Dolichyl-phosphomannose synthase from the Archae Thermoplasma acidophilum

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Introduction

Glycosyltransferases responsible for glycoprotein biosynthesis in eucaryotes are localized to internal membranes, particularly the endoplasmic reticulum and Golgi apparatus. Dolichyl-phosphomannose synthase (EC2.4.1.83) catalyzes the formation of dol-P-Man, which is a key intermediate in the biosynthesis of asparagine-linked oligosaccharides and GPI-anchors. High energy bond sugar-phosphoryl-isoprenoids are intermediates between NDP-sugars and acceptors where transfer of the sugar through a membrane is necessary. In prokaryotes, sugar is connected through phosphate to C55-undecaprenols (Strominger et al., 1972) while in eucaryotes, dolichyl-phosphate, a polyynol that is saturated in the first isoprene unit, is the preferred carrier (Kornfeld et al., 1985). For yeast Saccharomyces cerevisiae, as well as other fungal cells, it has been established that mannose transfer to hydroxyl amino acids and to α1→6 mannans is catalyzed in the endoplasmic reticulum and requires dolichyl-phosphoryl-mannose (dol-P-Man) as an intermediate (Tanner et al., 1987). In mycobacteria Man-phosphoryl-decaprenol is the donor for α1→6 mannan synthesis (Nakajima et al., 1974). This report shows eucaryotic functional characteristics for the dol-P-Man synthase of the archae, T.acidophilum.

The establishment of a separate kingdom for the archae was supported by sequence comparisons of 16S rRNA fragments from various organisms (Searcy et al., 1981). According to divergence in these homologous sequences, archae form an independent kingdom, having the same evolutionary distance from both eubacteria and eucaryotes (Searcy et al., 1981). Glycosylation of proteins has been traditionally studied in eucaryotes and was thought absent in prokaryotes. However, a number of reports now show archae to possess true N- and O-linked glycoproteins. Mescher et al. (Mescher et al., 1976) found preliminary evidence that Haloferax volcanii was C55-polyisoprenol-phosphate, a prokaryotic characteristic (Zhu et al., 1995). Also, there was no evidence for GlcNAc in either glycoproteins or glycolipids in Haloferax. In this report, we show characterization of dol-P-Man synthase from the archae Thermoplasma acidophilum, a mycoplasma-like thermophilic organism that grows optimally at 56°C and pH 1.7. Thermoplasma acidophilum is an organism of considerable interest from an evolutionary standpoint, having several features usually associated only with eucaryotes, including a large number of glycoproteins, a basic histone-like protein, a labile intracellular cytoskeleton and certain features of respiratory metabolism characteristic of eucaryotic cytoplasm, but not of mitochondria (Searcy et al., 1978; Yang et al., 1979). Thermoplasma acidophilum lives in an extremely acidic environment, yet its cytoplasm has been reported to be approximately neutral (Hsung et al., 1975), based on low passive proton influx and an active extrusion of protons. A further passive mechanism protecting against low pH was suggested to be based on structural characteristics of glycosylated membrane proteins and glycolipids (Yang et al., 1979). To further examine the evolutionary relationship of glycosylation systems of archae plasma membrane and the eucaryotic endoplasmic reticulum and Golgi, we focused attention on biosynthesis of N- and O-linked glycoproteins and glycolipids of T.acidophilum. A large number of proteins from T.acidophilum stain with periodic acid-Schiff reagent. One major membrane protein, molecular weight...
152,000, bears a carbohydrate consisting mainly of α-1→2 linked mannosyl residues (Yang et al., 1979). Lipopolysaccharide in *T. acidophilum* is composed of mannose and glucose in a ratio of 24:1 (Mayberry-Carson et al., 1974). Therefore we first chose to search for a polyisoprenyl-phospho-mannose or dolichyl-phosphomannose synthesis system.

