Polyisoprenyl phosphate specificity of UDP-GlcNAc:undecaprenyl phosphate N-acetylglucosaminyl 1-P transferase from E.coli

Jeffrey S. Rush, Paul D. Rick and Charles J. Waechter

Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536, USA and 1Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA

Introduction

E.coli and other gram-negative bacteria synthesize a complex cell-surface glycolipid, the enterobacterial common antigen (ECA; Makela et al., 1976; Mayer et al., 1979; Ramia et al., 1982; Kuhn, et al., 1988; Rick and Silver, 1996). The carbohydrate portion of ECA is a linear heteropolysaccharide containing a repeating trisaccharide unit (Lugowski et al., 1983; Mannel and Mayer, 1978) consisting of:

\[-[\text{D-Fuc} \rightarrow 4-\text{Ac}\alpha(1 \rightarrow 4)\text{D-ManNAcA-}\alpha(1 \rightarrow 4)\text{D-GlcNAc-}\alpha(1 \rightarrow 3)]\]

anchored to diacylglycerol by a phosphodiester linkage (Kuhn et al., 1983, 1988). The transfer of GlcNAc 1-P from UDP-GlcNAc to undecaprenyl phosphate (Und-P), catalyzed by GlcNAc 1-P transferase (GPT) is the first step in ECA biosynthesis (Rick et al., 1985; Barr and Rick, 1987). GlcNAc-P-Und is also believed to be the initial lipid-linked intermediate formed in the biosynthesis of several lipopolysaccharide O-antigens in a variety of gram-negative bacteria (Schmidt et al., 1976; Rick et al., 1994; Rick and Silver, 1996). The GPT of bacteria belonging to the family enterobacteriaceae is encoded by the rfe gene (Meier-Dieter et al., 1990, 1992). GPT activity has also been observed in membrane preparations obtained from the gram-positive bacteria, B.cereus, B.subtilis, B.megaterium, and M.luteus (Yamamori et al., 1978).

The assembly of N-linked oligosaccharides in eukaryotes is initiated by a reaction similar to that catalyzed by GPT; however, in this reaction GlcNAc 1-P is transferred from UDP-GlcNAc to dolichyl phosphate (Dol-P) forming GlcNAc-P-P-Dol (Lehrman, 1991). The structural differences in the glycosyl carrier lipids participating in these analogous enzymatic reactions catalyzed by GPTs in eukaryotes and prokaryotes are illustrated in Figure 1. The prokaryotic carrier lipid, Und-P contains 11 isoprene units and is fully unsaturated while the eukaryotic lipid cofactor, Dol-P, has 15–20 isoprene units including a characteristic saturated α-isoprene unit (Pennock et al., 1960). The functional significance of the use of fully unsaturated Und-P by prokaryotes and Dol-P, containing a saturated α-isoprene, by eukaryotes is unknown. However, several recent studies have shown that the presence of the reduced α-isoprene unit of the dolichyl moiety of Man-P-Dol is essential for all four known functions of the mannosylipid (Rush et al., 1993; DeLuca et al., 1994; D’Souza-Schorey et al., 1994; Kean et al., 1994; Dotson et al., 1995).

The cDNAs and the genes encoding eukaryotic GPT have been cloned from several sources (Rine et al., 1983; Scocca and Krag, 1990; Zhu and Lehrman, 1990; Rajput et al., 1992). Comparisons of the nucleotide sequences of these genes with the sequences of cDNAs encoding a variety of related N-acetylhexosamine 1-P transferase activities from hamster, yeast, Bacillus subtilis, and E.coli suggest a phylogenetic relationship (Lehrman, 1994). However, the enzymatic properties and subcellular distribution of bacterial GPTs have not been extensively characterized.

