Plant Physiol. (1978) 61, 25-29

Glycoprotein Synthesis in Plants

II. STRUCTURE OF THE MANNOLIPID INTERMEDIATE[1]

Received for publication July 25, 1977 and in revised form September 15, 1977

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ABSTRACT

This paper reports the purification and structural determination of the mannolipid shown previously (Ericson and Delmer 1977 Plant Physiol 59: 341-347) to serve as an intermediate in glycoprotein synthesis in cotyledons of *Phaseolus vulgaris*. The mannolipid was purified by chromatography in organic solvents on diethylaminoethyl-cellulose, followed by repeated steps of deacylation and rechromatography on Sephadex LH-20. Binding and elution behavior on diethylaminoethyl-cellulose was consistent with the presence of a monophosphate residue. Lability of the mannolipid to mild acid treatment as well as its resistance to hot phenol treatment or catalytic hydrogenation are consistent with the structure of a polyprenol having a saturated α-residue. After methanolysis, the chloroform-methanol-soluble portion of the mannolipid was analyzed by mass spectrometry. The fragmentation pattern obtained was nearly identical to that obtained from standard dolichol-phosphate. An intense ion at m/e 69 represented the ω-terminal isoprenoid residue, and repeating fragments separated by 68 mass units were observed up to m/e of > 1,200. All evidence supports the conclusion that the mannolipid is dolichol-monophosphate-mannose and thus provides further support for the concept that the processes involved in the glycosylation of protein in higher plants are similar to those known to occur in the animal kingdom.

In mammalian systems and yeast, phosphorylated polyprenols have been shown to function as acceptors for the formation of glycosylated lipid intermediates which can serve as glycosyl donors in the process of glycoprotein synthesis (11, 17). These phosphorylated polyprenols are of a class called dolichols, polyprenols containing from 16 to 22 isoprenoid units and further characterized by the presence of a saturated α-residue.

A variety of reports (1, 3, 4, 7, 13-16) have demonstrated the synthesis of compounds resembling glycosyl-phosphoryl-polyprenols in higher plants, although evidence in these reports for a direct involvement of such compounds in glycoprotein synthesis was not presented. However, recent evidence from Elbein's laboratory (8, 9) and our own (5) has provided strong support for the concept that such lipid intermediates do function in the glycosylation of plant proteins.

Until now, no reports have appeared which have demonstrated the purification and structural elucidation of the lipid moiety of these intermediates in plants. However, analyses of the properties of such plant glycolipids in the impure state have strongly suggested that the lipids are of the dolichol type (3, 4, 13, 14). By analogy with the results of the mammalian and yeast studies, these results do not seem surprising. Yet they are surprising in another sense because polyprenols have long been known to occur widely in the free or esterified form in plants, and the majority (there is one report of the occurrence of dolichol in one specialized plant tissue [12]) of these are not of the dolichol type; rather they are polyprenols which are fully unsaturated and contain only 6 to 13 isoprenoid units (11). Since a similar compound, phosphorylated undecaprenol, is the major lipid acceptor for extracellular polysaccharide biosynthesis in bacteria (11), it seemed possible that these smaller, allylic polyprenols of plants, rather than dolichol, would serve as the lipid carriers for glycoprotein synthesis in plants.

In this paper we report the purification and structural characterization of a mannolipid shown previously by us (5) to function as an intermediate in the glycosylation of protein in cotyledons of *Phaseolus vulgaris*. All properties examined, including analysis of the lipid moiety by MS, indicate that the lipid is indeed of the dolichol type.

