A PHOSPHATASE ACTING ON DOLICHYL PHOSPHATE IN MEMBRANES FROM NEURONAL PERIKARYA

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1. Introduction

Since the isolation of Dol-P-Glc in 1970 [1] significant progress has been made in the elucidation of how the carbohydrate moieties of the asparagine-type glycoproteins are synthesized. Glycosylation occurs via sugar lipid intermediates [2]. Some of the properties of most of the enzymes in this pathway have been studied [2]. There is however scant amount of information* available about Dol-P and Dol-P-P phosphatases.

The control mechanism operating in the dolichol pathway is yet to be elucidated. It has been proposed that the endogenous level of Dol-P could play a regulatory role in protein glycosylation [3]. By analogy with procaryotic systems [4–6], Dol-P, Dol-P-P phosphatases and dolichol kinase may be implicated in the control of the endogenous level of Dol-P.

Enzymes that split the phosphate bonds from prenyl phosphates have been described in mammalian systems [7–9], although in these cases Dol-P was not used as substrate and Mg^{2+} were always included in the incubation mixture. Here we report an enzymatic activity present in membranes from neuronal perikarya which removes the phosphate from Dol-P and requires no divalent cations.

Abbreviations: Dol-P, dolichyl phosphate; Dol-P-P, dolichyl pyrophosphate

* J. L. Strominger and J. F. Wedgwood reported the presence of phosphatases acting on Dol-P and Dol-P-P in lymphocytes (Second PAABS Congress, Caracas, Venezuela, 1978). In addition evidence was found for a Dol-P-P phosphatase activity in rat liver (E. Tábora, PhD Thesis, University of Buenos Aires, 1976)

2. Materials and methods

Wistar albino rats were used. Dol-P and dolichol were purchased from Sigma. Bovine serum albumin (fraction V) was from Sigma. Dol-P-[^{14}C]Gal was a kind gift from Nora I. de Iñón [10]. All other reagents were of the best analytical grade.

2.1. *Preparation of membrane fractions*

The cerebral cortices from 5 or 15 day-old rats were chopped with a razor blade and the neuronal cell bodies were isolated as in [11]. The neuronal cell bodies were homogenized in 0.25 M sucrose with several up and down strokes in the Dounce homogenizer, using a tight-fitting pestle. The membrane fraction results from spinning down the post-nuclear supernatant at 10 000 × *g* for 15 min. The fraction was resuspended in 0.25 M sucrose and stored frozen (−70°C) until used.

2.2. *Preparation of radioactive Dol-P*

Dolichol was tritiated according to [12], phosphorylated and purified as in [13] and [1], respectively.

2.3. *Incubation conditions for the measurement of Dol-P phosphatase activity*

The mixture, containing 60 000 cpm of [^{3}H]Dol-P, 125 mM Tris–maleate buffer (pH 7.1), 36 mM EDTA and 0.3% Triton X-100, was incubated at 37°C for 15 min. The reaction was stopped with chloroform–methanol–4 mM $MgCl_2$ (3:2:1). The organic phase was separated and washed with theoretical upper phase [14] [chloroform–methanol–4 mM $MgCl_2$ (3:48:47)]. The former was dried, dissolved in chloroform–methanol (2:1) and submitted either to DEAE-

cellulose column chromatography or thin-layer chromatography. In the first case, the percolate together with the washings were dried, dissolved in 4% Omnifluor in toluene and counted. In the second case, thin-layer chromatography was carried out on silica gel sheets (Eastman Kodak Co., Rochester, NY), using solvent system A or C. Under these conditions Dol-P stays at the origin whereas dolichol runs with a R_F of about 0.3 (A) or 0.36 (C). After the run, the sheets were cut into 5 mm pieces, added to a toluene Omnifluor scintillation liquid and counted. The following solvent systems were used for thin-layer chromatography: A, chloroform; B, isopropyl ether–petroleum ether (4:1); C, hexane–ethyl acetate (9:1); D, chloroform–methanol–formic acid–water (140:37:4:1).

Autoradiography was carried out on a LKB ultrofilm. The unlabeled lipids on the thin-layer chromatography plates were detected by an anisaldehyde spray [15]. Proteins were determined according to [16].

3. Results

[^{3}H]Dolichol was submitted to a procedure of chemical phosphorylation [13]. The identification of the resulting compound was based on the following facts.

(1) The labeled polyprenyl derivative was eluted from the DEAE-cellulose column with a salt concentration corresponding to Dol-P.
(2) When run on thin-layer chromatography using solvent A or D the synthetic sample comigrated with a standard of Dol-P.
(3) The dolichyl derivative was shown to be a glycosyl acceptor in an enzymatic system that utilizes UDP-Gal as precursor [10].