The results reported in this article describe the enzymatic properties of a membrane-bound form and a solublized form of

© Oxford University Press
J.S.Rush, P.D.Rick and C.J.Waechter

\[
\begin{align*}
(C55)\text{Poly-}P &= \text{CH}_3-C=\text{CH}-\text{CH}_2-[\text{CH}_2-C=\text{CH}-\text{CH}_2]_9-\text{CH}_2-C=\text{CH}-\text{CH}_2-	ext{P} \\
\text{UDP-GlcNAc} &\rightarrow \text{UMP} \\
(C55)\text{Poly-P-P-GlcNAc} &= \text{CH}_3-C=\text{CH}-\text{CH}_2-[\text{CH}_2-C=\text{CH}-\text{CH}_2]_9-\text{CH}_2-C=\text{CH}-\text{CH}_2-\text{P-P-GlcNAc} \\
\end{align*}
\]

\[\text{CH}_3\text{CH}_3\text{CH}_3\text{CH}_3\text{CH}_3\]

**PROKARYOTES**

\[
\begin{align*}
(C75-95)\text{Dol-}P &= \text{CH}_3-C=\text{CH}-\text{CH}_2-[\text{CH}_2-C=\text{CH}-\text{CH}_2]_{15-17}-\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-\text{P} \\
\text{UDP-GlcNAc} &\rightarrow \text{UMP} \\
(C75-95)\text{Dol-P-P-GlcNAc} &= \text{CH}_3-C=\text{CH}-\text{CH}_2-[\text{CH}_2-C=\text{CH}-\text{CH}_2]_{15-17}-\text{CH}_2-\text{CH}-\text{CH}_2-\text{CH}_2-\text{P-P-GlcNAc} \\
\end{align*}
\]

**EUKARYOTES**

Fig. 1. Enzymatic reactions catalyzed by prokaryotic (upper panel) and eukaryotic (lower panel) GPTs. The solid arrowhead indicates the position of the double bond in the α-isoprene unit of Und-P. In all structures, P represents a phosphoryl group and P-P a pyrophosphoryl group.

**E.coli GPT.** Both forms of the bacterial enzyme discriminate between the unsaturated α-isoprene of the natural substrate, Und-P, and the saturated α-isoprene unit of the eukaryotic carrier lipid, Dol-P.

**Results**

**Enzymatic properties of E.coli GPT activity**

In order to evaluate the polyisoprenyl phosphate substrate specificity of *E.coli* GPT, it was necessary to identify a detergent that effectively dispersed the various hydrophobic polyisoprenyl monophosphate substrates, without inhibiting GPT activity. A preliminary comparison of several detergents indicated that membrane-bound *E.coli* GPT activity was markedly inhibited by several non-ionic detergents, e.g., Triton X-100, Lubrol PX, Brij 35, Brij 58, and Tween 20 (Table I). However, GPT activity was stimulated by the presence of the zwitterionic detergent, 3-{(3-cholamidopropyl)-dimethylammonio}-1-propane-sulfonate (CHAPS) with maximal initial rates of membrane-bound *E.coli* GPT obtained between 0.5-1% (w/v) CHAPS (Figure 2).

GPT activity was maximal in the presence of 20-40 mM MgCl\(_2\). An apparent Km value of 5.6 μM was calculated for UDP-GlcNAc using the optimal concentration of CHAPS to disperse the hydrophobic substrate, Und-P (Figure 3).

**Polyisoprenyl phosphate specificity**

A variety of polyisoprenyl monophosphates were tested as substrates for *E.coli* GPT activity in order to evaluate both the importance of the unsaturated α-isoprene unit and the length of the polyisoprenyl chain. Figure 4A shows that GPT actively transfers GlcNAc 1-P to the natural substrate, Und-P, which is fully unsaturated. However, (C55)Dol-P, which has the same chain length, but which contains a saturated α-isoprene unit, is not an acceptable substrate.

