PHOSPHORYLATION OF DOLICHOL BY INSECT ENZYMES

The incorporation of phosphate from ATP into dolichyl phosphate mannose

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1. Introduction

Dolichyl phosphate has been found to be involved in the glycosylation of proteins in eucaryotes [1]. It has been detected in mammalian tissues [2], plants [3–5], insects [6], yeast [7,8] and other eucaryotic cells [1]. The biosynthesis of dolichyl phosphate may be envisaged to occur either by dephosphorylation of the pyrophosphate derivative (presumably formed from lower molecular weight precursors [9–12]) or by phosphorylation of free dolichol which is generally found in considerable amounts as compared with its phosphate [13].

This paper reports evidence indicating that extracts of insect larvae catalyze the transfer of ^{32}P from [γ-^{32}P]ATP to dolichyl derivatives.

2. Materials and methods

2.1. *Materials*

Larvae and pupae of *Ceratitis capitata* (Weidmann) (Meditarranean fruit fly) were provided by Ing. A. Turica from the Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina. Natural pig liver dolichyl phosphate was obtained as in [2]. Pig liver dolichol was a kind gift of Dr Josefina Martin-Barrientos. GDP-[^{14}C]Man (240 Ci/mol) was obtained from New England Nuclear. [γ-^{32}P]ATP was prepared according to [14]. Radioactive dolichyl derivatives used as standards were biosynthesized as in [15,16]. All organic solvents were distilled before use.

Abbreviation: Dol, dolichyl

2.2. *Enzyme preparation*

All the manipulations were carried out at 0–4°C. Day-7 larvae were washed first with sodium hipochlorite and then with distilled water. The material was then dried with filter paper, frozen by immersion in liquid N_2 and reduced to a fine powder in a mortar. This was suspended in 250 mM sucrose, 80 mM Tris–HCl buffer (pH 7.6), 1 mM EDTA-Mg, 4 mM 2-mercaptoethanol and 0.01% (w/v) 2,6-di-*tert*-butyl cresol, added as antioxidant, using a glass–Teflon homogenizer. Cell débris were removed by centrifugation at 500 × *g* for 10 min, and the supernatant fluid was centrifuged at 8000 × *g*. The lipid-containing layer at the top was discarded and the supernatant fluid was used as enzyme. Protein concentration was brought to 23-25 mg/ml (method [17]) by dilution with the above mentioned buffer. This extract was about 1/3rd as active in forming Dol-P-Mann (cpm/μg protein) as the corresponding microsome-enriched preparations [18]. Since no bactoprenyl derivative was detected by thin-layer chromatography [15] it was concluded that bacterial contamination from the digestive tract was negligible.

2.3. *Chromatography and electrophoresis*

Schleicher and Schüll 2043/a paper was employed. Thin-layer chromatography was carried out on cellulose (Eastman 6064) or silica gel (Eastman 6061) plastic sheets. Solvents were:

(A) 2-Propanol/conc. acetic acid/water (54/8/18);

(B) Isobutyric acid/1 M ammonium hydroxide (5/3);

(C) Chloroform/methanol/water (60/25/4);
(D) Chloroform/2-propanol/abs. ethanol/1 M acetic acid (2/2/3/1);
(E) 2-Propanol/water (4/1);
(F) 95% ethanol/1 M ammonium acetate buffer, pH 3.8 (15/6).

Column chromatographies were as in [6].

3. Results

Ceratitis capitata extracts supplemented with NaF (to decrease substrate degradation) and with GDP-Man (to trap the Dol-P as Dol-P-Man [6]) catalyzed the ^{32}P transfer from ^{32}P-labelled ATP to a lipidic material in which two compounds were characterized: one as dolichyl phosphate mannose; another behaving like dolichyl monophosphate.

3.1. *The formation of labelled products*

The ^{32}P-labelled lipidic material behaved as a dolichyl phosphate hexose in a cellulose thin-layer chromatography (fig.1). Optimal ^{32}P incorporation was found in the presence of 2.0 mM ATP and labelling was not affected by the addition of unlabelled 1 mM phosphate or 1 mM mannose-1-P.

Elution of the labelled material and rechromatography on cellulose plates with solvent C gave two radioactive spots, one of them behaving as Dol-P-Man and another which ran slower ($R_{\text{Dol-P-Man}}$ 0.88). A similar result was obtained by thin-layer chromatography on silica gel with solvent C ($R_{\text{Dol-P-Man}}$ 0.8, 1.0).

