Calmodulin-dependent enzymic phosphorylation of dolichol in *Tetrahymena pyriformis*

Chandrashekhar R. GANDHI and Roy W. KEENAN

Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78240, U.S.A.

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The Protozoan, *Tetrahymena pyriformis*, is capable of phosphorylating dolichol in the presence of CTP. Other nucleotides (ATP, UTP and GTP) were ineffective. The enzyme was activated independently by the bivalent cations Mg^{2+} , Mn^{2+} and Ca^{2+} . The Ca^{2+} stimulation of the enzyme activity was calmodulin-dependent. A substantial increase in the enzyme activity was seen in the presence of UTP. The apparent K_m values for CTP and dolichol, as calculated from the Lineweaver–Burk plot, were 3 mM and 70 μM respectively. Although the presence of detergent was essential, at higher concentrations there was a decrease in the enzyme activity. The enzyme had two pH optima (pH 7.4 and 9.0) and at both the activity was Ca^{2+} –calmodulin-dependent.

Many enzymes, immunoglobulins and most of the secreted proteins are glycoproteins (Struck & Lennarz, 1980). In eukaryotic cells, the glycosyl residues are assembled and transferred to protein in reactions that require phosphorylated dolichol as a coenzyme (Parodi & Leloir, 1976; Waechter & Lennarz, 1976; Hemming, 1977). The biologically active forms of the polyprenols are the mono- and di-phosphates. There is some evidence that the synthesis of glycoproteins is regulated by the level of dolichyl monophosphate (Lennarz, 1975; Lucas & Levin, 1977; Krag *et al.*, 1977). Availability of polyprenol phosphate in prokaryotes (Higashi *et al.*, 1970) and eukaryotes (Parodi & Leloir, 1976; Hemming, 1977) is believed to be governed by the relative activities of phosphatases and kinases.

Dolichol kinase, the enzyme catalysing the phosphorylation of dolichol, is reported to vary in cation requirement depending on the enzyme source employed. The phosphorylation of dolichol in rat liver (Rip & Carroll, 1980), bovine liver, mouse plasmacytoma and Chinese hamster ovary (Allen *et al.*, 1978) required Mg^{2+} , whereas enzyme from calf brain (Burton *et al.*, 1979) and sea-urchin embryo (Rossignol *et al.*, 1981) required Ca^{2+} . Thus far no evidence has been obtained to explain why the enzyme differs in cation requirement.

A lipid-linked pathway for the biosynthesis of glycoproteins (Keenan *et al.*, 1972a, 1973) and the presence of dolichyl phosphate phosphatase (Adrian & Keenan, 1979) were found in *Tetrahymena pyriformis*. It was considered worthwhile to de-

termine if this organism also has a dolichol kinase and to investigate its properties in order to learn more about this important enzyme and how it is regulated. The results reported in the present paper provide evidence that the phosphorylation of dolichol in *Tetrahymena pyriformis* is controlled by a system involving Ca^{2+} and calmodulin.

Materials and methods

$[\gamma\text{-}^{32}\text{P}]\text{CTP}$ was synthesized from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 2000 Ci/mmol) purchased from Schwarz–Mann, Spring Valley, NY, U.S.A., under the conditions similar to those described by Keenan *et al.* (1972b). Purified dolichol samples were a gift from the Polish Academy of Sciences. Calmodulin purified from beef brain was kindly provided by Dr. Marty Javors, UT Health Science Center, San Antonio, TX, U.S.A. Precoated silica gel-G plates were purchased from Analtech. All other reagents and chemicals used were of the highest available purity.

Enzyme preparation

Tetrahymena pyriformis (strain SB 255) originally supplied by Dr. Edvardo Orias was grown and isolated as described by Adrian & Keenan (1981a). A 20% homogenate was made from isolated cells in 0.25 M-sucrose/10 mM-Hepes, pH 7.4. The homogenate was centrifuged at 900g for 10 min. The supernatant fraction was further centrifuged at 20000g for 20 min. The 'high-speed' pellet was washed once with the buffer and suspended in it. The enzyme was stable for at least 15 days at -20°C .