**Table I. Effect of various detergents on *E. coli* GPT activity**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>GPT activity (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.7</td>
</tr>
<tr>
<td>CHAPS</td>
<td>19.8</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1</td>
</tr>
<tr>
<td>Brij 35</td>
<td>0.1</td>
</tr>
<tr>
<td>Brij 58</td>
<td>1.0</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.4</td>
</tr>
<tr>
<td>Octyl glucoside</td>
<td>0.6</td>
</tr>
<tr>
<td>Lubrol PX</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Spheroplasts were isolated and initial rates of GPT activity were assayed in the presence of the indicated detergent at a concentration of 0.5% as described in Materials and methods.
bacilli exhibited a striking preference for Und-P, the natural substrate. Thus, as in *E. coli*, at 30% of the rate observed when Und-P was used as substrate, membranes catalyzed the transfer of GlcNAc 1-P to (C55)Dol-P. *B. megaterium* preference for Und-P over Dol-P, GPT in membrane fractions from *B. cereus* *B. subtilis* as substrate. As seen in Table II, GPT activity associated with membrane preparations from all three bacilli was substantially increased GPT activity, fully unsaturated Poly-Ps containing 11, but not Und-P as substrate. GPT exhibited the opposite specificity utilizing (C55)Dol-P, as substrates for pig brain GPT. As seen in Figure 4B, pig brain GPT activity assayed in the presence of exogenous Und-P. Assay mixtures contained bacterial membranes (82 μg protein), 50 mM Tris-Cl (pH 8), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl2, 100 μM Und-P (dispersed by ultrasonication in 1% CHAPS), 50 mM sucrose, 5 μM UDP-N-acetyl-[6-3H]glucosamine, and the indicated concentration of CHAPS in a total volume of 0.05 ml. Following incubation at 37°C for 10 min, the incorporation of radioactivity into GlcNAc-P-P-Und was determined as described in Materials and methods.

In order to establish that the preference of *E. coli* GPT for Und-P was not simply an artifact of the physical state of the Dol-P dispersion in CHAPS, the same dispersions were tested as substrates for pig brain GPT. As seen in Figure 4B, pig brain GPT exhibited the opposite specificity utilizing (C55)Dol-P, but not Und-P as substrate.

To determine if the length of the polyisoprenyl chain influenced GPT activity, fully unsaturated Poly-Ps containing 11, 15, and 19 isoprene units were compared as substrates. From the results depicted in Figure 5, it can be seen that although the apparent affinity of GPT for the polyisoprenyl phosphate substrates increased with the polyisoprenyl chain length, the maximal velocities declined significantly. Of the three substrates tested, the natural substrate, (C55)Poly (Und-P), and (C75)Poly-P, were utilized by *E. coli* GPT with a higher catalytic efficiency compared to (C95)Poly-P.

To determine if other bacterial GPTs have a similar preference for fully unsaturated polyisoprenyl phosphate substrates, membrane fractions were prepared from *B. subtilis*, *B. megaterium*, and *B. cereus* and assayed using either Und-P or (C55)Dol-P as substrate. As seen in Table II, GPT activity associated with membrane preparations from all three bacilli was substantially higher when Und-P was used as substrate relative to identical incubations with (C55)Dol-P. While GPT in membrane fractions from *B. subtilis* and *B. cereus* exhibited an absolute preference for Und-P over Dol-P, GPT in *B. megaterium* membranes catalyzed the transfer of GlcNAc 1-P to (C55)Dol-P at 30% of the rate observed when Und-P was used as substrate. Thus, as in *E. coli*, the GPTs in the three strains of bacilli exhibited a striking preference for Und-P, the natural substrate.

![Fig. 2. Effect of CHAPS concentration on *E. coli* GPT activity assayed in the presence of exogenous Und-P. Assay mixtures contained bacterial membranes (82 μg protein), 50 mM Tris-Cl (pH 8), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl2, 100 μM Und-P (dispersed by ultrasonication in 1% CHAPS), 50 mM sucrose, 5 μM UDP-N-acetyl-[6-3H]glucosamine, and the indicated concentration of CHAPS in a total volume of 0.05 ml. Following incubation at 37°C for 10 min, the incorporation of radioactivity into GlcNAc-P-P-Und was determined as described in Materials and methods.](https://academic.oup.com/glycob/article-abstract/7/2/315/598180)

![Fig. 3. Dependence of particulate GPT activity on the concentration of UDP-GlcNAc. *E. coli* GPT activity was assayed in the presence of increasing concentrations of UDP-GlcNAc. Reaction mixtures and the assay procedure were essentially as described in Figure 2. Enzymatic reactions were carried out in the presence of CHAPS at a final concentration of 0.5% supplemented with exogenous Und-P (50 μM).](https://academic.oup.com/glycob/article-abstract/7/2/315/598180)

