plasma membrane	98	80.2	2.80	25.8	0.08	28.6	0.38	ND
Golgi apparatus	30	ND[2]	ND	11.8	0.03	202.1	10.80	200

[1]Relative specific activity: specific activity in the fraction/specific activity in the homogenate.
[2]Not detected.

other hand, ubiquinone content indicates that 7% of mitochondria contaminated the fraction rich in Golgi apparatus.

DISCUSSION

An acidic lipid acting as glucose acceptor was isolated from *P. zopfii* and fractionated by DEAE-cellulose column chromatography. The stability of the algal lipid to mild alkali rules out the possibility that it might be a fatty acid ester. The absence of an allylic linkage was suggested for the stability to mild acid of the algal acceptor and the stability to phenol hydrolysis and catalytic reduction of the glucosylated lipid (23).

The glucosylated lipid had chromatographic properties similar to Dol-P-glucose in three different systems, as well as an elution pattern from DEAE-cellulose column that was in agreement with polyprenyl monophosphate sugars from other systems (20).

All of this evidence suggests that the algal acceptor is an α-saturated polyprenyl phosphate, as Dol-P is. The properties of the biosynthesized [^{14}C]polyprenyl-P from algal preparations are consistent with those of the algal acceptor. Enzymic hydrolysis of the lipid with alkaline phosphatase confirmed the presence of a phosphate and liberated free alcohols. Reverse phase TLC of these alcohols indicated that it was a mixture of different chain lengths ranging from C_{90} to C_{105}, and being most abundant the C_{95} and C_{100} molecular species. Strong alkaline treatment confirmed these results.

The ability of the biosynthesized polyprenyl phosphate to act as glucose acceptor was confirmed by incubating the lipid with unlabeled UDP-glucose. The glucosylated lipid showed the same chromatographic properties as pig liver Dol-P-glucose and the algal glucosylated acceptor. These results confirm the polyprenylic nature of the glucose acceptor from algae, and the similarity with liver dolichol, in chain length and α-saturation. We do not know at present if the number and position of *cis* and *trans* double bonds is coincident.

From the data obtained from Dol-P biosynthesis by different subcellular fractions, it seems clear that both the mitochondria and the Golgi apparatus were sites of synthesis. Free dolichol (3, 16) and Dol-P (5) could be found in different concentrations in all subcellular fractions from rat or pig liver. However, this does not necessarily mean that it could be synthesized by all fractions. Mitochondria has been suggested as the site of dolichol synthesis (16). Another isoprenoid derivative, the side chain of ubiquinone, is also synthesized in mitochondria.

This is the first report not only on the presence of dolichol and its site of synthesis in algae, but also on the formation of its sugar derivatives in this system, as it has been demonstrated for bacteria, yeast, higher plants, insects, and animals (10).

From the evolutionary point of view, the fact that green algae, like other eukaryotic organisms, have Dol-P as sugar acceptor, is of considerable interest. It seems that very early in the evolution a change in the type of carrier lipids between prokaryotic and eukaryotic cells occurred.

Acknowledgments – We thank the members of the Departamento de Biología, Fundación Bariloche, for helpful discussions and criticism. The authors are grateful to L. F. Leloir and the Instituto de Investigaciones Bioquímicas, Fundación Campomar, for generous provision of chemicals and radioactive materials.

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