THE UNAMBIGUOUS CHARACTERIZATION OF DOLICHOL PHOSPHATE MANNOSE AS A PRODUCT OF MANNOSYL TRANSFERASE IN PIG LIVER ENDOPLASMIC RETICULUM

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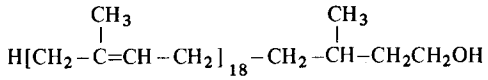
1. Introduction

Dolichol has been found freely distributed in many living systems [1, 2] but in the greatest amount in mammalian liver [3]. Its structure (fig. 1) has been established by the classical physical chemical techniques of infrared, NMR and mass spectroscopy. NMR studies showed that two of the internal isoprene residues were *trans* and the others *cis*, while the presence of an α-saturated residue has been shown, mainly by mass spectroscopy.

A metabolic function for the free alcohol, and for the majority of dolichol, that which is esterified to fatty acids [3] has yet to be confirmed. It has been proposed, however, that dolichol phosphate, the subcellular distribution of which is known [4], has an important role as an intermediate in the transfer of sugars from the nucleotide diphosphate derivatives to protein, in the biosynthesis of glycoproteins by various mammalian systems [5–7]. This role for dolichol phosphate is closely analogous to that of other polyprenol phosphates as intermediates in bacterial cell wall biosynthesis which has been firmly established, and reviewed in several articles [8, 9] and more recently in [10].

In earlier work [6, 11] evidence that the 'mannolipid intermediate' in the transfer of mannose from GDP-mannose to protein by pig liver endoplasmic reticulum was dolichol phosphate mannose, was based primarily on chromatographic evidence coupled with hydrolytic data, and incorporation of [^{3}H]dolichol phosphate. Characterization is now made more secure by being based also on physico-chemical evidence,



Dolichol-19

Fig. 1.

and by showing the mannolipid to be identical to synthetic dolichol monophosphate mannose.

2. Materials and methods

2.1. *Preparation and purification of [^{14}C]mannolipid*

Microsomes were isolated from 10 kg of fresh pig liver [6]. They were incubated with GDP [^{14}C]mannose (3.7 μmoles, 5.2 μCi) at 37° for 3 min. The incubation was terminated and the lipid extracted with chloroform:methanol (2:1, v:v). Approx. 40% of the mannose from GDP-mannose, was incorporated into mannolipid.

Mild alkaline saponification [12] was performed on the lipid and the product was chromatographed on silicic acid (Mallinckrodt) [13]. The rate of chromatography was aided by mixing an equivalent weight of silica gel (BDH 60–120 mesh). The radioactive lipid, which was eluted with chloroform:methanol (1:1, v:v) was further fractionated on silicic acid (Bio-Rad Biosil HA minus 325 mesh) essentially according to De Luca and Wolfe (private communication) using a chloroform:methanol gradient as eluant. The labelled fractions were bulked and fractionated on DEAE-

cellulose (acetate form) [12] using an ammonium acetate gradient. Following this step, the lipid was subjected to further mild alkali treatment [12].

The alkali treated lipid was further purified by reversed-phase partition column chromatography. The column was packed with Kieselguhr (BDH calcined Kieselguhr) soaked in paraffin suspended in chloroform:methanol:water (50:50:2.5, v:v:v, saturated with paraffin) solvent A. The lipid sample was added to the column adsorbed to paraffin saturated Kieselguhr (5:1, w:w, Kieselguhr:lipid). Fractionation was performed using solvent A as eluant. The labelled lipid was then re-chromatographed on silicic acid (Bio-Rad, see above). Purification was completed by preparative TLC on washed [14] Kieselgel G plates, in chloroform:methanol:water (65:25:4, v:v:v)-system B.

2.2. *Source of synthetic dolichol monophosphate mannose (DMP-mannose)*

We are extremely indebted to Dr. C.D. Warren, Massachusetts General Hospital, Boston, Mass. 02114, for supplying us with several mg of authentic DMP mannose synthesized chemically [15] from dolichol isolated from pig liver.