**Polyisoprenyl phosphate specificity**

*Recovery of a soluble form of GPT by disruption of spheroplasts in a French pressure cell*  

During the course of these studies, the *E. coli* spheroplast preparations were also disrupted in a French pressure cell, and the GPT activity was unexpectedly recovered in the soluble fraction (Table III). The majority (85%) of *E. coli* GPT activity was greatly enriched in the cytosolic fraction, cofractionating with β-galactosidase (90%). The remainder of the GPT activity was found in the 5,000 x g (2%) and 175,000 x g (13%) particulate fractions. GPT activity was not recovered in the periplasmic fraction which contained the characteristic marker enzyme alkaline phosphatase (54.3%).

GPT from *E. coli* does not appear to be an extrinsic membrane protein since resuspension and sedimentation of the particulate fraction in lysis buffer with 1 M NaCl did not release GPT activity in a soluble form. Although we cannot exclude the possibility that the "soluble" form of GPT is a proteolytic fragment of the membrane-bound enzyme produced during the disruption process, the presence of 4 μM pepstatin, leupeptin (25 μg/ml), and 1 mM PMSF did not affect the distribution of the GPT activity in the soluble and particulate fractions.

Upon gel filtration of the soluble GPT on a Bio Gel P-200 column, the enzyme activity was recovered in the void volume (results not included). In addition, the soluble fraction contains both phospholipids and another well-known membrane-bound enzyme, α-lactate dehydrogenase activity (43.6%; Table III). These observations suggest the possibility that the soluble GPT activity may have been released from the membrane fraction as a proteolipid complex. The GPT activity in the supernatant fraction may be associated with the low density vesicle fraction containing lipid-mediated sialyltransferase activity described by Whitfield et al. (1984).

Thus, the exact physical state of the GPT recovered in the
"soluble" fraction after disruption of spheroplasts in a French pressure cell remains to be determined. Nevertheless, the enzymatic properties of enzyme activity recovered in the 175,000 x g supernatant are identical to those of the membrane-bound form, most notably the strict specificity for the fully unsaturated polyprenyl phosphate substrate.

The level of GPT activity corresponds to expression of rfe gene
In order to verify that the "soluble" form of E. coli GPT is encoded by the rfe locus, cytosolic and membrane fractions obtained from three E. coli strains expressing different levels of GPT activity were prepared by French pressure cell treatment. Table IV shows that essentially no particulate or soluble GPT activity was detected in preparations obtained from E. coli 21548 (rfe::Tn10). Conversely, both membrane-bound and soluble GPT activities were markedly higher in E. coli PR4019, which contains the rfe gene on a multicopy plasmid, than in the wild type strain, AB1133. This result strengthens the conclusion that both the membrane-bound and the soluble form of GPT recovered in the 175,000 x g supernatant after disruption of spheroplasts in a French pressure cell are encoded by the rfe gene in E. coli.

Table II. Polysoprenyl phosphate substrate specificity of GPT activity from various bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>GPT activity Und-P (pmol/min/mg)</th>
<th>GPT activity (C55)Dol-P (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>10.2</td>
<td>3.1</td>
</tr>
<tr>
<td>B. cereus</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>1.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Membrane fractions were isolated from the various bacteria and GPT activity was assayed in the presence of exogenous Und-P or (C55)Dol-P as described in Materials and methods.

Fig. 4. Recognition of the unsaturated α-isoprene unit of Und-P by GPT from E. coli. Assay mixtures contained either E. coli membranes (58 μg protein) or pig brain microsomes (230 μg protein), 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl2, 0.5% CHAPS, 50 mM sucrose, 5 μM UDP-N-acetyl-[6-3H]glucosamine and the indicated amount of Und-P (•), or (C55)Dol-P (○) (dispersed by ultrasonication in 1% CHAPS) in a total volume of 0.05 ml. Following incubation at 37°C for 10 min, the enzymatic transfer of [3H]GlcNAc 1-P into GlcNAc-P-P-polyisoprenol was assayed as described in Materials and methods.

