Dolichyl phosphate phosphatase from *Tetrahymena pyriformis*

Gwen S. ADRIAN and Roy W. KEENAN

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78284, U.S.A.

(Received 9 March 1981/Accepted 17 March 1981)

A soluble dolichyl phosphate phosphatase from *Tetrahymena pyriformis* was purified about 68-fold. The enzyme appeared to be specific for dolichyl phosphate and existed in two interrelated forms, one of mol.wt. about 500 000 and the other of mol.wt. about 63 000. The enzyme was strongly inhibited by 5 mM-Mn²⁺ and was strongly stimulated by Mg²⁺. *Tetrahymena* in the exponential growth phase contained more of this enzymic activity than cells in stationary or lag phase. The dolichyl phosphate phosphatase may be loosely bound to mitochondrial membranes. Two roles proposed for this enzyme are (1) that of releasing dolichol from its phosphorylated biosynthetic form for its use in the cell as unesterified dolichol or dolichyl ester and/or (2) that of regulation of synthesis of glycoproteins or some other glycosylated compound.

Glycoproteins are currently receiving considerable attention because of their involvement in crucial biological processes such as embryological development, immunologic responses, cell–cell interactions and binding of hormones, viruses and bacteria to cell membranes. Biosynthesis of *N*-linked glycoproteins requires a lipid carrier for sugars; this lipid carrier is dolichyl phosphate. Several workers have suggested that the rate of synthesis of glycosylated peptides may be controlled by the amount of available dolichyl phosphate (Lucas & Levin, 1977; Krag *et al.*, 1977; Harford *et al.*, 1977; Carson & Lennarz, 1979). If so, an enzyme that can increase or decrease the cellular concentrations of dolichyl phosphate may be important in regulation of this pathway. We published preliminary work on a dolichyl phosphate phosphatase from *Tetrahymena pyriformis* (Keenan *et al.*, 1978; Adrian & Keenan, 1979). More recently, Wedgwood & Strominger (1980), Idoyaga-Vargas *et al.* (1980) and Burton *et al.* (1980) have also reported finding such activities in cultured human lymphocytes, neuronal perikarya from rats and calf brain membranes respectively.

This paper reports the further characterization of the dolichyl phosphate phosphatase activity from *T. pyriformis* to determine more about the nature, specificity and possible role of this enzyme in *T. pyriformis*.

Experimental

T. pyriformis starter cultures were a gift from Dr. Guy A. Thompson. The organisms were cultured and stored as described previously (Adrian &

Keenan, 1979). Alumina was obtained from E. Merck (Rahway, NJ, U.S.A.), high-pressure-liquid-chromatography solvents from Fisher Chemical Co. (Fairlawn, NJ, U.S.A.), *p*-nitrophenyl phosphate from Boehringer Mannheim Corp. (San Francisco, CA, U.S.A.), and proteose peptone and yeast extract from Difco Laboratories (Detroit, MI, U.S.A.). Protein concentrations were determined by the microbiuret method (Munkres & Richards, 1965) with bovine serum albumin as standard.

[³H]Dolichol and [³H]dolichyl phosphate were prepared as described previously (Adrian & Keenan, 1979). Dolichyl phosphate was determined by phosphorus analysis (Itaya & Ui, 1966). [³²P]-Phosphatidic acid was synthesized utilizing *Escherichia coli* diacylglycerol kinase, [³²P]ATP and diacylglycerol by the method of Schneider & Kennedy (1976). Incubation of the reaction product in dilute alkali converted over 90% of the radioactivity into a water-soluble form, as expected with [³²P]phosphatidic acid. The reaction product also migrated as standard phosphatidic acid on t.l.c. [³²P]Phosphatidic acid was quantified by calculation of the conversion of [³²P]ATP of known specific radioactivity.

[¹⁴C]Phytanol was phosphorylated by the method of Rupar & Carroll (1976). The product exhibited the expected i.r. spectrum, and its concentration was determined by phosphorus assay.