Protein was estimated by the method of Lowry *et al.* (1951).

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; h.p.l.c., high-pressure liquid chromatography.

Assay of dolichol phosphorylation

All the assays were carried out in duplicate and the indicated values are their means. The variation was within 10%. Unless otherwise stated, the incorporation of radioactivity from [γ - 32 P]CTP into dolichol was measured in a 50 μ l incubation mixture containing the final concentrations of 230 μ M-dolichol (mixture isolated from human liver), 0.5% Triton X-100, 8 mM-[γ - 32 P]CTP (sp. radioactivity 7 Ci/mol), 20 mM-MgCl₂, 5 mM-CaCl₂, 10 mM-NaF, 20 mM-UTP, 100 mM-Tris/maleate, pH 7.4, and enzyme suspension containing 0.4–0.6 mg of protein. The reaction mixture was incubated for 15 min at 37°C and the reaction was terminated by the addition of 800 μ l of chloroform/methanol (2:1, v/v). After 30 min at room temperature, the mixture was washed once with 125 μ l of water followed by three washes with 300 μ l of chloroform/methanol/water (3:48:47, by vol.). The lower organic phase was dried and the radioactivity measured in 10 ml of toluene scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Characterization of the reaction product

Thin-layer chromatography. The reaction product was identified as dolichyl phosphate by t.l.c. on silica gel-G plates. The plates were developed in (i) chloroform/methanol/water (13:5:1, by vol.); (ii) chloroform/methanol/conc. NH₄OH (13:7:1, by vol.) and (iii) di-isobutyl ketone/acetic acid/water (60:45:6, by vol.). In all the solvent systems, the reaction product co-chromatographed with authentic dolichyl phosphate.

High-pressure liquid chromatography. The reaction product was mixed with carrier dolichyl phosphate and analysed by h.p.l.c. as described by Keenan *et al.* (1982) using the solvent system devised by Professor F. W. Hemming (personal communication) of propan-2-ol/methanol (13:7, v/v) + 0.11 g of 88% phosphoric acid. The results of h.p.l.c. are shown in Fig. 1. It can be seen that the radioactivity corresponds to the elution profile of the carrier dolichyl phosphate.

Results

Experiments were carried out to optimize the conditions for the assay of dolichol kinase. The enzyme preparation used in these assays was tested in a parallel experiment to determine dolichyl phosphate phosphatase activity. We substituted dolichyl [32 P]phosphate for dolichol and replaced [γ - 32 P]CTP by unlabelled CTP. Fluoride was not included. It was observed that the phosphatase activity was inhibited up to 58% in the presence of Ca²⁺ levels used routinely in the kinase assays. The activity of dolichol kinase was further elevated by

Fig. 1. The high-pressure liquid chromatography of 32 P-labelled dolichyl phosphate

The lipid extract from *T. pyriformis* particulate fraction, incubated with dolichol and [γ - 32 P]CTP, was mixed with carrier synthetic dolichyl phosphate and chromatographed on a reversed-phase column. Portions of the column eluate were collected and the radioactivity was determined. The numbers over the peaks refer to the numbers of isoprene units in the synthetic dolichyl phosphate. All conditions and procedures were as described in the Materials and methods section.

the inclusion of 10 mM-NaF, which is presumed to be an inhibitor of dolichyl phosphate phosphatase activity (Allen *et al.*, 1978), and 20 mM-UTP, which probably inhibited the breakdown of the radioactive CTP.

A linear increase in dolichyl phosphate was observed up to 15 min (Fig. 2) and the optimum

Fig. 2. *The effect of time on the activity of dolichol kinase*
Aliquots from the incubation were taken at the times indicated and the radioactivity was measured in the lipid extract. All other conditions were as described in the Materials and methods section.

Fig. 3. *The effect of protein concentration on the activity of dolichol kinase*

Protein was added at the concentrations indicated. Other procedures and conditions were as described in the Materials and methods section.

protein concentration was 7–10 mg/ml (Fig. 3). The enzyme activity increased steadily with increasing concentrations of CTP and dolichol, the saturating levels of which were 10 mM and 250 μ M respectively (Figs. 4a and 4b). Apparent K_m values of 3 mM and 70 μ M were calculated from the Lineweaver–Burk plots for CTP and dolichol respectively (Figs. 4a and 4b, insets).