Fig. 5. Effect of chain length of the polyisoprenyl moiety of the Poly-P substrate on GPT activity. Assay mixtures contained E. coli membranes (116 μg protein), 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl2, 0.5% CHAPS, 50 mM sucrose, 5 μM UDP-N-acetyl-[6-3H]glucosamine, and the indicated amount of either Und-P (•), (C75)Poly-P (○), or (C95)Poly-P (■), dispersed by ultrasonication in 1% CHAPS, in a total volume of 0.05 ml. Following incubation at 37°C for 10 min, the enzymatic transfer of [3H]GlcNAc 1-P into GlcNAc-P-P-polyisoprenol was assayed as described in Materials and methods.

---

The text is discussing the recognition of the unsaturated α-isoprene unit of Und-P by GPT from E. coli. Assay mixtures contained either E. coli membranes or pig brain microsomes, along with various components including Tris-HCl, 2-mercaptoethanol, EDTA, MgCl2, CHAPS, sucrose, UDP-N-acetyl-[6-3H]glucosamine, and the indicated amount of Und-P or (C55)Dol-P. Following incubation at 37°C for 10 min, the enzymatic transfer of [3H]GlcNAc 1-P into GlcNAc-P-P-polyisoprenol was assayed. The level of GPT activity corresponds to expression of the rfe gene. The soluble form of GPT was detected in preparations obtained from E. coli 21548 (rfe::Tn10), whereas higher levels were found in E. coli PR4019, which contains the rfe gene on a multicopy plasmid, compared to the wild type strain, AB1133. The results support the conclusion that both the membrane-bound and soluble GPT recovered after disruption of spheroplasts in a French pressure cell are encoded by the rfe gene in E. coli.

Table II presents the polysoprenyl phosphate substrate specificity of GPT activity from various bacterial strains. The table includes E. coli, B. megaterium, B. cereus, and B. subtilis, along with their respective GPT activities in terms of Und-P and (C55)Dol-P. Membrane fractions were isolated from each strain, and GPT activity was assayed in the presence of exogenous Und-P or (C55)Dol-P as described in Materials and methods.

---
Table III. Comparison of GPT activity with various bacterial marker enzyme activities in fractions prepared from E. coli spheroplasts following French pressure cell treatment

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>GPT activity (pmol/min)</th>
<th>β-Gal activity (pmol/min)</th>
<th>LDH (nmol/min)</th>
<th>Alk. Phos. (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periplasm</td>
<td>0</td>
<td>1511.3 (17.4)</td>
<td>1781.2 (51.4)</td>
<td>384 (54.3)</td>
</tr>
<tr>
<td>5000 x g pellet</td>
<td>323 (2.4)</td>
<td>1662 (12.5)</td>
<td>166.3 (4.8)</td>
<td>2.3 (3.2)</td>
</tr>
<tr>
<td>175,000 x g pellet</td>
<td>129 (6.2)</td>
<td>129.9 (6.2)</td>
<td>1781.2 (51.4)</td>
<td>197.4 (27.9)</td>
</tr>
<tr>
<td>175,000 x g sup.</td>
<td>11296 (85)</td>
<td>185.5 (89.8)</td>
<td>1511.3 (43.6)</td>
<td>124 (17.4)</td>
</tr>
</tbody>
</table>

The different fractions were prepared and the indicated enzyme activities were assayed as described in Materials and methods. GPT, GlcNAc 1-phosphate transferase; β-Gal, β-galactosidase; LDH, d-lactate dehydrogenase; Alk. Phos., alkaline phosphatase.

Discussion

Polysisoprenyl phosphates function as glycosyl carrier lipids in the assembly of many prokaryotic and eukaryotic glycoconjugates (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Waechter, 1989). Two structural features differentiate the prokaryotic and eukaryotic glycosyl carrier lipids: the prokaryotic carrier lipids are fully unsaturated and contain approximately 11 isoprene units, while the eukaryotic carrier lipids have 15–19 isoprene units and contain a characteristic saturated α-isoprene unit (Penncoc et al., 1960).