High-pressure liquid chromatography of dolichols was performed as described by Adrian & Keenan (1979). The reversed-phase column was eluted with propan-2-ol/methanol/hexane/water (65:30:5:1, by vol.) as described by Tavares *et al.* (1977).

Enzyme preparation

Fresh *Tetrahymena* pellets or freeze-dried *Tetrahymena* were homogenized in 0.05 M-succinate/0.1 M-KCl buffer, pH 6.0. Fresh cells were disrupted by the use of a Tekmar Tissumizer with two 30-s bursts at about 17 500 rev./min. The homogenates were centrifuged at 37 000 g for 20 min. The supernatant, which contained the majority of the enzyme activity, was stored at -20°C until required. All operations were carried out at 4°C unless otherwise indicated.

Enzyme assays for hydrolysis of dolichyl phosphate, [^3H]dolichyl phosphate and [^{14}C]phytanyl phosphate

The hydrolysis of non-radioactive dolichyl phosphate and [^3H]dolichyl phosphate was determined as previously described (Adrian & Keenan, 1979), except that dolichol was eluted from the alumina column with chloroform/methanol (1:1, v/v) instead of acetone. [^{14}C]Phytanyl phosphate hydrolysis was measured in the same manner as [^3H]dolichyl phosphate hydrolysis. Typical incubations were in a total volume of 0.125 ml with 14 mM- MgCl_2 in 0.05 M-succinate buffer, pH 6.0. Incubations were usually run at 35°C for 5, 10 or 15 min, depending on the enzyme concentration. Reactions were usually run with 0.3 mM-substrate, the calculated K_m for both dolichyl phosphate and phytanyl phosphate, because of technical problems in solubilizing dolichyl phosphate. Therefore, velocities measured were about one-half the apparent $V_{\max}$. One unit of enzyme activity equals $1\mu\text{mol}$ of substrate hydrolysed/min.

[^{32}P]Phosphatidic acid phosphatase assays

[^{32}P]Phosphatidic acid hydrolysis was measured by a method similar to that of Flynn *et al.* (1977). The [^{32}P]phosphatidic acid substrate as prepared contained Triton X-100, and additional detergent in the assay inhibited the reaction. [^{32}P]Phosphatidic acid dissolved in chloroform was pipetted into tubes, the solvent removed under an N_2 stream, and the residue was suspended by sonication in 0.05 M-succinate buffer, pH 6.0. Enzyme was then added and samples were incubated for 5 and 10 min at 35°C . Incubations were carried out with 10 μM - or 0.3 mM-phosphatidic acid (40 000 d.p.m./assay) in a total volume of 0.125 ml. The reaction was stopped by adding the incubation mixture to a Brinkman tube containing 0.19 ml of chloroform, 0.05 ml of methanol and 0.045 ml of dilute HCl in 0.4% NaCl. After thorough mixing and centrifugation, a portion of the upper aqueous phase was counted for radioactivity in 10 ml of Beckman scintillant. Enzymic activity was determined from the known specific radioactivity of the [^{32}P]phosphatidic acid.

Column chromatography

A Biogel A 1.5 column (50 cm $\times$ 2.3 cm) was equilibrated with 0.04 M-succinate/0.1 M-KCl buffer, pH 6.0. The void volume of the column was 78.5 ml. The column was calibrated with ovalbumin (mol.wt. 44 000–46 000), aldolase (mol.wt. 140 000) and bovine γ -globulin (which elutes as a protein of mol.wt. 250 000, because of its elliptical shape). Protein solutions were eluted with 0.05 M-succinate/0.1 M-KCl buffer, pH 6.0.

The $\text{Ca}_3(\text{PO}_4)_2$ -cellulose kindly provided by Dr. Jonathan Nishimura was packed into a column and equilibrated with 0.05 M-succinate buffer, pH 6.0. Protein solutions were loaded and several column volumes of the succinate buffer were run through the column eluting much of the protein. Dolichyl phosphate phosphatase then was eluted with 0.3 M-phosphate buffer, pH 6.7.

Subcellular fractionation

The method of Nozawa & Thompson (1971) was used for subcellular fractionation of fresh *Tetrahymena*. The fractions were checked by electron microscopy, and they compared well with the electron micrographs published by Nozawa & Thompson (1971).

