The Biosynthesis of Gram-negative Endotoxin

A NOVEL KINASE IN ESCHERICHIA COLI MEMBRANES THAT INCORPORATES THE 4'-PHOSPHATE OF LIPID A*

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Extracts of Escherichia coli contain an enzyme that generates the β,1→6 linkage of lipid A from fatty-acetylated monosaccharide precursors, according to the reaction 2,3-diacyl-GlcN+UDP + UDP-2,3-diacyl-GlcN → 2,3-diacyl-GlcN(β,1→6)2,3-diacyl-GlcN+1-P + UDP (Ray, B. L., Painter, G., and Raetz, C. R. H. (1984) J. Biol. Chem. 259, 4852–4859). We now describe a membrane-bound kinase that phosphorylates the 4'-position of the above tetraacyldisaccharide 1-phosphate product. The lipid A 4'-kinase is distinct from the diglyceride kinase of E. coli. When crude membrane preparations are employed, several nucleoside triphosphates are able to support the phosphorylation of the tetraacyldisaccharide 1-phosphate, but ATP is the most efficient. The 4'-kinase requires Mg** and is stimulated by phospholipids, especially cardiolipin. Under optimal conditions the specific activity in crude extracts is 0.5 nmol/min/mg. The enzyme is rapidly inactivated by preincubation in the presence of detergents, such as Nonidet P-40 or octylglucoside, but phosphoenolpyruvate and glycerol stabilize the enzyme. The product generated in vitro has been characterized by fast atom bombardment mass spectrometry and by ¹H and ³¹P NMR spectroscopy. These analyses confirm that the 4'-hydroxyl is the site of phosphorylation. The 4'-kinase reported here is likely to represent a key step in the de novo biosynthesis of lipid A.

The lipid A domain of lipopolysaccharide is a hydrophobic molecule that constitutes the outer monolayer of the outer membrane of Gram-negative bacteria and causes shock when introduced into the circulation of most mammals (1–3). The predominant molecular species of lipid A found in the Escherichia coli envelope (4) is shown in Fig. 1. Prior to 1983 (5, 6), the biosynthesis of lipid A was unknown, since the true covalent structure of lipid A (Fig. 1) (4, 7–10) was not correctly resolved. The evidence from the enzymological and radiochemical labeling studies clearly demonstrates that UDP-2,3-diacylglycerol (UDP-GlcNAc, as shown in the upper half of Fig. 1). The evidence from the enzymological and radiochemical labeling studies clearly demonstrates that UDP-2,3-diacylglycerol is a precursor of 2,3-diacylglycerol 1-phosphate (Fig. 1) (17).

The verification of the existence of UDP-2,3-diacylglycerol and 2,3-diacylglycerol 1-phosphate as metabolites in living cells of wild-type E. coli (15) raised the question of how these compounds are generated from known biosynthetic intermediates. Recently, we have discovered enzymes in E. coli, distinct from the glycerol-3-phosphate acyltransferase (17, 18), that are capable of acylating UDP-GlcNAc, as shown in the upper half of Fig. 1. The evidence from the enzymological and radiochemical labeling studies clearly demonstrates that UDP-2,3-diacylglycerol is a precursor of 2,3-diacylglycerol 1-phosphate (Fig. 1) (17).

We now present further support for this biosynthetic scheme by demonstrating the existence of a 4'-kinase. This enzyme catalyzes the ATP-dependent phosphorylation of the tetraacyldisaccharide 1-phosphate intermediate (Fig. 1) generated by the lipid A disaccharide synthase. Under certain conditions the 4' phosphorylation reaction proceeds to about 60% of completion, generating a tetraacyldisaccharide-1,4'-bisphosphate (Fig. 1). Fast atom bombardment mass spectrometry and NMR spectroscopy have been employed for the structural analysis. The product generated by the 4'-kinase in vitro is identical to the predominant lipid A precursor that accumulates in vivo at 42 °C in temperature-sensitive mutants of Salmonella typhimurium, deficient in KDO1 biosynthesis (19–23).

EXPERIMENTAL PROCEDURES

Materials—³²P, and [γ-³²P]ATP were products of New England Nuclear. Glass-backed plates (5 x 20 cm), coated with a 250-µm layer of Silica Gel 60, were obtained from E. Merck, Darmstadt, West Germany. Glass-backed plates (5 x 20 cm), coated with a 250-µm

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1The abbreviations used are: KDO, 3-deoxy-D-manno-octulosonic acid; EPPS, N-(2-hydroxyethyl)pipecarazine-N'-3-propanesulfonylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FAB, fast atom bombardment; HPLC, high performance liquid chromatography.