This article describes examples of prokaryotic GPTs that discriminate between the natural, fully unsaturated polysisoprenyl phosphate substrate and the eukaryotic substrate, Dol-P. The difference in the polysisoprenyl phosphate specificity of the prokaryotic and eukaryotic enzymes catalyzing the transfer of GlcNAc 1-P from UDP-GlcNAc to the respective glycosyl carrier lipids is illustrated in Figure 1.

The major focus of this study was E.coli GPT, which has been shown to be the rfe gene product by Rick and coworkers (Rick et al., 1985; Barr and Rick, 1987; Meier-Dieter et al., 1992; Rick and Silver, 1996). The E.coli enzyme and other bacterial GPTs from B.subtilis, B.megaterium, and B.cereus have been shown to exhibit an impressive preference for Und-P over Dol-P as a polysisoprenyl phosphate substrate.

A list of several other examples of prokaryotic and eukaryotic glycosyltransferases and their polysisoprenyl phosphate substrate specificities is compiled in Table V. It is well-established that many of the eukaryotic enzymes involved in the biosynthesis of lipid-linked saccharide intermediates recognize the presence of the saturated α-isoprene unit in Dol-P (Mankowski et al., 1975, 1977; Pless and Palamarczyk, 1978; Villemez and Carlo, 1979; Palamarczyk et al., 1980; Jankowski et al., 1989; McLachlan and Krag, 1992, 1994; Szkopinska et al., 1992). Other reports suggest that fully unsaturated Poly-P is the preferred acceptor substrate of several bacterial glycosyltransferases (Jankowski et al., 1974; Mankowski et al., 1977; Romero et al., 1977). Some bacterial glycosyltransferases are less discriminating, and will efficiently glycosylate Dol-P (Lechner et al., 1985; Hartmann et al., 1993; Rush et al., 1993). Of the glycosyltransferases with an established substrate specificity, the eukaryotic GPT, which initiates the synthesis of GlcMan9GlcNAc2-P-P-dolichol in the N-glycosylation pathway by transferring GlcNAc 1-P from UDP-GlcNAc to Dol-P forming GlcNAc-P-P-Dol, exhibits a high degree of specificity for Dol-P over Poly-P (Mankowski et al., 1977; Pless and Palamarczyk, 1978; Palamarczyk et al., 1980; McLachlan and Krag, 1992; Jankowski et al., 1989).

It is not difficult to envision how prokaryotic and eukaryotic glycosyltransferases would discriminate between the stereochemical configuration of the allylic phosphate headgroup of Und-P and the more flexible saturated isoprenyl phosphate of Dol-P. However, the functional significance of this specificity is not clear. One possibility is that since the allylic phosphate bond of Und-P is more acid-labile, the reduction of the α-isoprene unit may provide chemical stability to the polysisoprenyl phosphate linkage when the glycosyl carrier lipid is exposed to the relatively acidic environments of endosomal or lysosomal compartments during intracellular trafficking. Further comparisons of the biophysical properties of Poly-P and Dol-P may yield some insight into the functional advantage of having longer polysisoprenyl glycosyl carrier lipids in eukaryotes. Why the mechanism for enzymatic reduction of the polysisoprenyl phosphate carrier lipids evolved and the reactive sites of the glycosyltransferases changed correspondingly remains an intriguing question. Domain swapping studies may reveal the structural basis for the recognition of the saturated and unsaturated α-isoprenyl phosphate units by the various prokaryotic and eukaryotic glycosyltransferases.

In any case, the fact that the saturated α-isoprene unit of Man-P-Dol and Glc-P-Dol is critical for the function of these glycolipid intermediates (Rush et al., 1993; DeLuca et al., 1994; D’Souza-Schorey et al., 1994; Kean et al., 1994; Dotson et al., 1995) emphasizes the importance of understanding the mechanism and regulation of the enzymatic reduction of the α-isoprene unit. A recent report indicates that the free allylic alcohol is reduced by a microsomal enzyme utilizing NADPH as the reductant (Sagami et al., 1993). It will be interesting to see if this enzymatic reaction can be demonstrated for other tissues, and if the gene which encodes the polyenyl reductase is altered in the CHO mutant (Lec9) which exhibits a phenotype expected for a defective or missing reductase (Rosenwald et al., 1993).