Dol-P-Man synthase has been partially purified from rat liver microsomes (Jensen et al., 1981), porcine aorta (Heifetz et al., 1977), *Acanthamoeba castellanii* (Carlo et al., 1979), murine mitochondria (Gasnier et al., 1992), and has been purified from cultures of *E. coli* carrying the yeast (*S. cerevisiae*) gene for this enzyme (Schutzbach et al., 1993). We report here an 1070-fold purification of this enzyme and show some novel properties including a higher molecular weight than those reported for eucaryotic enzymes and its surprising preference for C$_{85-105}$ or C$_{55}$ dolichyl-phosphate as an acceptor, the first such observations in archaea. Although no reports of isolation or characterization of dolichols have been reported in Archae, due to the enzyme's remarkable preference for this substrate, we refer to it in this article as dol-p-Man synthase.

**Results**

Purification of dol-P-Man synthase. Several detergents, viz., Triton X-100, NP-40, sodium deoxycholate, Brij 99, CHAPS, and polidocanol, were tested at different concentrations for solubilization of dol-P-Man synthase, and among these, 1% polidocanol gave the best results. Solubilized enzyme fraction (100 ml), was concentrated with Amicon P-10 membrane and chromatographed on Sephacryl S-200 (Figure 1). The active fractions were pooled and applied to a Cibacron blue 3GA-agarose column and the enzyme was eluted with a gradient of 0–4 M NaCl (Figure 2). Hydrophobic interaction chromatography on Octyl-Sepharose was used as a third step (Figure 3), and the active fractions were eluted with a gradient of 4 to 0 M NaCl. The concentrated enzyme was dialyzed with buffer C, applied on a hydroxylapatite column, and the enzyme was...
eluted with a 0–0.3 M \( \text{Na}_2\text{PO}_4 \) gradient (Figure 4). The summary of the purification data is presented in Table I. Dol-P-Man synthase was purified 1070-fold with a recovery of 4%. The purified enzyme was stable for two weeks at \(-20^\circ\text{C}\) in phosphate buffer pH 6.0, containing 20 mM Mg\(^{2+}\), 0.2% polidocanol, and 20% glycerol. SDS-gel electrophoresis of partially purified dol-P-Man synthase is shown in Figure 5A.

Localization of dol-P-MAN synthase activity on SDS-polyacrylamide gels. Partially purified dol-P-Man synthase (Figure 5A) was incubated with sodium lauryl sarcosine (SLS) (Chatterjee et al., 1992) instead of sodium dodecyl sulfate for 120 min. at 4°C. One lane together with a standard lane was stained for protein with Coomassie blue, and on the other lane the bands were cut according to Coomassie blue stains. Each gel slice was analyzed for dol-P-Man synthase activity. Upon electrophoresis with SDS under reducing conditions, a weak band with an apparent molecular mass of 42 kDa (arrow in Figure 5B) showed high enzyme activity.

Properties of purified dol-P-Man synthase

This experiment used transfer of mannose from GDP\(^{[3}\text{H}]\)-Man to endogenous lipids as a baseline, and measured mild acid labile \(^{[3}\text{H}]\)-mannose labeled lipid products. Figure 6 shows that dolichyl phosphate was a dramatically better acceptor than undecaprenyl (C\(_{55}\)-polyisoprenyl) phosphate. The optimum condition for incorporation of GDP-\(^{[3}\text{H}]\)Man into endogenous and exogenous lipid acceptors was pH 6.2, and 65°C, and 60°C for protein acceptors. The enzyme showed an almost absolute requirement for divalent cation. The best divalent metal ion condition was 20 mM Mg\(^{2+}\). Figure 7 shows that amphomycin, an inhibitor of dol-P-Man synthesis (Storm et al., 1973), blocked mannosyl transfer to the endogenous lipid, protein, and to dol-P; 100 \( \mu \text{g/ml} \) amphomycin inhibited mannosyl transfer to dol-P by 97%, and 1.0 \( \text{mg/ml} \) inhibited 82% of mannosyl transfer to endogenous lipids and 80% of mannosyl transfer to endogenous proteins. The enzyme exhibited typical Michaelis-Menten saturation kinetics to C\(_{55}\)-dolichyl phosphate. The apparent \( K_m \) for dol-P was 2.6 \( \mu \text{M} \) (data not shown). The purified enzyme solution catalyzed the synthesis of dolichyl-phosphate mannose, was free of dolichyl-phosphate galactose synthase and only contained a trace of dolichyl-phosphate glucose synthase activity (data not shown).