3. Results and discussion

3.1. *Chromatography*

[^{14}C]Mannolipid and authentic DMP-mannose co-chromatographed on Kieselgel G plates in the two systems; system B (see above) R_f value 0.3–0.35, and diisobutyl ketone:acetic acid:water (20:15:2, v:v:v)-system C R_f value 0.4–0.45. The lipids stained identically with anisaldehyde [16]. These R_f values are similar to those reported for prenol monophosphate sugars [17, 6, 14].

3.2. *Acid and alkali treatment*

Acid treatment [12] of the lipids in both cases brought about hydrolysis which yielded a lipid portion that co-chromatographed on TLC, in systems B and C, with synthetic dolichol phosphate [26] and radioactive water soluble products which corresponded to mannose and methyl mannoside on TLC [6]. Staining of the chromatograms failed to reveal the presence of any other sugars.

Both the mannolipid fractions were stable to mild alkali treatment [12].

The acid lability, alkali stability and the chromatographic properties of the acid hydrolysis products are consistent with a phosphate link between the sugar and dolichol [6, 17].

Stronger alkaline treatment, 0.1 N NaOH in propanol at 67°, yielded in both cases, dolichol phosphate [26] and mannose as shown by TLC [6] and electrophoresis essentially according to [18].

3.3. *Reducing sugar and phosphate determinations*

The ratio of reducing sugar [19] to phosphate [20] was shown to be 1.2:1, for the [^{14}C]mannolipid. This supports the presence of a monophosphate link between the isoprenoid and the sugar residue and coupled with the release, on acid treatment, of mannose but not dimannan, it is also consistent with the idea that a single sugar residue is attached to the polyprenol monophosphate (see e.g. [21]).

3.4. *Catalytic reduction*

This was performed, essentially as [22]. There was no evidence of any hydrogenolysis in either lipid as would be expected from an allylic alcohol phosphate derivative, under identical conditions [18, 22]. To ensure that the system was functional, lutein was added, and this was found to be completely reduced to a colourless material.

3.5. *Infrared spectroscopy*

Infrared spectra [23] were obtained of the [^{14}C]mannolipid, authentic DMP mannose and authentic dolichol phosphate. They compared favourably with the spectrum obtained for dolichol, which has been well documented [23].

At 3.33 μm there was a large absorption band with shoulders at 3.3 and 3.4 μm. These are due to C–H stretching of methylene and methyl groups. At 7.29 μm there was a band which is due to the deformation of methyl groups. This showed up also in a larger band at 6.85 μm, which is also consistent with deformation of methylene groups. An absorption band at 6.0 μm shows unconjugated C=C stretching while a large band at 11.95 μm is consistent with the double bonds being tri-substituted, a characteristic feature of all polyisoprenoids. At 9.5 μm there was a broad band of absorption which is due to the stretching of

a C–O bond, indicating, as in dolichol that the terminal isoprene residue is saturated. In solanesol in which the α-isoprene is unsaturated, the absorption band for the C–O bond is at 10.00 μm. The bond at 9.5 μm in the mannolipid is consistent with the derivative of a simple primary alcohol ($-H_2C-H_2C-O-$) and that at 10.00 μm in solanesol with the derivative of an allylic alcohol ($-C=HC-H_2C-O-$) in which a double bond is β to the C–O bond.

In the spectrum of dolichol there is also an absorption band at 2.9 μm. This is due to the stretching of the terminal O–H bond. In dolichol phosphate this was not evident; however in [^{14}C]mannolipid and DMP-mannose the band was very large, and is due to extra hydroxyls from the mannose present.

In the [^{14}C]mannolipid and DMP mannose there were broad bands of absorption at 8 μm and 8.5 μm. These were not evident in dolichol and were weak in dolichol phosphate.

In the spectra of [^{14}C]mannolipid, DMP mannose and dolichol phosphate there was a weak band at 5.8 μm, which was absent in dolichol.

3.6. *NMR spectroscopy*

To obtain NMR spectra, the samples were dissolved in deuterochloroform. However, due to the small amount of [^{14}C]mannolipid (< 1 mg) available, and its low solubility in this solvent, it was necessary to accumulate 450 spectra (using a digital signal averager) before a useful NMR spectrum (60 Hz) was obtained. This technique inevitably resulted in peak broadening but the main features of the spectrum were unambiguous.

