EVIDENCE FOR A COMPOUND WITH THE PROPERTIES OF 2,3-DEHYDRODOLICHYL PYROPHOSPHATE

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1. Introduction

The dolichols are a family of long chain polyisoprenols, usually consisting of 16–21 isoprene units linked in a linear, head-to-tail sequence. All but 4 or fewer of the double bonds are in the *cis* configuration, and the α-isoprene unit is saturated [1]. The monophosphate ester of dolichol functions as a carrier of glycosyl units in their transfer from sugar nucleotide donors to protein acceptors [2–4]. In spite of the physiologic importance of dolichyl phosphate, little is known concerning its biosynthesis, although a pathway has been postulated [5].

Evidence has been reported for the biosynthesis of dolichyl monophosphate, utilizing isopentenyl pyrophosphate as substrate and a 25 000 × *g* supernatant fraction from pea epicotyl as enzyme source [6]. Subsequently, different membrane fractions from rat or chicken liver were reported to form dolichyl monophosphate, and the results suggested that mitochondrial and Golgi membranes are the primary sites of synthesis [7]. Similar findings have been reported in a study of algae membrane fractions [8]. Apparently these enzyme preparations contain the enzymes required to produce the long-chain polyprenyl phosphate, including the enzyme(s) responsible for selective reduction of the α-isoprene unit.

Evidence has been reported for the biosynthesis of 2,3-dehydrodolichyl monophosphate, utilizing isopentenyl pyrophosphate as substrate and a 1000 × *g* particulate fraction from hen oviduct as enzyme source [9]. They were unable to demonstrate reduction of the α-isoprene unit.

In this report we present evidence for the in vitro biosynthesis of an allylic long-chain polyprenyl pyrophosphate with properties compatible with the structure of 2,3-dehydrodolichyl pyrophosphate. Although this compound is a likely precursor in the biosynthesis of dolichyl monophosphate, its existence has not previously been reported.

2. Materials and methods

2.1. *Materials*

[1-^{14}C]Isopentenyl pyrophosphate (57 mCi/mmol) and [1-^{3}H]dolichol (12.1 Ci/mmol) were obtained from Amersham/Searle Corp. Dolichyl monophosphate (grade III) was obtained from Sigma Chemical Corp. Dolichyl pyrophosphate was prepared as in [10]. *Trans, trans*-farnesyl pyrophosphate was a generous gift from Dr Hans Rilling, Department of Chemistry, University of Utah. Fractogel, OR-PVA 6000, is a product of E. Merck Labs, and was purchased from Whatman Ltd. Day-old white Leghorn chicks were obtained from Babcock Industries, Ithaca, NY and fed Purina lab chick chow ad libitum. Hydromix liquid scintillation fluid was purchased from Yorktown Research, South Hackensack, NJ. All other reagents were of analytical grade unless otherwise noted.

2.2. *Enzyme preparation*

Seven-day-old chicks were administed 2.5 mg diethylstilbesterol (25 mg/ml in sesame oil) daily by

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subcutaneous injection. After 10 days of hormone treatment, the birds were sacrificed and the livers removed. Subsequent steps were carried out at 0–4°C. The livers were minced with 2 vol. (ml/g tissue) of a buffer containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 0.9% NaCl and 1 mM EDTA. The preparation was then homogenized with 10 passes of a Potter-Elvehjem homogenizer (1325 rev./min) and the resulting homogenate was centrifuged at 600 × *g* for 15 min. The pellet was rehomogenized with 2 vol. buffer and centrifuged at 600 × *g* for 15 min. The supernatant fractions were combined and subjected to centrifugation at 6700 × *g* for 15 min. The resultant supernate was centrifuged at 40 000 × *g* for 15 min. The 40 000 × *g* supernate was centrifuged at 200 000 × *g* for 1 h, and the clear supernate (beneath the lipid float) was taken as enzyme source. The preparation contained 15–17 mg protein/ml as determined by the Lowry method [11].