Modulation of solubilized dol-P-Man synthase by synthetic phospholipids. Effect of synthetic phospholipids and other compounds on synthase activity is shown in Table II. Dol-P-Man synthase was purified 1070-fold with a recovery of 4%.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total vol. (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity (cpm/min \cdot mg)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate (8000 \times g)</td>
<td>300</td>
<td>10700</td>
<td>1030</td>
<td>100</td>
</tr>
<tr>
<td>30 mm supernatant</td>
<td>70</td>
<td>755</td>
<td>13137</td>
<td>90</td>
</tr>
<tr>
<td>Polidocanol extract</td>
<td>100,000 \times g pellet</td>
<td>90</td>
<td>30</td>
<td>220420</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>50</td>
<td>7</td>
<td>598282</td>
<td>38</td>
</tr>
<tr>
<td>Cibacron Blue</td>
<td>5</td>
<td>1.5</td>
<td>808206</td>
<td>11</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
<td>2</td>
<td>0.4</td>
<td>1102100</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The enzyme activity assay was described under Materials and methods.
Fig. 6. Incorporation of $[^3]$Hmannose from GDP$[^3]$Hmannose into products from endogenous lipid and protein acceptors, and exogenous acceptors. Enzyme activity for exogenous acceptor was assayed in a solution contained GDP$[^3]$HMan (0.1 μCi, 100,000 c.p.m.), 3 μl dolichyl (C₈₀,₀₃)-phosphate, or 3 μl C₅₅-polyisoprenol phosphate (1 μg/μl in 1% Triton X-100), 1 μl purified enzyme solution (5 μg protein), and 20 mM Na-phosphate buffer pH 6.0, 10 mM MgCl₂ in a total volume of 60 μl. Enzyme activity for endogenous lipid acceptors were assayed in the presence of 10 μl of the cell lysate (50 μg protein), and the same buffer as above in total volume of 60 μl. A, Endogenous lipids (as a baseline), B, C₅₅-polyisoprenol phosphate; C, C₈₀,₀₃ dolichyl-phosphate. Man synthase activity was increased 3-fold (exogenous acceptor) and 2-fold (endogenous acceptor) by glycerol. Phospholipids tested either had no significant effect or decreased the enzyme activity.

Fig. 7. Effect of amphomycin on the transfer of $[^3]$Hmannose from GDP$[^3]$Hmannose to endogenous and exogenous acceptors. Incubation mixtures consisted of cell lysate (50 μg of protein), 20 mM P-buffer pH 6.0, GDP$[^3]$H-Man (0.1 mCi, 100,000 c.p.m.), 20 mM MgCl₂. Dol-P (3 μg) was added as exogenous acceptor. A range of 0 to 0.4 mg/ml amphomycin was used for enzyme inhibition assays. The reaction mixture were incubated for 2 h at 37°C. A, Exogenous lipid; B, endogenous protein acceptors; and C, endogenous lipid.

Table II. Activity of *T. acidophilum* dol-P-Man synthase in synthetic phospholipids

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>O-phosphoryl-ethanolamine</td>
<td>69</td>
</tr>
<tr>
<td>L-α-Lysophosphatidyl-ethanolamine</td>
<td>80</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>89</td>
</tr>
<tr>
<td>L-α-phosphatidic acid</td>
<td>52</td>
</tr>
<tr>
<td>L-α-phosphatidyl-choline</td>
<td>85</td>
</tr>
<tr>
<td>L-α-Lysophosphatidyl-choline</td>
<td>100</td>
</tr>
<tr>
<td>Glycerol</td>
<td>298</td>
</tr>
<tr>
<td>L-α-glycerophosphate</td>
<td>94</td>
</tr>
<tr>
<td>L-α-glycerophosphoryl-choline</td>
<td>120</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-glycerol-3-phosphate diphenyl ester</td>
<td>56</td>
</tr>
<tr>
<td>L-α-phosphatidyl-NN-dimethyl-ethanolamine dipalmitoyl</td>
<td>94</td>
</tr>
</tbody>
</table>