MATERIALS AND METHODS

***In Vitro* Synthesis of Mannolipid.** Preparation of extracts and production of mannolipid were scaled up from the general procedure of Ericson and Delmer (5). Developing seeds of *P. vulgaris* (14-17 mm in length) were harvested and the seed coats and embryos removed. The cotyledons (220 g fresh wt) were chopped with a razor blade at 4 C, and ground in a chilled mortar with 110 ml of 0.05 M tris-HCl (pH 8.5) containing 1 mM EDTA. The homogenate was centrifuged at 800*g* for 10 min and the supernatant used for synthesis of mannolipid. One-quarter of the extract was used for preparation of high specific radioactivity mannolipid and the remainder for synthesis of larger quantities of low specific radioactivity mannolipid. Conditions for synthesis of the high specific radioactivity preparation were: crude extract, 28.5 ml; 0.2 M $MnCl_2$, 1.5 ml; and [GDP-^{14}C]mannose (New England Nuclear, specific radioactivity 210 mCi/mmol, 20 μCi/ml), 0.075 ml. For the low specific radioactivity preparation, conditions were: crude extract, 120 ml; 0.2 M $MnCl_2$, 6 ml; [GDP-^{14}C]mannose (2.03 mCi/mmol, 9.09 μCi/ml), 0.22 ml. The reactions were incubated for 15 min at 23 C and terminated, and the mannolipid extracted into chloroform-methanol as described previously (5) except that the extractions were scaled up appropriately in volume. The low specific radioactivity preparation was pooled with the high specific radioactivity preparation. This pool was the starting material for further purification and contained 546,500 cpm and a minimum of 35 μg of mannolipid. Because it was unknown what the intracellular concentration of previously existing mannolipid was at the time of synthesis, this value of 35 μg can only represent a minimum estimate. By comparison of the ion intensities in MS

[1] Supported by Contract EY-76-C-02-1338 from the United States Energy Research and Development Administration.

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of the *P. vulgaris* lipid with that of standard dolichol phosphate, it is estimated that such a purification yielded approximately 100 μg of mannolipid. When not undergoing purification, all mannolipid preparations were stored under N_2 at −21 C.

Purification of Mannolipid. The steps in purification are listed in Table I. Concentration of mannolipid was always performed by evaporation at 37 C under N_2. DEAE-cellulose (Whatman DE52) was prepared by washing in glacial acetic acid and drying; the resin was resuspended in glacial acetic acid, poured to form a column (2 × 43 cm), washed successively with glacial acetic acid, methanol, chloroform, and finally was equilibrated for use in chloroform-methanol (2:1). The initial mannolipid preparation, dissolved in chloroform-methanol (2:1), was applied to the column and the column washed with 200 ml of the same solvent, then with 200 ml of methanol, and finally, the mannolipid was eluted with 500 ml of a linear 0 to 0.1 M ammonium acetate gradient in methanol. The flow rate was 20 ml/hr; fraction sizes were 2.4 ml.

Deacylation was performed by incubation of the mannolipid preparation for 10 min at 23 C in 0.1 N NaOH in methanol. After neutralization of the mixture with 1 N acetic acid, 42 ml of 0.9% NaCl in 0.01 N HCl and 46 ml of chloroform were added. After thorough extraction, phases were separated by centrifugation. The mannolipid remained in the lower phase.

Sephadex LH-20 was purchased from Pharmacia, equilibrated in 1 mM ammonium acetate in chloroform-methanol (1:1), and poured with upward flow to form a column (3 × 29 cm). Mannolipid pooled from the DEAE-cellulose column was concentrated to 2 ml and applied with upward flow. The flow rate was 20 ml/hr; fraction size was 2 ml/fraction. Polyethylene glycols of various average mol wt (Baker Chemical Co.) were dissolved in the column solvent, applied, and run through the column in separate runs but under identical conditions to those for chromatography of the mannolipid. The standards were detected by monitoring the refractive index of the eluate with a Pharmacia refractive index monitor. For the last two steps of purification, glass-distilled high purity solvents were used. Large scale preparation of the mannolipid was repeated once, and the analyses, including MS, were similar for both preparations.

Catalytic Reduction. The mannolipid was reduced by the procedure of Wright *et al.* (21).