Starvation and refeeding of *Tetrahymena*

The method of Cameron & Jeter (1970) was used for starving and refeeding of *Tetrahymena*. Exponential-phase *T. pyriformis* (strain WH 14; 2 litres), containing approx. 55 000 cells/ml, were centrifuged at 600 g for 8 min and washed with sterile 5 mM-phosphate buffer containing 2 mM- MgSO_4 . The cells were resuspended in the sterile phosphate buffer in two low-form flasks that contained 1 litre of phosphate buffer each and incubated with shaking for 22 h. These starved cells were then centrifuged at 600 g for 8 min, washed with water and one-quarter of the cells was added to each of four low-form flasks containing 1 litre of the proteose peptone medium described above. Growth of the cells was followed by cell counts using a haemocytometer. One flask was harvested during lag phase (after 4 h), one at mid-exponential phase (20.8 h), one in late-exponential phase (28 h) and one in stationary phase (52 h).

Results

The *Tetrahymena* dolichyl phosphate phosphatase activity was partially purified, and a dolichyl phosphate phosphatase preparation was obtained that would not hydrolyse *p*-nitrophenyl phosphate. Dolichyl phosphate phosphatase, which had a specific activity of 2.0×10^{-3} units/mg in the initial buffer extract, yielded enzyme preparations with

specific activities of 1.7×10^{-2} units/mg and 4.3×10^{-2} units/mg respectively, after fractionation on first a Biogel A 1.5 column and second a $\text{Ca}_3(\text{PO}_4)_2$ -cellulose column. A 22-fold purification was calculated from these specific activities. However, since the enzyme from the $\text{Ca}_3(\text{PO}_4)_2$ -cellulose column was eluted in phosphate buffer and phosphate inhibited dolichyl phosphate phosphatase, the actual purification was about 68-fold, i.e. the final phosphate concentration in the assay was 0.18 M and 0.18 M-phosphate was shown in independent experiments to cause 68.5% inhibition of dolichyl phosphate phosphatase activity.

Dolichyl phosphate phosphatase was eluted consistently from the Biogel column in two peaks as shown in Fig. 1. The molecular weights of the two peaks were estimated to be 500 000 and 63 000.

Three fractions of the high-molecular-weight peak, fractions 21, 22, and 23 shown in Fig. 1, were pooled and again chromatographed on the same Biogel column. As shown in Fig. 2, the dolichyl phosphate phosphatase again eluted in two peaks of activity, and only 55% of the dolichyl phosphate phosphatase activity loaded on the column was recovered in the eluate. These results indicate that this enzyme may exist in two forms and the smaller form may be less active than the larger.

Hydrolysis of dolichyl phosphate was compared with hydrolysis of two other lipid phosphates, [^{32}P]phosphatidic acid and [^{14}C]phytanyl phosphate. The results of assays of subcellular fractions are shown in Table 1. The Biogel fractions shown in Fig. 2 were also assayed for hydrolysis of phosphatidic acid and phytanyl phosphate. These ac-

Fig. 1. Separation of dolichyl phosphate phosphatase and *p*-nitrophenyl phosphatase activities on Biogel A 1.5. About 150 mg of protein in a volume of 22 ml was loaded on the column. The protein was a buffer extraction of 1 litre of strain WH 14 *Tetrahymena* cells. Fractions (6.5 ml) were collected and assayed for [^3H]dolichyl phosphate and *p*-nitrophenyl phosphate hydrolysis as described in the Experimental section.

Fig. 2. Refractionation of dolichyl phosphate phosphatase on Biogel A 1.5. Fractions 21, 22 and 23, which contained the larger-molecular-weight dolichyl phosphate phosphatase peak from the fractionation shown in Fig. 1, were pooled, and the resultant 18.9 ml, which contained 7.8 mg of protein, was rechromatographed on the Biogel A 1.5 column. Dolichyl phosphate phosphatase activity again emerged in two peaks.