When larvae extracts were incubated with [γ-^{32}P]ATP and GDP-[^{14}C]Man a double-labelled dolichyl-P-hexose was similarly detected (fig.2A). Another ^{14}C-labelled compound containing only traces of ^{32}P was also found. This compound had the mobility of dolichyl diphosphate oligosaccharide [6].

3.2. *Acid hydrolysis*

After mild acid treatment of the double-labelled material (0.01 M HCl at 100°C for 30 min) the bulk of ^{14}C radioactivity became soluble in water whereas most of the ^{32}P-labelled material remained soluble in the organic phase. The labelled water-soluble substances behaved like mannose and an oligosaccharide (fig.2B) and the ^{32}P-labelled lipidic material behaved like dolichyl phosphate both in silica gel thin-layer (fig.2B) and paper chromatography (fig.2C).

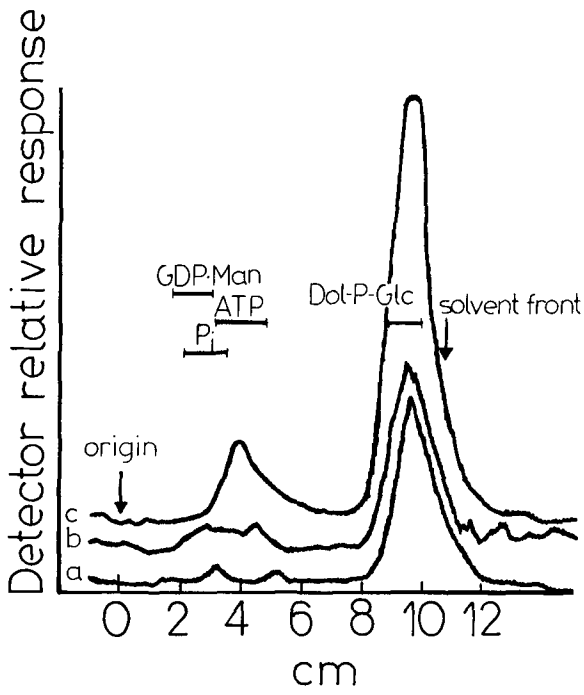

Fig.1. Phosphorylation of endogenous lipids by [γ-^{32}P] ATP. Incubation mixtures contained: (a) 40 mM Tris-maleate buffer (pH 7.45); 8 mM $MgCl_2$; 2 mM $MnCl_2$; 1 mM EDTA; 3 mM NaF, 0.18% Triton X-100; 30 mM 2-mercaptoethanol; 1 mM GDP-Man; 1.6 × 10^8 cpm [γ-^{32}P]ATP (initial spec. act. ~10^9 cpm/μmol) and enzymatic extract (3 mg protein) in final vol. 150 μl. P_i, 1 mM, was added to incubation (b) and 1 mM ATP to incubation (c) (final conc. 2.06 mM). The mixtures were then incubated at 25°C for 30 min, stopped and processed by the method in [19]. The lower phase was washed 10–15 times with 'theoretical' upper phase containing 1 mM ATP, concentrated under N_2 and chromatographed on thin-layer cellulose plates with solvent B. Radioactive spots were located by means of a radiochromatogram scanner.

3.3. *Alkaline hydrolysis*

The double-labelled material was subjected to mild alkaline treatment (0.1 M KOH in 50% *n*-propanol at 69°C for 60 min) and some of the radioactivity became water-soluble. This labelled material was submitted to high-voltage paper electrophoresis and after scanning for radioactivity, two negatively-charged, double-labelled compounds were detected (fig.3). One of them had the mobility of mannose-1-P and the other was slower and had a much lower $^{32}P/^{14}C$ ratio, as expected for an oligosaccharide-phosphate.