Phenol Treatment. The mannolipid (30,000 cpm) was evaporated to dryness, resuspended in 0.48 ml of 50% (w/v) phenol, and incubated for up to 3 hr at 70 C and the water and phenol phases were separated as described previously (10). Susceptibility of the [^{14}C]mannolipid to degradation by either catalytic reduction or phenol treatment was measured by determining the fraction of radioactivity which was rendered water-soluble after treatment.

Mass Spectrometry. Mannolipid, purified as described above, was placed in a vial, evaporated to dryness, and 1 ml of 0.15 N methanolic HCl was added. The vial was sealed and heated at 100 C for 5 min. After cooling, 1 ml of H_2O, 1 ml of methanol, and 2 ml of chloroform were added and the mixture was extracted and centrifuged to separate the layers. Approximately 95% of the radioactivity was released into the upper phase by this treatment. The lower layer was concentrated and used for mass spectral analysis. One hundred μg of porcine liver dolichol phosphate (Sigma) was similarly treated and analyzed. Low resolution MS was performed on a Varian CH5 mass spectrometer at 70 e.v. Repetitive scanning to m/e 1,400 was performed as the probe temperature was raised gradually to 350 C.

Table I
Purification of Mannolipid

Step	Total Radioactivity Recovered	Recovery
	cpm	%
Initial Chloroform: Methanol Extract	546,500	100
DEAE Cellulose	278,880	51
Deacylation	234,080	43
Sephadex LH-20 (I)	240,720	44
Rerun LH-20 (II)	239,060	44
Deacylation and rerun on LH-20 (III)	196,000	36

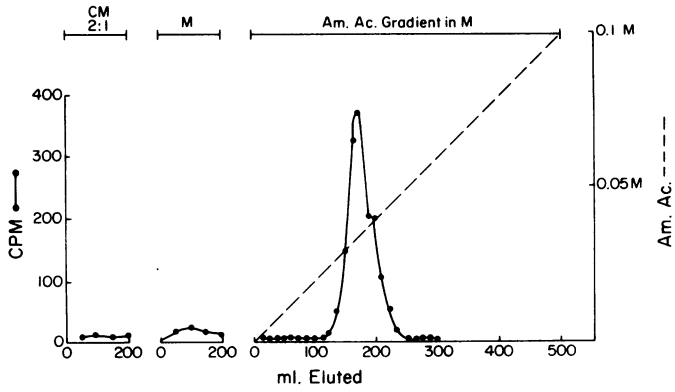

FIG. 1. Chromatography of mannolipid on DEAE-cellulose. A large scale preparation of mannolipid in chloroform-methanol (2:1) was applied to the column which was equilibrated in the same solvent. The column was washed with chloroform-methanol (2:1), then with methanol, followed by a 0.01 M gradient of ammonium acetate in methanol. C: chloroform; M: methanol; AmAc: ammonium acetate.

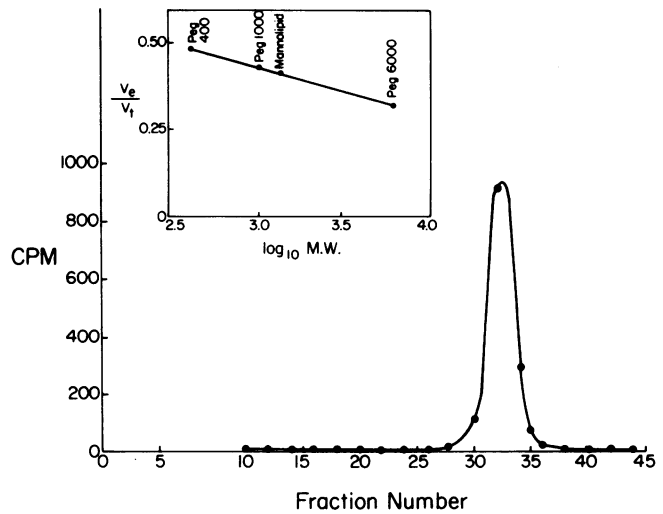

FIG. 2. Chromatography of mannolipid on Sephadex LH-20. The pooled fractions of the mannolipid eluted from DEAE-cellulose were concentrated and applied to an upward flow LH-20 column equilibrated in chloroform-methanol (1:1) containing 1 mM ammonium acetate. Inset shows a plot of V_e/V_t *versus* mol wt of various PEG standards of average mol wt as well as that of the V_e/V_t of the mannolipid. V_e: elution volume; V_t: total bed volume.