Table IV. Distribution of GPT activity in fractions prepared from spheroplasts of three strains of E. coli following French pressure cell treatment

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fraction</th>
<th>GPT activity specific (pmol/min/mg)</th>
<th>GPT activity total (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21548</td>
<td>Supernatant</td>
<td>0.1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AB1133</td>
<td>Supernatant</td>
<td>5.7</td>
<td>335.6</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>7.6</td>
<td>56.4</td>
</tr>
<tr>
<td>PR4019</td>
<td>Supernatant</td>
<td>42.3</td>
<td>2589.3</td>
</tr>
<tr>
<td></td>
<td>Pellet</td>
<td>61.6</td>
<td>577.3</td>
</tr>
</tbody>
</table>
necessary, with 1 M sucrose to a final concentration of 0.25 M sucrose and resuspended in buffer A to a density of 320 g, x 30 min on ice, unbroken cells and debris were removed by centrifugation (5000 x g, 1 h). The 175,000 x g supernatant fraction was stored at -20°C until analysis. Bacterial membranes were resuspended in buffer A to a membrane protein concentration of 10-25 mg/ml and stored at -20°C until analysis.

Preparation of pig brain microsomes

Microsomes were prepared from fresh pig brain gray matter as described previously (Sumbilla and Waechter, 1985).

In vitro assay of GPT activity in soluble and particulate fractions

GPT assay mixtures contained 10-100 μg of bacterial protein (or pig brain gray matter microsomes), 50 mM Tris–HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl₂, 0.5% CHAPS, and 5 μM UDP-N-acetyl-[6-3H]glucosamine (80-150 c.p.m./pmol), 50 mM sucrose, and the indicated concentration of the appropriate polyisoprenyl monophosphate (dispersed by ultrasonication in 1% CHAPS) in a total volume of 0.05 ml. Following incubation at 37°C for 5-10 min, the enzymatic reaction was stopped by the addition of 40 vol of CHCl₃/CH₃OH (2:1) and the incorporation of N-acetyl-[6-3H]glucosaminyl-1-P into [3H]GlCNAC-P-P-polyisoprenyl determined as described previously (Waechter and Scher, 1981). When membranes from B. cereus were incubated with UDP-[3H]GlCNAC, the enzymatically labeled glycolipid products were chromatographed on silica gel G plates by developing with CHCl₃/CH₃OH/H₂O/NH₃OH (65:35:4:4) to resolve [3H]GlCNAC-P-P-Polyisoprenyl from [3H]GlCNAC-P-P-Und. The radioactive zones were detected using a Bioscan System 200 Imaging Scanner, and the distribution of radioactivity between the two products was determined to calculate initial rates for GPT.

Analytical methods

β-Galactosidase activity was determined exactly as described in the Worthington Manual using o-nitrophenyl-β-D-galactosynitrate as substrate. Protein concentrations were determined by the method of Rodriguez-Vico (Rodriguez-Vico et al., 1989) using the Pierce BCA Protein Assay Reagent. The lipid-phosphorus content of the polyisoprenyl phosphate substrates was determined by the method of Bartlen (1959).

Acknowledgments

We thank Dr. Mark Lehrman for his helpful suggestions during the preparation of the manuscript. This work was supported by NIH Grant GM36065 (C.J.W.) and GM52882 (P.D.R.).
Abbreviations

ECA, entero bacterial common antigen; Und-P, undecaprenyl phosphate; Dol-P, dolichol monophosphate; Poly-P, fully unsaturated polypropenyl monophosphate; GPT, UDP-GlcNAc:polyisoprenyl phosphate GlcNAc 1-P transferase; CHAPS, 3-[3-cholamido-propyl]dimethylammonio]-1-propane-sulfonate.

References


Received on August 16, 1996; revised on September 23, 1996; accepted on September 30, 1996