Table 1. *Phosphatase activity in subcellular fractions*

Abbreviations used: Dol-P, dolichyl phosphate; P.A., phosphatidic acid; Phy.P., phytanyl phosphate; PNPP, *p*-nitrophenyl phosphate.

Fraction	Protein content		10 ⁴ × Specific activity (μmol/min per mg)				Total activity (%)			
	(mg)	(%)	Dol-P	P.A.	Phy.P.	PNPP	Dol-P	P.A.	Phy.P.	PNPP
Cilia	16.7	3.0	3.95	35.9	0.45	632	1.9	1.4	0.2	3.7
Pellicle	25.4	4.6	3.89	26.7	2.10	615	2.7	1.6	1.4	5.4
Mitochondria, lysosomes and peroxisomes	236.6	43.3	4.50	31.9	1.30	584	30.4	18.4	8.8	47.9
Microsomes	33	6.0	3.66	42.2	9.49	1280	3.4	3.3	8.9	14.7
Microsomal supernatant	234.6	42.9	9.24	133.0	11.90	348	61.7	75.2	80.4	28.3

tivities were not eluted in parallel, and the fraction with the most total units of enzyme differed for each of the three activities.

Evidence that the lipid phosphates were hydrolysed by different enzymes was also obtained by inhibition experiments. Phosphatidylcholine (1.5 mM) caused 91–100% inhibition of phosphatidic acid hydrolysis and only 53–70% inhibition of dolichyl phosphate hydrolysis. Phytanyl phosphate (0.1 mM) did not inhibit hydrolysis of up to 0.3 mM-dolichyl phosphate, and 0.1 mM-phosphatidic acid did not inhibit hydrolysis of up to 0.2 mM-dolichyl phosphate. Phosphatidic acid (0.1 mM) did inhibit hydrolysis of 0.3 mM- and 0.4 mM-dolichyl phosphate by 10% and 12% respectively. These experiments were difficult because of the low solubilities of the substrates. Hydrolysis of all three lipid phosphates was stimulated by MgCl₂. Hydrolyses of dolichyl phosphate and phosphatidic acid were both inhibited by Ca²⁺, F⁻ and vanadium oxide sulphate.

To determine at what growth stage dolichyl phosphate phosphatase was most abundant, cultures that had been initially synchronized by starvation were refed and harvested at four different times during the cell growth cycle. As shown in Fig. 3, the dolichyl phosphate phosphatase activity was highest during exponential phase, whereas *p*-nitrophenyl phosphate-hydrolysing activity was highest during the stationary phase.

To determine if the dolichyl phosphate phosphatase activity that remained with the pellet (see Fig. 3) was loosely or tightly bound to membrane, homogenized fresh cells were extracted repeatedly with 0.05 M-succinate/0.1 M-KCl, buffer, pH 6.0, to determine what proportion of the enzyme could be solubilized. Four extractions solubilized 80–90% of the total activity, indicating that the enzyme was loosely bound. The effects of various cations on the hydrolysis of dolichyl phosphate are shown in Table 2.

Fig. 3. *Dolichyl phosphate phosphatase and p-nitrophenyl phosphate phosphatase content of Tetrahymena at different stages in the cell growth cycle*

Specific activities for hydrolysis of [³H]dolichyl phosphate and *p*-nitrophenyl phosphate of cells that were harvested at different phases of the cell growth cycle. Homogenized cells were extracted with buffer twice, and the 37000 g supernatants were pooled to prepare the soluble enzyme of these experiments. Abbreviation used: exp., exponential.

Discussion

An enzyme activity was found in *T. pyriformis* that appeared to be quite specific for dolichyl phosphate. This activity was partially purified and was definitely different from the non-specific acid phosphatases that hydrolyse *p*-nitrophenyl phosphate as shown by our previous work (Adrian & Keenan, 1979) and the physical separation of the two activities by the use of a Biogel A 1.5 column. The dolichyl phosphate phosphatase is also most likely to be different from the enzymes that hydrolyse phosphatidic acid and phytanyl phosphate as indicated by (1) different distributions of the activities in subcellular fractions, (2) different