2.3. *Enzyme assay*

Incubations were carried out for 90 min at 37°C and contained, in 250 μl final vol. 72.5 μM [1-^{14}C]-isopentenyl pyrophosphate, 50 mM Tris–acetate buffer (pH 7.5), 24 μM *trans, trans*-farnesyl pyrophosphate, 0.5% Triton X-100, 1 mM $MgCl_2$, 1 mM ATP, and 0.8 mg crude enzyme. The reaction was terminated by the addition of 5 ml $CHCl_3$–CH_3OH (2:1), an internal standard of [^{3}H]dolichol was added, and the mixture was centrifuged in a clinical centrifuge. The pellet was re-extracted with 5 ml $CHCl_3$–CH_3OH (2:1), and the combined organic phases were washed with 1/5th vol. H_2O, followed by 1/5th vol. CH_3OH–H_2O (1:1). The organic phase was dried under a stream of N_2.

2.4. *Chromatography*

Fractogel chromatography was carried out as in the legend to fig.1.

DEAE-cellulose was prepared in the acetate form according to [12], and chromatography was carried out as in the legend to fig.3. Dolichyl phosphate eluting from the column was assayed by the lipid phosphorus determination [13].

SG-81 paper was treated with EDTA according to [14] prior to use. Paper chromatography employed the following solvent systems:

(A) $CHCl_3$–CH_3OH–H_2O) (60:25:4);
(B) $CHCl_3$–CH_3OH–NH_4OH–H_2O (60:25:4:4).
Dolichyl phosphate standards were detected by I_2 or the anisaldehyde spray reagent [15].

3. Results

Incubation of [1-^{14}C]isopentenyl pyrophosphate with chick-liver enzyme preparations results in the incorporation of radiolabeled material into the $CHCl_3$–CH_3OH phase (6.0–7.5 × 10^5 cpm). A portion of the ^{14}C-labeled $CHCl_3$–CH_3OH-soluble (6000–10 000 cpm) elutes from a molecular seive column (Fractogel) in the same region as [^{3}H]dolichol (fig.1). Fractions 14–26 were pooled and used for all further experiments.

The ^{14}C-labeled material isolated from the Fractogel column gives the pattern shown in fig.2A when subjected to SG-81 paper chromatography in solvent system A. The major peak has a mobility relative to dolichyl phosphate (R_{DP}) of 0.61. Dolichyl pyrophosphate and farnesyl pyrophosphate have R_{DP} values of 0.60 and 0.10, respectively. In solvent system B (not shown) the major peak has an R_{DP} of 0.55; dolichyl pyrophosphate has an R_{DP} of 0.57. A minor peak runs near the solvent front and well

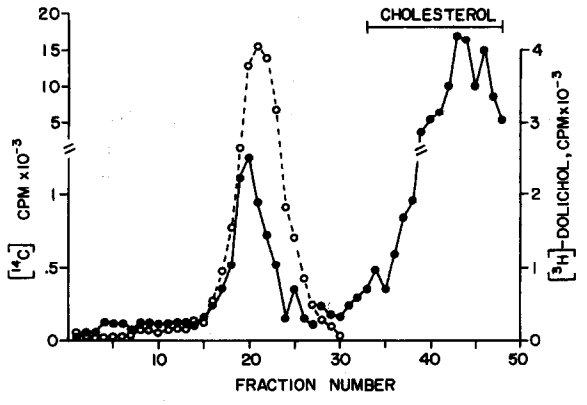

Fig.1. Gel filtration of $CHCl_3$–CH_3OH-soluble products. ^{14}C-Labeled material (——) and [^{3}H]dolichol (. . . . .) were mixed and chromatographed on a 1 × 43 cm column of Fractogel equilibrated in $CHCl_3$–CH_3OH (2:1) containing 0.20 M ammonium acetate, as in [9]. An initial 13 ml solvent was collected, followed by 0.25 ml fractions. No radioactivity appeared in the initial 13 ml eluant.