Thin-layer chromatography in solvent system D separates Dol-P-[^{14}C]Gal from the ^{3}H-labeled synthetic sample (fig.1A). After incubation a new product labeled with tritium appeared (fig.1B). This compound exhibited identical R_F value as that of Dol-P-[^{14}C]Gal. All the evidence presented above strongly indicates that the chemical phosphorylation of [^{3}H]dolichol resulted in the formation of [^{3}H]Dol-P.

The incubation of membranes obtained from neuronal perikarya with radioactive Dol-P resulted in the appearance of a labeled compound soluble in chloroform–methanol (2:1), which was not retained by a column of DEAE-cellulose, thus behaving as a neutral lipid presumably dolichol (see below). This property

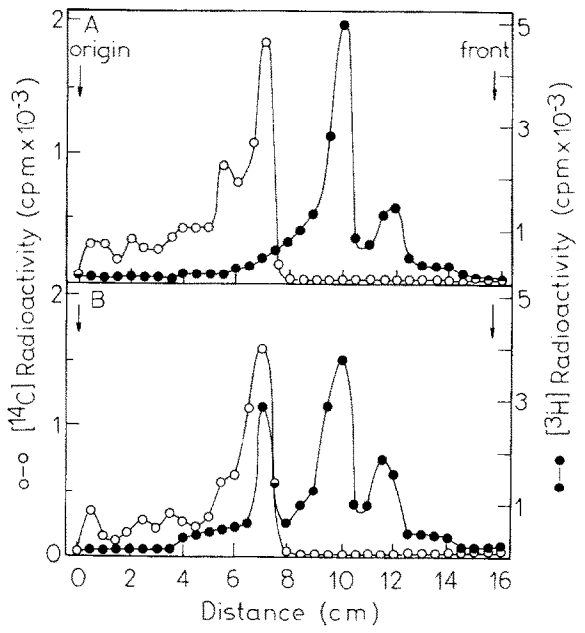

Fig.1. Acceptor capacity of the tritium labeled synthetic dolichyl derivative using a membrane preparation from *Acetobacter xylinum*. (A) Chromatogram of the incubation mixture, stopped at 0 time, containing the ^{3}H-labeled synthetic sample, [^{14}C]Dol-P-Gal (as internal standard), unlabeled UDPGal and other components as in [10]. (B) Thin-layer chromatography in the same conditions as in (A) after the enzymatic reaction had taken place.

was used to separate the substrate from the product and as a method for measuring the enzyme.

Attempts were made to characterize the neutral compound. The organic phase obtained after stopping the reaction with chloroform–methanol–water (3:2:1) was washed with theoretical upper phase [14] partially purified DEAE-cellulose and run on thin-layer chromatography with solvent system A, B and C (fig.2). The control (not incubated) was not submitted to the DEAE-cellulose step. This sample showed a single component comigrating with authentic Dol-P. In contrast, in the incubated samples a single new compound appeared, which comigrated with liver dolichol.

The reaction proceeds linearly up to 10 min incubation and within the concentration range of 30–140 μg membrane proteins. The optimum pH (for the enzyme activity) was between 7.1 and 7.4 (results not shown). The enzyme remains practically unchanged for at least 1 month when stored at −70°C.

The effects of several cations are shown in table 1.

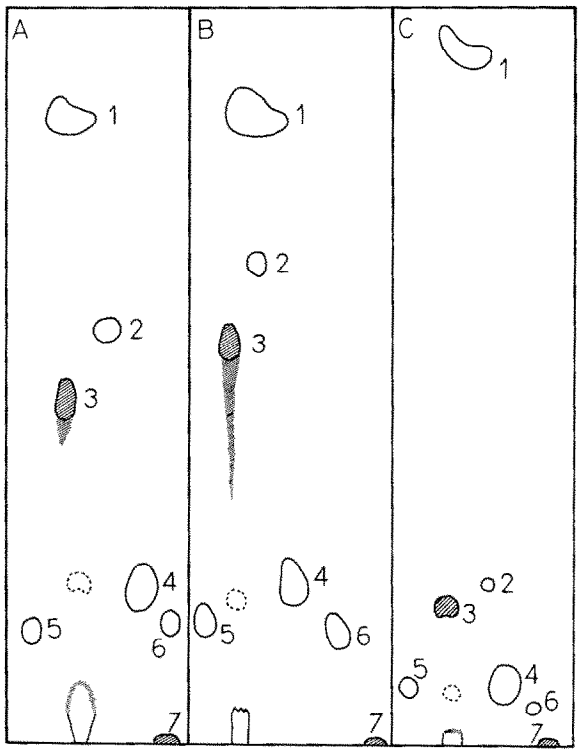

Fig.2. Cochromatography of the enzymatically labeled neutral lipid with a standard of dolichol on thin-layer chromatography. The ^{3}H-labeled product of the phosphatase reaction was prepared as in section 2 and mixed with liver dolichol. The mixture was chromatographed on preactivated plates of silica gel 60 and developed with solvent system A (panel A), B (panel B) and C (panel C). Unlabeled lipids (empty spots) were detected by the anisaldehyde spray [15], labeled lipids (shaded areas over the unlabeled lipids) were detected by autoradiography. The lipids used as standard were: 1, squalene; 2, ubiquinone; 3, dolichol; 4, geraniol; 5, cholesterol; 6, β-sitosterol; 7, [^{3}H]Dol-P.