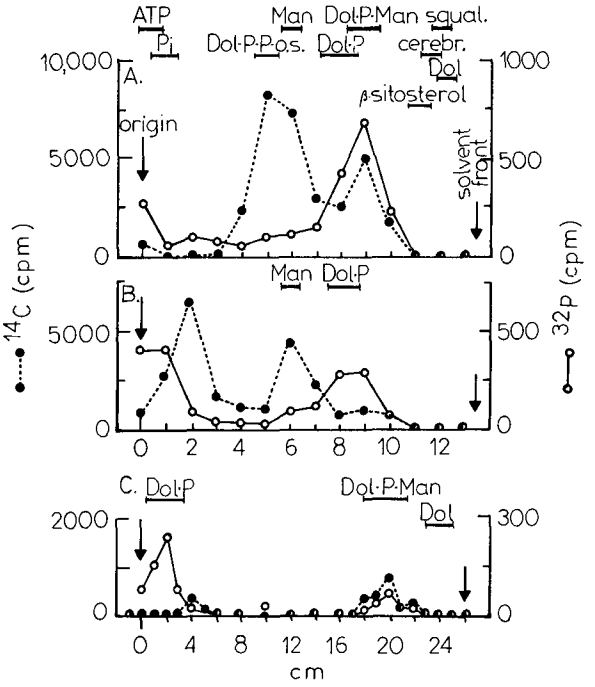

Fig.2. Incorporation of ^{32}P from [γ-^{32}P]ATP into Dol-P-Man. The same incubation mixture as in fig.1(c) was used, but with 2.0×10^5 cpm GDP-[^{14}C]Man (~9 μM) instead of unlabelled GDP-Man. The mixture was then processed and phospholipids were chromatographed together with standards in silica gel G sheets with solvent D [15], after which the latter were cut in 1 cm strips and counted in a liquid scintillator using toluene–4% Omnifluor. Dol-P was detected by the enzymatic test in [15]. (A) Non-hydrolyzed; (B) treated with 0.01 M HCl for 30 min at 100°C; (C) strips 7, 8, 9 and 10 (corresponding to Dol-P) of experiment B were washed with toluene (to remove the scintillator), the silica gel was scraped off and extracted with 0.6 N HCl in chloroform/methanol (2/1) [15]. The extract was then chromatographed on paper using solvent E and radioactivity was located as above. *Abbreviations:* Squal., squalene, Dol-P-P-o.s., dolichyl diphosphate oligosaccharide, cerebr., cerebrosides

In addition, two compounds labelled with ^{14}C but not with ^{32}P were observed: one remained at the origin and the other migrated slightly to the cathode like a mannose standard. The fast anionic peak was eluted from the paper, treated with 0.1 N HCl for 5 min at 98°C and submitted to paper electrophoresis. It comigrated again with Man-1-P. After treatment with *E. coli* alkaline phosphatase, the ^{14}C-label ran like mannose on silica gel thin-layer chromatography with solvent D. The ^{32}P-label behaved as P_i in paper

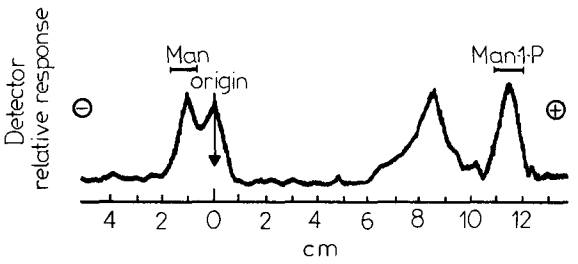

Fig.3. Electrophoresis of water-soluble substances obtained from mild alkaline treatment of double-labelled phospholipids. Paper electrophoresis was performed at 20 V/cm for 1.5 h with 0.07 M acetic acid/10% pyridine, at pH 6.5. Radioactive spots were located by means of a scanner afterwhich the corresponding strips were cut and counted in a liquid scintillator.

chromatography with solvent F. Since identical alkaline treatment of insect Dol-P-Man released mannose-2-P (because of the β-configuration of mannose in the glycolipid [18]), the double-labelled 'fast' anionic compound was assumed to be [^{14}C]mannose-2-^{32}P originated from dolichyl-^{32}P-[^{14}C]Man. No double-labelled substances other than dolichyl-P-Man and polyprenyl-bound oligosaccharide could be detected.