Table 2. *Effectors of dolichyl phosphate phosphatase*

Effector	Activity (% of control value)	Effector	Activity (% of control value)
No added effector*	100	(NH ₄) ₂ SO ₄	
		0.88 M	54
		1.66 M	29
MnCl ₂ (noMg ²⁺)		NaCl	
5 mM	2	0.1 M	67
14 mM	2	0.3 M	55
30 mM	4		
MnCl ₂ (14 mM-Mg ²⁺)		KCl	
5 mM	0	0.1 M	51
25 mM	0	0.2 M	33
		0.3 M	27
CaCl ₂ (noMg ²⁺)		Sodium phosphate buffer	
5 mM	0	0.05 M	61
14 mM	3	0.15 M	37
30 mM	0	0.3 M	19
CaCl ₂ (14 mM-Mg ²⁺)		Na ₄ P ₂ O ₇	
5 mM	50	8 mM	60
25 mM	8		

* Mg²⁺ (14 mM) was used in all reactions except where otherwise stated.

distributions of the activities in Biogel fractions and (3) inhibition experiments.

The *Tetrahymena* dolichyl phosphate phosphatase differed from the other dolichyl phosphate phosphatases reported (Wedgwood & Strominger, 1980; Idoyaga-Vargas *et al.*, 1980; Burton *et al.*, 1980) in that it was stimulated by Mg²⁺ and much of it was readily solubilized. The dolichyl phosphate phosphatase from rat neuronal perikarya (Idoyaga-Vargas *et al.*, 1980) was inhibited by Mn²⁺ and Ca²⁺ as was the *Tetrahymena* enzyme.

Several experiments support some speculations about the role of the dolichyl phosphate phosphatase from *Tetrahymena*. The fact that dolichyl phosphate phosphatase was most prevalent in exponential-phase cells and not in stationary-phase cells probably indicates that the dolichyl phosphate phosphatase is not simply a degradative enzyme, but rather an enzyme involved in some process of optimally growing and dividing cells.

Since most dolichol is found in nature in the non-esterified or esterified form (Hemming, 1974) and dolichol is biosynthesized as a phosphorylated compound (Beytia & Porter, 1976), there is obviously a phosphatase and/or pyrophosphatase that can remove the phosphate residues and release dolichol for whatever functions it has in the cell. The enzyme described in the present work could serve such a role in actively growing *Tetrahymena pyriformis*, since, as shown by this work and that of others (Allen *et al.*, 1967; Daleo & Pont Lezica, 1977), at least some, if not all, non-specific phosphatases have very low activities in dolichyl phos-

phate hydrolysis. This role for dolichyl phosphate phosphatase may also be indicated by the fact that about 60% of the enzyme was found in the soluble fraction of the cell, which is also the probable site for the enzymes that synthesize dolichyl pyrophosphate.

Experimental evidence indicated that the membrane-bound dolichyl phosphate phosphatase was rather loosely bound to mitochondrial membranes. Since mitochondria are active in the synthesis of dolichyl phosphate sugars and oligosaccharide lipids in *Tetrahymena* (Keenan *et al.*, 1975) and rat and rabbit liver (Martin & Thorne, 1974; Gateau *et al.*, 1978; Coolbear *et al.*, 1979), this location of the enzyme may be due to its possible role in regulation of synthesis of these glycoprotein intermediates. Our data suggest two possible control mechanisms for the enzyme: (1) two apparently interchangeable forms of the enzyme exist, and the smaller form may be less active than the larger; (2) the enzyme is strongly inhibited by Ca²⁺ and stimulated by Mg²⁺.

Attempts were made to show an effect of dolichyl phosphate phosphatase *in vitro* on synthesis of dolichyl phosphate mannose, but owing to technical difficulties it was not possible to show a clear-cut effect of the enzyme on dolichyl phosphate mannose synthesis *in vitro*. It is possible that the endogenous dolichyl phosphate is imbedded in the membranous mannosyltransferase preparation and was not accessible to the soluble dolichyl phosphate phosphatase added to the reaction.