Reaction mixtures contained 100 μg total phospholipid. The lipids were sonically dispersed in buffer A as described in Materials and methods; 100 μg of *T. acidophilum* cell lysate was added for endogenous acceptor assay and 5 μl of partially purified enzyme and 3 μg dol-P for exogenous lipid acceptor.

Characterization of the lipid products. Endogenous manno-lipids were hydrolyzed under mild acid condition (0.02 N HCl, 100°C for 15 min.). The proportion of $[^3]$H-Man and $[^3]$HMan-lipids in organic and aqueous phases after mild acid hydrolysis shows that 85% of manno-lipids were hydrolyzed in 10 min. The aqueous phase was concentrated and applied on a Bio-gel P-2 (1 x 60 cm) column, and the sample was eluted with H₂O as shown in Figure 8. Four radioactive peaks from hydrolyzed manno-lipids corresponded in size with glucose, lactose, maltotriose, and maltotetraose, suggesting that manno labeled oligosaccharides up to dp 4 were assembled on lipid intermediates.
Discussion

*Thermoplasma acidophilum* belongs in the kingdom archaea (Fox et al., 1980; Woese et al., 1980) lacking a murein cell wall. Archae retain the procaryotic phenotype lacking nuclear membrane and internal organelles (Woese et al., 1977; Fox et al., 1980), save perhaps a primitive cytoskeleton. However, they possess true N-linked glycoproteins with Asn-x-Thr glycosylation code and O-linked glycoproteins (Yang et al., 1979) in the manner of eucaryotes. Being evolutionarily halfway between procaryotes and eucaryotes based on sequences of 16S ribosomal RNA, their cell walls consist of glycosylated proteins rather than the peptidoglycan structure in eubacteria, and their membrane lipids are unique in consisting of derivatives of dialkylglycerol, diphytanylglycerol diethers (Langworthy, 1982a). It is now apparent that the synthetic machinery for true glycoproteins evolved much earlier than that of the endoplasmic reticulum and Golgi apparatus. Thus, plasma membranes of archae are likely to bear progenitor mechanisms for glycosylation of proteins, including proteins for transport and elongation of sugar chains. Study of these early glycosylation mechanisms may lead to an understanding of development of the control of topology and distribution of anabolic glycosylation enzymes within the eucaryotic endoplasmic reticulum/Golgi. To deduce the path for synthesis of mannose-containing glycoproteins in *T. acidophilum*, we first chose to study an enzyme that had been well studied in procaryotes and eucaryotes and had been cloned and sequenced (Beck et al., 1990) but never purified from the host, namely mannosyl-phosphoryl-dolichol synthase.

The partially purified enzyme could be precisely located in a polycrylamide gel band since Man-p-dol-synthase has a higher molecular weight at 42 kDa than the 30 kDa yeast enzyme (Gasnier et al., 1992). Amphomycin at 0.1 μg/ml blocks 100% of dol-P-Man synthesis, in accord with other reports of inhibition of pathways dependent on polyisoprenol lipids (Storm et al., 1973). However, 35% of labeling of endogenous protein and lipid were retained in incubation mixtures containing as much as 0.4 μg/ml amphomycin, indicating that GDP-mannose has some direct glycosylation functions in *T. acidophilum*, as in eucaryotes.