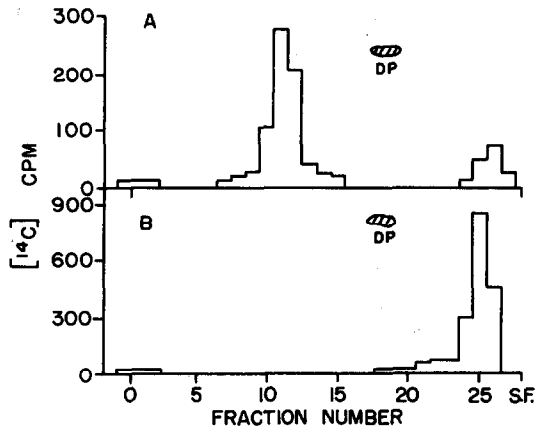

Fig.2. SG-81 paper chromatography. Fractions 14–26 in fig.1 were pooled and subjected to chromatography on SG-81 paper using solvent system A. The pooled Fractogel fractions were washed with 1/5th vol. H_2O followed by 1/5th vol. CH_3OH–H_2O (1:1). (A) 1100 cpm ^{14}C-labeled material and (B) 2200 cpm mild-acid hydrolyzed ^{14}C-labeled material were chromatographed. Dolichyl phosphate (DP) was included as a standard (0, origin; S.F., solvent front).

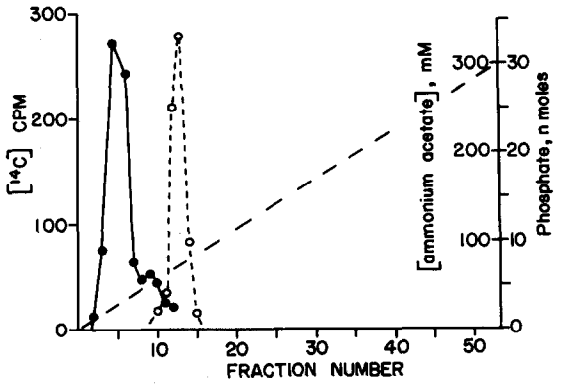

Fig.3. DEAE-cellulose chromatography. The ^{14}C-labeled products eluting in fractions 14–26 from Fractogel (fig.2) were chromatographed on a 1.5 × 9 cm column of DEAE-cellulose (acetate) equilibrated in $CHCl_3$–CH_3OH–H_2O (10:10:3). Three bed volumes of solvent were eluted, followed by a 240 ml linear gradient of 0–300 mM ammonium acetate in $CHCl_3$–CH_3OH–H_2O (10:10:3). Each fraction contained 4 ml eluant, and was washed with 1 ml H_2O. A 20% aliquot of the organic phase was taken for liquid scintillation counting, and 35% was removed for lipid phosphate determination. The remaining material was pooled and used for SG-81 paper chromatography as in the legend to fig.4. ^{14}C-Labeled material (- - - - -); phosphate (——).

ahead of dolichyl phosphate in both solvent systems A and B.

The chromatographic pattern shown in fig.2B is obtained when the material isolated from the Fractogel column is subjected to mild acid hydrolysis ($CHCl_3$–CH_3OH–0.8 N HCl (10:10:3) for 1 h at 80°C). The major peak apparent in fig.2A disappears and virtually all the radioactivity moves near the solvent front. Under these hydrolytic conditions, dolichyl monophosphate is stable (its chromatographic mobility is unchanged) and dolichyl pyrophosphate is converted into dolichyl monophosphate [10]. The results shown in fig.2B are consistent with the major peak shown in fig.2A containing an allylic group.

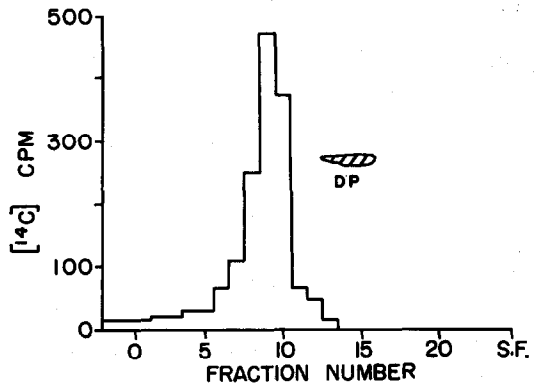

Fig.4. SG-81 paper chromatography of material eluted from DEAE-cellulose. Fractions 2–7 (shown in fig.3) containing lipid phosphate, and fractions 11–15 containing ^{14}C-labeled material were pooled separately and concentrated under a stream of N_2. They were then subjected to SG-81 paper chromatography in solvent system A. Dolichyl phosphate (DP) was included as a standard (0, origin, S.F. solvent front).