3.4. *Addition of dolichyl phosphate*

An isotopic dilution test was performed by incubating insect extracts with [γ-^{32}P]ATP and GDP-[^{14}C]Man with or without 50 nmol purified pig liver Dol-P. The incorporation of ^{32}P into Dol-P-[^{14}C]Man decreased 18-fold suggesting a competition between the exogenous Dol-P and the labelled endogenous acceptor (table 1). Analysis by thin-layer

Table 1
Effect of Dol-P on ^{32}P-labelling of Dol-P-Man

	$^{32}P/^{14}C$ ratio in	
	Dol-P-man	Dol-P-P-oligo-saccharides
Without addition	0.350	0.023
+ Dol-P (50 nmol)	0.019	0.056

Incubations were as indicated in legend of fig.2 except for the lipid which was pre-dried under N_2 together with salts. Triplicates were processed, chromatographed and counted as indicated

chromatography (solvent D) of the products of incubations containing exogenous Dol-P showed a radioactive peak somewhat different from the control in the absence of Dol-P. It was a ^{32}P-labelled substance of intermediate mobility (R_F 0.55) between that of Dol-P-Man (R_F 0.60) and that of polyprenyl-bound oligosaccharides (R_F 0.44). In this position, Dol-P is found together with other phospholipids: hence we might assume that Dol-^{32}P had accumulated due to the presence of unlabelled Dol-P.

3.5. *Addition of dolichol*

Addition of pig liver dolichol to the ATP-containing incubation mixtures stimulated the biosynthesis of both ^{14}C-labelled Dol-P-Man and ^{32}P-labelled Dol-P (table 2). Since stimulation depended on the amount of endogenous insect dolichol (0.6–1.4 μg/g protein in *Ceratitis* membranes), extracts with low levels of dolichol should be necessary.

Table 2
Effect of dolichol addition

Additions	cpm	% Stimulation
A. Biosynthesis of Dol-P-[^{14}C]Man		
None	4232	–
2.3 mM ATP	4964	17.2
50 μg dolichol	4413	4.0
2.3 mM ATP + 5 μg dolichol	5185	22.5
2.3 mM ATP + 15 μg dolichol	5642	33.3
2.3 mM ATP + 50 μg dolichol	5867	38.6
B. Biosynthesis of Dol-^{32}P		
Exp. 1		
None	907	–
15 μg dolichol	1743	92.2
Exp. 2		
None	1444	–
15 μg dolichol	2875	99.1

The same incubation mixture as in fig.1 was used with the following modifications: part A: labelled ATP was omitted and ATP and/or dolichol were added as indicated. Triton X-100 was 0.03%. Data were the mean of 2 experiments. Part B: detergent was omitted and 1.6×10^5 cpm of GDP-[^{14}C]Man (~9 μM) was used instead of unlabelled GDP-Man. Experiments 1 and 2 were performed with different enzymes. The samples were processed and chromatographed as indicated in fig.2. Radioactivity was located by cutting 0.5 cm strips which were counted in a liquid scintillator

4. Discussion

Our results indicate that most of the ^{32}P incorporated appears in dolichyl derivatives probably due to a low level of glycero-phospholipids synthesis at days 6–8 of the *Ceratitis* life cycle [20] and to a slow rate of turnover of some of these lipids in insects [21].

Calliphora eggs have been shown [22] to synthesize [^{14}C]dolichol by incubating them with DL-[2-^{14}C]mevalonic acid lactone. Insect and other eucaryotic polyprenols biosynthesis would be similar to that of bacterial undecaprenols [9]. Thus, a prenyl pyrophosphate precursor would be condensed head-to-tail with successive isopentenyl pyrophosphate units giving (after saturation of the α-unit) dolichyl pyrophosphate, which would be then dephosphorylated to give dolichyl phosphate [10–12].

Mevalonic acid phosphate(s) or isopentenyl pyrophosphate were not detected in our incubation mixtures. Moreover, dolichol addition stimulated Dol-P and Dol-P-Man formation. Thus, the results in this paper would indicate that some of the phosphates in dolichyl derivatives could be attributed to the activity of a polyprenyl kinase similar to that described for bacteria [23]. However, ^{32}P in dolichyl derivatives might be partly provenient indirectly from [γ-^{32}P]ATP through a way starting in mevalonic acid phosphorylation. Attempts to purify the kinase activity from insect tissue extracts (with organic solvents) were not successful, unlike the results reported for procaryotic polyprenyl kinase [23].

However, the possible role of other nucleoside triphosphates as phosphate donors cannot be discarded.

The existence of a dolichol kinase would suggest the possibility of a regulatory step within the pathway of dolichyl derivatives synthesis in eucaryotes.

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