RESULTS AND DISCUSSION

Purification of Mannolipid. Table I shows a summary of the steps used in the purification of the mannolipid along with the recoveries obtained for each step. The mannolipid eluted as a single peak from DEAE-cellulose at a salt concentration of < 0.05 M (Fig. 1). The fact that the lipid bound to DEAE-cellulose, but eluted at a low salt concentration is indicative of a monophosphate, rather than a pyrophosphate, linkage between the lipid moiety and the mannose (10, 20). Also indicative of a

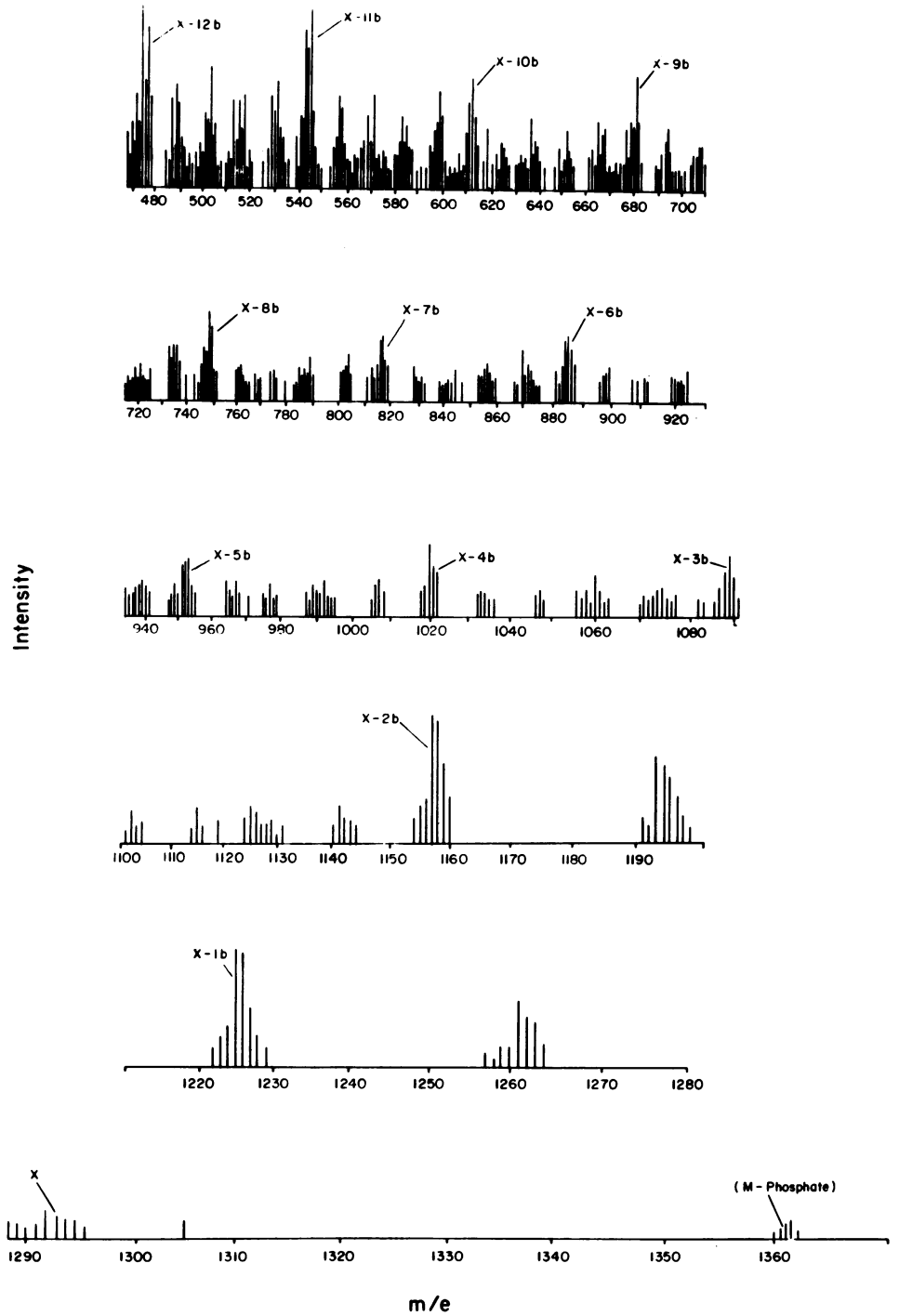

FIG. 3. Partial mass spectrum of *P. vulgaris* lipid moiety. Purified mannolipid was subjected to methanolysis as described under "Materials and Methods," and the chloroform-methanol-soluble material was concentrated under N_2 and analyzed by MS on a Varian CH5 mass spectrometer at 70 e.v. ionization energy, using a direct probe. This spectrum was taken at approximately 310 C. M, X, and b are defined in Figure 4.

monophosphate linkage is our previous result (5) showing that synthesis of the mannolipid is reversed by addition of excess GDP but not GMP to the reaction. The stability of the mannolipid to the mild alkaline conditions of deacylation is also consistent with the properties of a mannosyl-phosphoryl-polyprenol (11, 18), and was used to advantage in purification as a means to eliminate contamination by other phospholipids susceptible to degradation by deacylation. The mannolipid also eluted as a single peak from Sephadex LH-20 (Fig. 2). In addition to purification, a very crude estimate of the mol wt was obtained by this procedure by comparing the elution volume of the mannolipid to that of standard samples of polyethylene glycols of known average mol wt (Fig. 2, inset). This procedure yielded an estimated mol wt of slightly greater than 1,300. This must be considered as a crude estimate, since, among other possible variables, the presence of *cis*-isoprene residues would result in a more folded structure for the molecule causing a lowering of the molecular radius and thus of the apparent mol wt.

Acid Stability and Chromatographic Properties of Manno-