*Thermoplasma* is a mycoplasma-like organism lacking a cell wall; thus, the only barrier between the cellular interior and the environment is a 4–5 nm thick plasma membrane (Brock, 1978). The plasma membrane lipids are mainly diglycerol tetraethers, which provide *Thermoplasma* with a lipid ’monolayer’ membrane (Langworthy, 1982b). Modification of the activity of solubilized enzyme by phospholipid could be expected to be different than that reported from other organisms (Jensen et al., 1989; Schutzbach et al., 1992). Therefore, this enzyme has unique properties in membrane lipid interaction. Only glycerol gave a significant stimulating effect. Purified lipid fractions from *T. acidophilum* were not available for testing. The *T. acidophilum* Man-p-dol-synthase is different from the yeast mitochondrial enzyme in molecular weight and effect of phospholipids (Schutzbach et al., 1992) but similar in its unsaturated dolichol acceptor specificity.

The steps in synthesis of lipid-linked oligosaccharide precursors in eucaryotes have been reviewed (Kornfeld et al., 1985). The oligosaccharide is assembled in the endoplasmic reticulum on the lipid carrier dolichol phosphate. The sugars are added in a stepwise fashion with the first seven sugars derived from the nucleotide sugars, presumably on the cyto-

plasma side after which translocation to the lumen occurs and protein glycosylation occurs in the lumen of the rough endoplasmic reticulum (Kornfeld et al., 1985), topologically similar to the outside of the archae plasma membrane. No archae lipid intermediates have been characterized. In a previous report from our laboratory (Zhu et al., 1995), *Haloflexax volcanii* Glc-
P-polyprenol synthase showed a procaryotic preference for C55-polyisoprenol, and no lipid intermediates larger than a monosaccharide were detected indicating a lack of assembly of glucose-labeled oligosaccharides on lipid intermediates (Zhu et al., 1995). Although the endogenous lipid acceptors from archae have never been isolated or characterized, in this report the preferred acceptors for a lipid-p-Man synthase in *T. acidophilum* were C45, and C55-105 dolichol-phosphates, showing eucaryotic enzyme characteristics. Mannose-labeled oligosaccharides were found on acid labile lipids, also an eucaryotic characteristic. *T. acidophilum* has also been reported to possess GlcNAc attached to Asn in its glycoproteins (Yang et al., 1979). Lipid-linked mannosyl oligosaccharides (up to four sugars, Figure 8) are probably assembled in the cytoplasm and transferred to protein across the plasma membrane which conducts usual functions of the eucaryotic ER and Golgi. It now appears that some species of archaea such as *Haloflexax volcanii* retain procaryotic properties of glycosylation systems (Mescher et al., 1976; Lechner et al., 1985; Zhu et al., 1995) while *T. acidophilum* appear much closer to eucaryotes in glycosylation specificity and mechanisms. Further study of glycosylation in *T. acidophilum* may shed an interesting light on the evolution of eucaryotic glycoprotein synthetic systems.

Materials and methods

Materials

GDP[3H]-mannose (5–15 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. Phospholipids, dolichyl(C40)-phosphate, Octyl-Sepharose, synthetic phospholipids, and Cibacon blue 3GA were purchased from Sigma Chemical Co. Sephacryl S-200 was purchased from Pharmacia Biotech, Uppsala, Sweden. Amphomycin, C45-dolichyl-phosphate, and C55-polyisoprenol phosphate were a gift from Drs. Salvatore J. Turec, Charles I. Wächter, and Jeffrey S. Rush (Department of Biochemistry, University of Kentucky Medical Center, Lexington, KY).

Cell culture

*Thermoplasma acidophilum* cells were a kind gift from Dr. Dennis Searcy (University of Massachusetts, Amherst, MA). Cells were cultured aerobically at 56°C and pH 1.7, and stopped in the stationary phase at an A540 of 1.2.

Membrane preparation and solubilization

All preparation steps were carried out at 0–4°C. Wet cells (50 g) were lysed with 300 ml 0.2 M Tris–HCl buffer pH 8.0, 1 mg DNase and PMSF (1 mM final concentration) were added, and the mixture was stirred for 60 min. The lysate was centrifuged at 13,000 × g for 30 min. The pellet was discarded and the supernatant fraction was centrifuged at 100,000 × g for 90 min. Membranes were collected and solubilized with 20 mM phosphate buffer pH 6.0 with 1% polidocanol (polyoxyethylene 9 lauryl ether), 10 mM MgCl2 for 2 h and centrifuged at 100,000 × g for 90 min. The pellet was discarded, and the supernatant fraction containing the solubilized dol-P-Man synthase was used for further purification.