It can be seen that Mg^{2+}, Ca^{2+} and Mn^{2+} inhibit product formation as compared with the control. This inhibition was observed at both cation concentrations tested (10 or 58 mM). The addition of EDTA to the incubation mixture increased the phosphatase activity ~2-fold over that of the control.

The specificity of the phosphatase was investigated by competition with various metabolites. These compounds were used in concentrations highly in excess (5 mM) of that of Dol-P ($<10^{-6}$ M). As shown in fig.3, the addition of ATP, ADP, AMP, P_i, PP_i, glucose 6-P and glycerol-P did not decrease significantly the

Table 1
Effect of cations on Dol-P dephosphorylation

Additions	Conc. (mM)	Radioactivity in the product (cpm)	Conversion of substrate (%)
A.			
1. None	–	2668	8.8
2. EDTA	45	5380	12.6
3. Mg^{2+}	10	1800	6.0
4. Ca^{2+}	10	1680	5.6
5. Mn^{2+}	10	348	1.2
B.			
1. None	–	2750	6
2. EDTA	45	4364	9
3. Mg^{2+}	58	1063	2.1
4. Ca^{2+}	58	795	1.6
5. Mn^{2+}	58	n.d.	0

With the exception of expt. 1,3,4 and 5 (which did not contain EDTA) the enzyme reaction was carried out as in section 2. n.d., non-detectable. Expt. A and B correspond to different enzymatic preparations

amount of Dol-P converted into dolichol. In fact, glucose 6-P and the adenosine phosphates activated the enzyme.

Similar results were obtained by measuring the reaction product either on thin-layer chromatography or DEAE-cellulose column chromatography.

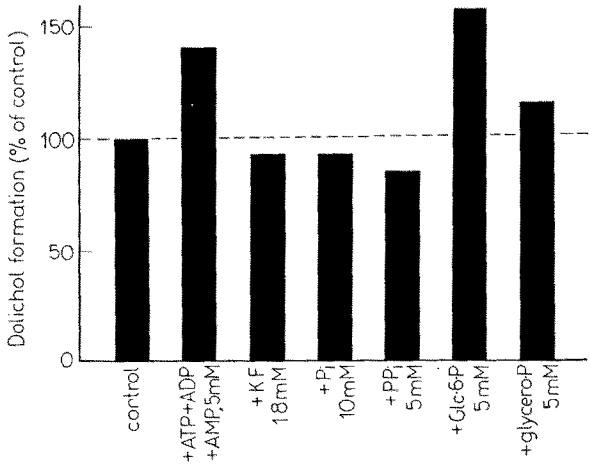

Fig.3. Effect of various metabolites on Dol-P dephosphorylation. The enzyme reaction was carried out as in section 2. The different metabolites were added at the concentrations shown in the figure.

4. Discussion

The results obtained in this work strongly indicate the presence of a Dol-P phosphatase in membranes from neuronal perikarya. The enzyme activity is enhanced by the addition of EDTA and appears to be specific.

Preliminary results indicate the presence of two additional enzymes in membranes from neuronal perikarya (results not shown).

(1) The incubation of tritiated Dol-P-P led to the formation of Dol-P and dolichol. Further characterization of this enzyme is in progress.
(2) A dolichol kinase which uses CTP as a phosphate donor was also found. This enzyme is present in other eukaryotic cell membranes [17–19].

It has been firmly established that the dolichol pathway is involved in the biosynthesis of glycoproteins of asparagine type [2]. However, the regulation of this pathway is presently unknown. It has been proposed that the rate of protein glycosylation depends on the level of endogenous Dol-P [3]. Therefore, any enzyme(s) that modify the level of Dol-P may have a regulatory role on protein glycosylation. One possibility is that the dolichol kinase and both of the phosphatases control the level of Dol-P in the cell. However, it is not possible at present to exclude a similar function for the transferases. It was reported that inhibition of the early steps of dolichol formation resulted in decreased amounts of dolichyl saccharide synthesis and lower rates of protein glycosylation [20]. The measurement of each of these enzymatic activities in different physiological conditions of the cell will help to increase knowledge of the regulatory mechanisms underlying protein glycosylation.

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