Fig. 4. *The effect of CTP and dolichol concentrations on the activity of dolichol kinase*

CTP and dolichol were added at the concentrations indicated. All other conditions were as described in the Materials and methods section. The apparent K_m values for CTP and dolichol were 3 mM and 70 μ M respectively as calculated from Lineweaver–Burk plots (insets a and b). Dolichol and [γ -³²P]CTP were added at 230 μ M and 8 mM concentrations respectively in the incubations (a) and (b). Dolichyl phosphate formed is expressed as c.p.m. $\times 10^{-3}$ /mg of protein per 15 min incubation.

Nucleotide, detergent and pH

Dolichol phosphokinase activity was very specific for CTP. The results of isotopic dilution studies are shown in Table 1. When unlabelled 20mM-UTP, -CTP, -GTP and -ATP were included in the incubation medium in addition to [γ - 32 P]CTP, only CTP depressed the incorporation of radioactivity into the lipid nearly as much as predicted from the dilution of the radioactive coenzyme. On addition of UTP the enzyme activity was found to increase by 19%. No such effect was, however, seen in the presence of ATP or GTP. As is evident from Fig. 5, there is very low enzyme activity in the absence of detergent. Optimum concentration of the detergent (Triton X-100) was found to be 0.5% and a decrease in the enzyme activity at higher concentrations was observed.

The enzyme had two pH optima, one around pH 7.4 (Tris/maleate buffer) and the other around 9.0 (glycine buffer) (Fig. 6). A similar but not identical pH effect was seen by Allen *et al.* (1982) in bovine liver microsomes. Dolichol kinase activity at both pH values was found to be Ca^{2+} -calmodulin-dependent (results not shown).

Table 1. Effect of nucleotides on the dolichol kinase activity

Results are expressed as c.p.m./mg of protein per 15 min incubation. The enzyme assay was carried out as described in the Materials and methods section. Unlabelled nucleotides were added at 20mM concentration.

Nucleotide	Dolichyl phosphate radioactivity	
	(c.p.m.)	(%)
None	8480	100
+UTP	10200	119
+CTP	3500	41
+ATP	8600	101
+GTP	8590	101

Table 2. Effect of bivalent metal ions on the dolichol kinase activity

Results expressed as c.p.m./mg of protein per 15 min incubation. The chloride salts of Mg^{2+} , Mn^{2+} and Ca^{2+} were added at 20mM, 15mM and 5mM concentrations respectively. All other conditions were as described in the Materials and methods section.

Cation added	Dolichyl phosphate radioactivity (c.p.m.)
None	590
Mg^{2+}	3290
Mn^{2+}	8200
Ca^{2+}	11600
$\text{Mg}^{2+} + \text{Mn}^{2+}$	7430
$\text{Mg}^{2+} + \text{Ca}^{2+}$	14800
$\text{Mn}^{2+} + \text{Ca}^{2+}$	10500

Fig. 5. The effect of detergent concentration on the activity of dolichol kinase

Triton X-100 was added at the levels indicated and the other conditions were as described in the Materials and methods section.

Fig. 6. The effect of pH on the activity of dolichol kinase

Buffer was added at 100mM final concentration. ♦, Acetate buffer; ●, Tris/maleate buffer; ■, glycine buffer. All other conditions were as described in the Materials and methods section.

Effect of ions

The effect of various ions on the activity of dolichol kinase is demonstrated in Table 2. The cations were added at their optimum concentrations. The enzyme showed maximum activity in the presence of Ca^{2+} as compared with equimolar

Table 3. Effect of EGTA washing on the activity of dolichyl phosphate

Results are expressed as c.p.m./mg of protein per 15 min incubation. Enzyme preparations were washed once with 0.25 M-sucrose/10 mM-Hepes containing 5 mM-EGTA, pH 7.4. All other conditions were as described in the Materials and methods section. Calmodulin was added at 10 μ M.