lipid. More than 98% of the radioactivity of the mannolipid was released as water-soluble material after treatment in 0.01 N HCl at 100 C for 10 min. The radioactivity released migrated coincident with mannose in paper chromatography. If methanolysis was performed instead of hydrolysis, the product released was the methyl mannoside. Such acid lability is characteristic of glycosyl-phosphoryl-polyprenols (11, 18). Similarly, the previously reported chromatographic properties of this mannolipid (5) in three separate solvent systems are indicative of such compounds.

Resistance of Mannolipid to Catalytic Hydrogenation and Phenol Treatment. Glycosyl-phosphoryl derivatives of allylic polyprenols have been shown to be degraded by treatment with phenol (50%, 68–70 C, 3 hr) or by catalytic hydrogenation, whereas similar derivatives of dolichol, which contain the saturated α-residue, are stable (10, 11, 14, 20). Under conditions where more than 80% of the allylic derivatives are degraded, we find that the *P. vulgaris* mannolipid is > 95% resistant to either phenol treatment or catalytic hydrogenation. Such a result has been reported for similar presumed lipid intermediates in other plant systems (3, 14) and do suggest that the α-residue of these plant lipids is saturated.

Mass Spectrometry. The purified mannolipid was subjected to methanolysis and the material which remained soluble in chloroform-methanol (2:1) was concentrated and subjected to MS. It is not proven that the phosphate group was retained by the lipid in this procedure, but we assume this to be the case. If the α-residue is saturated, the phosphate residue was probably not susceptible to release (11, 18), and the fact that a very high temperature during MS (> 300 C) was needed before maximal ion intensities were observed suggests that the phosphate residue was still present. Evans and Hemming (6) reported that at the probe temperature used by them (value not given), dolichol phosphate did not give a satisfactory spectrum, although solanesol phosphate did undergo dephosphorylation in the spectrometer to yield solanescene.