In the experiments described here, phosphorylated pig liver dolichol was used as a substrate. We

have found that *Tetrahymena* does contain dolichols and therefore also dolichyl phosphate, which could serve as an endogenous substrate for the *Tetrahymena* dolichyl phosphate phosphatase (Adrian & Keenan, 1981).

It would be of interest to determine if the *Tetrahymena* dolichyl phosphate phosphatase will also hydrolyse dolichyl pyrophosphate. If so, this enzyme could neatly fit into the proposed cycle for synthesis of glycoproteins in that it might hydrolyse dolichyl pyrophosphate released from lipid oligosaccharide to dolichyl phosphate, which would then be available for another cycle of glycoprotein synthesis, or it might hydrolyse the biosynthetically produced dolichyl pyrophosphate to the free poly-prenol, a necessary but as yet undescribed step in dolichol biosynthesis.

Nancy D. Rice's very capable technical assistance with this work is greatly appreciated. Marge Jones generously prepared the [^{32}P]phosphatidic acid used in these experiments. We are indebted to Dr. Erle Adrian and Sue Turner for performing the electron microscopy. The project was supported financially by NIH Grant no. AM 17897-06.

References

- Adrian, G. S. & Keenan, R. W. (1979) *Biochim. Biophys. Acta* **575**, 431–438
- Adrian, G. S. & Keenan, R. W. (1981) *Biochim. Biophys. Acta* **663**, 637–644
- Allen, C. M., Alworth, W., Macrae, A. & Bloch, K. (1967) *J. Biol. Chem.* **242**, 1895–1902
- Beytia, E. D. & Porter, J. W. (1976) *Annu. Rev. Biochem.* **45**, 113–142
- Burton, W. A., Scher, M. G. & Waechter, C. J. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1901
- Cameron, I. L. & Jeter, J. R. (1970) *J. Protozool.* **17**, 429–431
- Carson, D. P. & Lennarz, W. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5709–5713
- Coolbear, T., Mookerjee, S. & Hemming, F. W. (1979) *Biochem. J.* **184**, 391–397
- Daleo, G. R. & Pont Lezica, R. (1977) *FEBS Lett.* **74**, 247–250
- Flynn, T. J., Deshmukh, D. S. & Pieringer, R. A. (1977) *J. Lipid Res.* **18**, 777–780
- Gateau, O., Morelis, R. & Louisot, P. (1978) *Eur. J. Biochem.* **88**, 613–622
- Harford, J. B., Waechter, C. J. & Earl, F. L. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1036–1043
- Hemming, F. W. (1974) in *Biochemistry of the Lipids* (Goodwin, T. W., ed.), pp. 39–97, University Park Press, Baltimore
- Idoyaga-Vargas, V., Belocopitow, E., Mentaberry, A. & Carminatti, H. (1980) *FEBS Lett.* **112**, 63–66
- Itaya, K. and Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
- Keenan, R. W., Kruczek, M. & Fusinato, L. (1975) *Arch. Biochem. Biophys.* **167**, 697
- Keenan, R. W., Rice, N. E. & Adrian, G. S. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1644
- Krag, S. S., Cifone, M., Robbins, P. W. & Baker, R. M. (1977) *J. Biol. Chem.* **252**, 3561–3564
- Lucas, J. J. & Levin, E. (1977) *J. Biol. Chem.* **252**, 4330–4336
- Martin, H. G. & Thorne, K. J. I. (1974) *Biochem. J.* **128**, 281–289
- Munkres, K. D. & Richards, F. W. (1965) *Arch. Biochem. Biophys.* **109**, 466–479
- Nozawa, Y. & Thompson, G. A. (1971) *J. Cell Biol.* **49**, 712–721
- Rupar, C. A. & Carroll, K. K. (1976) *Chem. Phys. Lipids* **17**, 193–206
- Schneider, E. G. & Kennedy, E. P. (1976) *Biochim. Biophys. Acta* **441**, 201–212
- Tavares, I. A., Johnson, N. J. & Hemming, F. W. (1977) *Biochem. Soc. Trans.* **5**, 1771–1773
- Wedgwood, J. F. & Strominger, J. L. (1980) *J. Biol. Chem.* **255**, 1120–1123