	Dolichyl phosphate radioactivity	
	(c.p.m.)	(%)
Control	17 200	100
EGTA washed	400	
EGTA-washed + Mg ²⁺	4530	24
EGTA-washed + Ca ²⁺	1340	6
EGTA-washed + calmodulin	600	1
EGTA-washed + Mg ²⁺ + calmodulin	4490	24
EGTA-washed + Ca ²⁺ + calmodulin	12 500	72
EGTA-washed + Mg ²⁺ + Ca ²⁺	4150	22
EGTA-washed + Mg ²⁺ + Ca ²⁺ + calmodulin	15 400	87

concentrations of Mg²⁺ or Mn²⁺ (results not shown). Higher rates of reaction were observed with Mn²⁺ than with Mg²⁺. A cumulative increase in the phosphorylation of dolichol was observed when both Mg²⁺ and Ca²⁺ were present in the medium. No other combination of ions tested showed such an effect. EDTA inhibited the enzyme activity again, indicating the requirement for bivalent cations.

Effect of Ca²⁺-calmodulin

Table 3 and Figs. 7(a) and 7(b) show the effect of calmodulin on the enzymic phosphorylation of dolichol. For these experiments, the enzyme preparation was washed with 0.25 M-sucrose/10 mM-Hepes, pH 7.4, containing 5 mM-EGTA. Using these preparations, the enzyme activity was stimulated in the presence of Mg²⁺ but not Ca²⁺. The addition of calmodulin to the incubations containing Ca²⁺ markedly increased activity, but no change was observed when calmodulin was added to the incubation containing Mg²⁺. This clearly indicates that dolichol kinase from *T. pyriformis* was activated in two different ways, namely a Ca²⁺-calmodulin-dependent and -independent mechanism which required Mg²⁺.

Furthermore, Ca²⁺-calmodulin-dependent enzymic phosphorylation of dolichol was strongly inhibited by chlorpromazine and trifluoperazine. The enzyme activity was inhibited 50% at 60 μ M and 80 μ M concentrations of trifluoperazine and chlorpromazine respectively (Fig. 8).

Specificity to polyprenol

It is evident from Fig. 1 that, from the mixture containing dolichol-17, -18, -19 and -20, dolichol

Fig. 7. The effect of calmodulin (a) and Ca²⁺ (b) concentrations on the activity of dolichol

The enzyme activity was determined at the indicated levels of calmodulin (a) and Ca²⁺ (b) in the presence of 5 mM-Ca²⁺ and 10 μ M-calmodulin respectively. Mg²⁺ was not included in the incubations. All other conditions were as described in the Materials and methods section.

kinase from *Tetrahymena pyriformis* prefers dolichol-18 and dolichol-19 as substrates. In another experiment, we found that even a short-chain polyprenol (dolichol-11) was also an excellent substrate for this reaction. The major polyprenols in *Tetrahymena* are dolichol-14 and dolichol-15 (Adrian & Keenan, 1981b) and therefore is was assumed that, from the mixture, the enzyme would prefer dolichol-17 and/or dolichol-18 over dolichol-19 and -20. However, this observation is not unusual, since

Fig. 8. Inhibition of dolichol kinase by chlorpromazine and trifluoperazine

Enzyme activity was determined at the indicated levels of chlorpromazine (■) and trifluoperazine (●). Mg^{2+} was not included in the incubations. All other conditions were as described in the Materials and methods section.

rat liver dolichol kinase showed marked preference for dolichol-16 and dolichol-19 (Keller *et al.*, 1982), although the major constituent of rat liver dolichols is dolichol-18 (Gough & Hemming, 1970).

Discussion

The protozoan, *Tetrahymena pyriformis*, is capable of phosphorylating dolichol in the presence of CTP as phosphoryl donor. The enzyme had two pH optima, required Mg^{2+} and Ca^{2+} for maximum activity and was found to be dependent on calmodulin.

The product of the reaction was confirmed to be dolichyl monophosphate by t.l.c. in three different solvent systems and by h.p.l.c.