The mass spectrum of the *P. vulgaris* lipid moiety, obtained using a probe temperature from 300 to 350 C, is consistent with the interpretation that the lipid moiety is of the dolichol class. The most prominent ions in the spectrum were at m/e 69 and 81, representing the ω-terminal isoprenoid residue which retains the charge upon fragmentation (19) and most probably an ion containing the terminal residue and one more carbon of the chain. Figure 3 shows a partial mass spectrum at the higher masses. To aid in interpretation of this spectrum, Figure 4 outlines a probable cracking pattern (19) which is consistent with the fragmentation of a dolichol phosphate containing 18

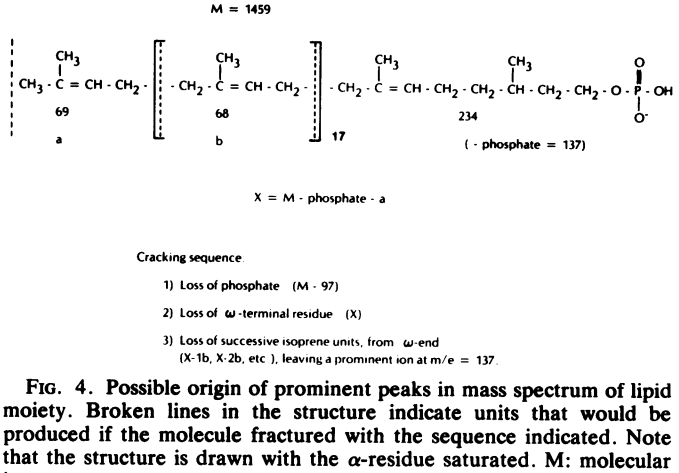

FIG. 4. Possible origin of prominent peaks in mass spectrum of lipid moiety. Broken lines in the structure indicate units that would be produced if the molecule fractured with the sequence indicated. Note that the structure is drawn with the α-residue saturated. M: molecular ion.

internal isoprenoid residues and a saturated α-residue. The occurrence of these expected repeating fragments is observed as indicated in Figure 3. Although the molecular ion was not observed, the continuation of the fragment ion series to m/e values of > 1,200 shows that the lipid is within the mol wt range of a dolichol. In addition, a separate analysis of standard dolichol phosphate (Sigma) yielded a spectrum quite similar to that of the *P. vulgaris* lipid (data not shown); these two spectra were also quite similar to the published spectrum of dolichol (2). The single notable difference in the spectrum of the *P. vulgaris* lipid from that of standard dolichol phosphate is the occurrence in the spectrum of Figure 3 of groups of prominent ions at m/e values between 1,190 to 1,200 and 1,255 to 1,265. Although these ion groups are also separated from each other by 68 mass units, their presence is not understandable from the fragmentation pattern and structure outlined in Figure 4, and, at present, we have no explanation to account for them.

CONCLUSION

This report represents the first direct demonstration by MS that a mannolipid which serves as an intermediate in the glycosylation of proteins in plants does in fact contain a lipid moiety of the dolichol type. All other determined properties of the mannolipid are consistent with the conclusion obtained from the mass spectral data. Thus, even though the majority of free and esterified polyprenols of plants are allylic and smaller than dolichol, it appears that the process of mannosylation of protein in plants draws from a pool of dolichol derivatives for use as lipid intermediates. These results provide further support that the mechanisms of glycosylation of both plant and animal proteins are strikingly similar.

Acknowledgments—The authors acknowledge the assistance of B. Soltmann and the Mass Spectrometer Facility, Department of Biochemistry, Michigan State University for the mass spectral analyses. We also thank P. Trinity for his help in development of the purification procedures and C. A. Sweeley for helpful discussions concerning the mass spectrometry.

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