The paper chromatographic evidence also suggests that the major peak in fig.2A contains a pyrophosphate moiety. To test this hypothesis, the material obtained from Fractogel was subjected to chromatography on DEAE-cellulose in $CHCl_3$–CH_3OH–H_2O (10:10:3). Two products are obtained from the column. The first product elutes without the addition of salt to the solvent and runs near the solvent front when subjected to SG-81 paper chromatography in solvent system A. The second product is eluted by a linear salt gradient at 75 mM ammonium acetate

(fig.3); dolichyl monophosphate elutes at 30 mM salt. The ^{14}C-labeled material eluting from the DEAE-cellulose column with ammonium acetate has an R_{DP} of 0.60 on SG-81 paper in solvent system A (fig.4). The phosphate-containing material eluting in fractions 2–7 is unmodified dolichyl phosphate as judged by paper chromatography. The results of DEAE-cellulose chromatography are consistent with the major peak of fig.2A containing a pyrophosphate moiety.

4. Discussion

The evidence presented here indicates that high-speed supernatant fractions of livers from diethylstilbestrol-treated chicks incorporate [^{14}C]isopentenyl pyrophosphate into a compound with the properties of 2,3-dehydrodolichyl pyrophosphate. The dolichols are lipids of relatively high molecular weight, and chromatography on a molecular sieve column indicates that the ^{14}C-labeled product has a molecular weight similar to dolichol. In fact, the compound seems to have a molecular weight slightly greater than dolichol, which suggests it contains additional groups.

That the compound contains a pyrophosphate group is evidenced by its behavior on DEAE-cellulose chromatography, where it elutes at a salt concentration greater than that required for the elution of dolichyl monophosphate. The mobility of the product on SG-81 paper chromatography is virtually identical to that of dolichyl pyrophosphate. The results obtained upon mild acid hydrolysis suggest that the pyrophosphoryl group is linked to the ^{14}C-labeled moiety by an allylic bond.

The biosynthesis of dolichol is believed to occur by the head-to-tail condensation of isoprene units, and 2,3-dehydrodolichyl pyrophosphate has been pointed out [5] to be a logical precursor to dolichyl monophosphate. This is the first report presenting evidence for the existence of such a compound. In vivo, the α-isoprene unit of 2,3-dehydrodolichyl pyrophosphate may be specifically hydrogenated and the resultant dolichyl pyrophosphate hydrolyzed to the monophosphate. Conversely, the pyrophosphate may be converted into the monophosphate and then hydrogenated. Our evidence, coupled with that in [9], supports the latter pathway.

Several publications have indicated that dolichol can be directly converted into dolichyl monophosphate utilizing either CTP [16] or ATP [17]. The direct phosphorylation of dolichol may reflect the cell's ability to salvage dolichol or to shift dolichol from various pools such as fatty acyl dolichol to the glycosyl carrier form, dolichyl monophosphate. It is also possible that the de novo synthesis of dolichol is favored by the removal of the pyrophosphate moiety releasing free dolichol. Free dolichol would then have to be converted into the monophosphate ester. With either biosynthetic route (conversion of pyrophosphate into monophosphate or conversion of pyrophosphate into alcohol followed by phosphorylation) the 2,3-dehydrodolichyl pyrophosphate is a likely intermediate.

This is also the first report concerning the in vitro biosynthesis of a dolichol-like compound utilizing a supernatant fraction from a high-speed centrifugation. It is interesting to note that this finding parallels the results obtained with several bacterial systems in which undecaprenyl mono- and pyrophosphate, allylic C_{55} polyisoprenoids, are synthesized by high-speed supernatant fractions [18–20].

The biosynthesis of dolichol and its derivatives in eukaryotic organisms is of obvious importance because of the role dolichyl monophosphate plays in the glycosylation of proteins. However, the biosynthetic route from isopentenyl pyrophosphate is obscure. This report adds an additional compound to the postulated sequence.

Acknowledgements

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