Ultrafiltration

All steps were carried out at 4°C. The solubilized enzyme was concentrated with Amicon P-10 membrane and kept frozen at −20°C.

*Sephacryl S-200 column chromatography

A Sephacryl S-200 column (1.5 x 100 cm bed volume) was equilibrated with 0.02 M phosphate, pH 6.0, 10 mM MgCl2 and 0.2% polidocanol (buffer A).
The solubilized enzyme was dialyzed, applied to the column and eluted with the same buffer. Fractions were collected and analyzed for protein and dol-P-Man synthase activity.

**Cibacron blue 3GA-agarose column chromatography**
A Cibacron blue 3GA column (2.5 x 10 cm) was preequilibrated with buffer A. Pooled active fractions from Sephacryl S-200 were applied and eluted with a 0-4 M NaCl gradient. The activity and protein content were determined in individual fractions.

**Octyl-Sepharose column chromatography**
The pooled active fractions from Cibacron blue gel chromatography were dialyzed against 20 mM phosphate, pH 6.0, 10 mM MgCl₂, 0.2% polidocanol containing 4 mM NaCl (buffer B) and applied to a 2.5 x 5 cm Octyl-Sepharose column, equilibrated with buffer B. The enzyme was eluted with a gradient of NaCl in a range 4-0 M in buffer A. Active fractions were pooled and stored at -20°C.

**Hydroxylapatite column chromatography**
The Octyl-Sepharose pooled eluate was dialyzed against 5 mM phosphate, pH 6.0, 5 mM MgCl₂, 0.1% polidocanol (buffer C) and applied to a 1.0 x 3.0 cm hydroxylapatite column equilibrated with buffer C. The active fractions were eluted with 0-0.3 M Na₂HPO₄ gradient, concentrated using Centricon 10 microconcentrators to a volume of about 0.5 ml and stored at -80°C.

**Enzyme activity assay**
Exogenous acceptor. Dol-P-Man synthase was routinely assayed with exogenous acceptor dolichyl(C₂₀)-phosphate (Sigma Chemical Co.) by the following procedure. The incubation mixture contained GDP-[³²P]Man (0.1 μCi/100,000 c.p.m.), 3 μl dol-P (1 μg/μl in 1% Triton X-100), 1 - 10 μl enzyme solutions (homogenate or purified enzyme), and 20 mM Pi-buffer pH 6.0, 10 mM MgCl₂ in a total volume of 60 μl. After incubation at 22°C for 60 s, the incubation mixture was extracted described as above, [³²P]-dolichol was substituted. H]-labeled lipid products were extracted described as above, [³²P]-labeled glycoproteins were precipitated with 10% TCA, and proteins were dissolved in 0.2 N NaOH for radioactivity measurement by scintillation spectrometry.

**In-gel activity analysis**
One hundred microliters of partially purified enzyme was incubated with 4% sodium lauryl sarcosine (SLS) (Chatterjee et al., 1992), 10% 2-mercaptoethanol, 0.002% bromphenol blue at 4°C for 2 h. Sample was loaded onto two lanes of 10% SDS–polyacrylamide gel. One sample lane and protein standard lane were stained with Coomassie blue; the bands were cut, macerated, and extracted three times with buffer A from an unstained lane according to Coomassie blue stained bands, and the extracts assayed for enzyme activity.

**Abbreviations**
NDP-sugar, nucleotide diphasphate-sugar; GDP-mannose, guanosine-diphosphate-β-mannose; dol-P-Man, Dolichyl phosphate mannose; Polidocanol, poloxethylene 9 lauryl ether; ER, endoplasmic reticulum. PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; SLS, sodium lauryl sarcosine; SDS, sodium dodecyl sulfate. Enzymes: dolichyl-phosphate mannose synthase (EC2.4.1.83) and dolichyl-phosphate galactose synthase (EC2.4.1.117).

**References**


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