The amount of dolichyl phosphate formed increased for at least 30 min and the rate was linear up to 15 min. A similar kinetics for this enzyme was reported in rat liver microsomes (Rip & Carroll, 1980) and mammalian cell homogenates (Allen *et al.*, 1978). Burton *et al.* (1979) found that the enzymic phosphorylation of dolichol by calf brain membrane fraction was greatest in the presence of

Ca^{2+} but less with Mn^{2+} and least with Mg^{2+} . On the other hand, Rip & Carroll (1980) reported very low enzyme activity in the presence of either Ca^{2+} or Mn^{2+} with rat liver microsomes. They observed a partial inhibition of the Mg^{2+} -stimulated enzyme activity when Ca^{2+} was added to the medium. Dolichol kinase from *Tetrahymena* showed stimulation with Mg^{2+} and Ca^{2+} and a cumulative increase in the enzyme activity when Ca^{2+} and Mg^{2+} were both present in the medium. Combinations of Mg^{2+} and Mn^{2+} or Mn^{2+} and Ca^{2+} , however, failed to produce such an effect. With EGTA-washed enzyme preparations, there was stimulation of the enzyme activity only with Mg^{2+} but not with Ca^{2+} . This led us to conclude that the enzyme may be stimulated in two different ways, one dependent on Mg^{2+} stimulation and the other on Ca^{2+} . Many enzyme systems utilizing Ca^{2+} also require calmodulin (Means & Dedman, 1980), a heat-stable low-molecular-weight protein first reported by Cheung (1970). When Ca^{2+} and calmodulin were added to the incubation with EGTA-washed enzyme, the original activity was restored (Table 3). The loss of enzyme activity in the presence of either Ca^{2+} or calmodulin alone and inhibition of the enzyme activity in the presence of trifluoperazine and chlorpromazine, inhibitors of calmodulin stimulation, substantiate the requirement of calmodulin for dolichol kinase in *Tetrahymena*.

The reaction appeared to be saturated with respect to dolichol at 250 μM and CTP at 10 mM concentrations respectively. An apparent K_m for dolichol was found to be 70 μM. A similar K_m for dolichol was reported in rat liver microsomes (Rip & Carroll, 1980). The high K_m for CTP, as observed in these investigations, was also found for the enzyme from bovine liver (Allen *et al.*, 1978) and rat liver (Rip & Carroll, 1980). A decrease in K_m for CTP from 3 mM to 0.3 mM was observed by Allen *et al.* (1978) when they included 15 mM-ATP in their incubations. They suggested that ATP provided substrate for phosphatases, thereby protecting CTP from breakdown. Burton *et al.* (1979) observed a K_m of 6.5 μM for CTP in the presence of UTP and using endogenous polyprenols as substrate for this reaction. In our experiments, no change in the enzyme activity was noted in the presence of either ATP or GTP, but a 19% increase in the enzyme activity was seen in the presence of UTP. Also, when the incubation was deprived of UTP, the K_m for CTP almost doubled (results not shown). These results indicate that UTP is better for protecting the CTP against phosphatases than ATP or GTP in *Tetrahymena*.

To our knowledge, only one enzyme from *Tetrahymena*, guanylate cyclase, has been shown to be Ca^{2+} -calmodulin-modulated (Kakiuchi *et al.*, 1981). They reported that the activation of guanyl-

ate cyclase from *Tetrahymena* was specifically attributable to calmodulin from *Tetrahymena*, whereas calmodulin from other sources was ineffective. On the other hand, *Tetrahymena* calmodulin stimulated bovine brain phosphodiesterase, although not quite as effectively as brain calmodulin (Jamieson *et al.*, 1979). Since calmodulin used in these investigations was from bovine brain, it appears that *Tetrahymena* dolichol kinase is not specific for its own modulator protein. It will be interesting to study the enzymic reaction in the presence of *Tetrahymena* calmodulin to determine if it is more effective than the brain calmodulin used in these experiments.

Finally, as the glycoprotein biosynthesis is probably regulated by the availability of dolichyl phosphate and a major portion of the dolichol kinase activity is calmodulin-dependent, it is probable that glycoprotein biosynthesis, in *T. pyriformis*, may be modulated by calmodulin.

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