Two large peaks at 8.32 τ (methyl protons adjacent to double bonds) and 7.99 τ (methylene protons adjacent to double bonds) and a smaller broader peak, centred at 4.88 τ (olefinic protons) showed the compound to be isoprenoid [24]. However the intensity of the resonance in the region 6.0–6.5 τ was greater than expected for dolichol alone, and was consistent with the presence also of a mannose residue in the molecule (see e.g. [25]).

The relative intensities of resonance in the regions 4.5 → 5.0, 6.0 → 6.6, 8.0, 8.3 τ were observed to be 0.3:0.15:1.00:0.79, which is in good agreement with the calculated values for dolichol-19, plus mannose, based on [24, 25] of 0.27:0.11:1.00:0.81.

The NMR spectrum also established the polyisoprenoid chain as predominantly *cis* [24] for the methyl resonance was at 8.32 τ with just a small shoulder at 8.40 τ. A spectrum of solanesol phosphate (an all *trans* polyisoprenoid) obtained under the same conditions, gave the methyl resonance at 8.40 τ with a small shoulder at 8.32 τ (T. Chojnacki and F.W. Hemming, unpublished 1971). It is relevant that the only predominantly *cis* polyisoprenoid compound hitherto characterized in pig liver is dolichol.

An NMR spectrum of synthetic DMP mannose phosphate mannose obtained in the same way, was identical in all essential details, with that of the [^{14}C]mannolipid.

3.7. *Mass spectroscopy*

Attempts were made to obtain the mass spectra of the [^{14}C]mannolipid, authentic DMP mannose, authentic dolichol phosphate, and authentic dolichol. Dolichol gave a satisfactory spectrum (c.f. [23]) but the other three samples did not. On the other hand, the allylic phosphate, solanesol monophosphate has been shown to give a spectrum of solanesene (T. Chojnakci and F.W. Hemming, unpublished work 1971) due to dephosphorylation in the spectrometer. It appears that prenol phosphate derivatives may be insufficiently volatile to produce a mass spectrum of the whole molecule, but the ready dephosphorylation of allylic phosphates releases the volatile isoprenoid portion as a hydrocarbon. The failure of this to occur in the case of dolichol phosphate derivatives may be related to the absence of the β-double bond.

3.8. *Exchange of mannose between GDP [^{14}C]mannose and authentic dolichol phosphate mannose in a pig liver microsomal preparation*

Due to the reversibility and rapidity of the transfer of mannose from GDP-mannose to lipid acceptor, to form mannolipid, in pig liver systems [6] it would be expected that an increase in the pool size of the mannolipid or lipid acceptor, would lead to the trapping of a larger proportion of the [^{14}C]mannose in the mannolipid fraction. When authentic unlabelled DMP mannose (100 μg) and GDP [^{14}C]mannose (0.05 μCi, 1.56 μM) were incubated with pig liver microsomes [6] after 4 min there was a slight increase (10%) in the incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into the lipid fraction, while after 15 min the level of incorporation into lipid was very

much greater (50–100%), when compared to the incorporation (34,000 and 36,000 dpm) of control incubations in the absence of the authentic DMP mannose. This provides additional evidence for the identity of the [^{14}C]mannolipid with dolichol phosphate mannose.

4. Conclusion

The chromatographic properties of the [^{14}C]mannolipid and its lability to dilute acid, but stability to dilute alkali are consistent with its containing mannose linked to a polyprenol through phosphate. The production on dilute acid hydrolysis of a lipid chromatographically identical to dolichol monophosphate and of mannose, coupled with the presence of equimolar proportions of mannose and phosphate in the mannolipid suggest the structure of dolichol monophosphate mannose. Infrared and nuclear magnetic resonance spectroscopy provide strong qualitative and quantitative support for this structure. As expected for such a structure, the mannolipid was resistant to hydrogenolysis.

The fact that the above properties of the mannolipid are identical to those of authentic dolichol phosphate mannose render this characterization unambiguous.

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