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**Fig. 1.** Postulated scheme for the biosynthesis of lipid A in *E. coli*. In the figure, R designates a \(\beta\)-hydroxyacrylmoiety and \(U\) designates uridine. Evidence for the fatty acylation of UDP-GlcNAc and for the precursor-product relationship between UDP-2,3-diacyl-GlcNAc and lipid X has been presented by Anderson et al. (17). The \(\beta\)-hydroxyacrylmoiety is generated by the 4'-kinase reported in this manuscript, and it is also the predominant acidic lipid A precursor of the \(\alpha\beta\gamma\delta\) (pgsB) gene (16), which is necessary for cell growth and outer membrane biogenesis in *E. coli*.

UDP-GlcNAc

\[
\begin{align*}
\text{UDP-GlcNAc} & \rightarrow \text{Acetate} \\
\beta\text{-Hydroxyacrylmyristoyl-ACP} & \rightarrow \beta\text{-Hydroxyacrylmyristoyl-ACP} \\
(\text{UDP-3-Monoacyl-GlcNAc}) & \rightarrow \text{ATP} \\
(\text{Diacyl-disaccharide-1,4'-bis-P}) & \rightarrow \text{Lauroyl and myristoyl moieties} \\
\text{KDO} & \rightarrow \text{KDO} \\
(\text{KDO})_2 & \rightarrow \text{E.coli K12 Lipid A with KDO disaccharide} \\
\end{align*}
\]

**Preparation of Cell Extracts for Enzymatic Assays—** *E. coli* strains R2029 (dgk-6) and R477 (dgkH) were described previously (30). Cells (1 liter) were grown in LB broth (31) at 37 °C, harvested at the end of log phase (A_{600} = 4), resuspended in 15 ml of 50 mM Tris-HCl, pH 8, and disrupted using a French pressure cell at 18,000 psi. Unbroken cells were removed by centrifugation at 8,000 × g for 10 min. Membranes were prepared by ultracentrifugation of the French press extracts at 150,000 × g for 1 h. Membranes were resuspended in 3 ml of 50 mM Tris-HCl, pH 8, yielding a concentration of 15–20 mg/ml of protein. All steps in the preparation were performed at 4 °C. Samples were stored at −70 °C. Protein concentration was determined by the method of Lowry et al. (32) using a bovine serum albumin standard.

**Conditions for Fast Atom Bombardment Mass Spectrometry and NMR Spectroscopy—** Fast atom bombardment mass spectrometry was done at the National Science Foundation Regional Instrumentation Facility at The Johns Hopkins University, as described previously (33–35). 31P NMR spectra were acquired at 270 MHz with a WH-270 Fourier transform superconducting spectrometer interfaced with a Nicolet 1180 computer. 31P NMR spectra were acquired at 80 MHz on a Nicolet NT-200 Fourier transform superconducting spectrometer equipped with a Nicolet 1280 computer. The spectra were usually recorded at 30 °C at a sample concentration of 5–10 mg/ml in CDCl3,CDOD (10:1, v/v). Decoupling experiments were performed at 500 MHz on a Bruker AM6500 Fourier transform superconducting spectrometer.

**Preparation of the Product from the Lipid A 4'-Kinase Reaction—** A 120-ml reaction mixture was prepared as described in the legend to Fig. 3, except that nonradioactive tetraacyldisaccharide 1-phosphate (0.25 mg/ml) was employed. The system was incubated for 2 h at 37 °C and then an additional 240 mg of P-enolpyruvate was added. Three hours later, the reaction was stopped by the addition of 300 ml of methanol. Next, 150 ml of chloroform was added. The resulting one-phase system was centrifuged at low speed to remove debris. The supernatant was converted to a two-phase system by the addition of 150 ml of chloroform and 150 ml of 0.3 M HCl. Centrifugation at low speed was required to separate the phases. The lower (chloroform) phase was concentrated to 15 ml on a rotary evaporator. This was diluted to 100 ml in chloroform:methanol:water (23:1, v/v). Then the sample was applied to a DEAE-cellulose column (125-ml bed volume) prepared in the same solvent, and the product was eluted as described by Raetz et al. (22) for the purification of the precursor IVa. The solvent and salts were removed by acidic Bligh-Dyer partitioning (22), followed by rotary evaporation. About 10 mg of kinase product was recovered. Thin layer chromatography, however, revealed that this material was contaminated to approximately 30% with a cardiolipin-like molecule.

Final purification was achieved by HPLC, using an Altich C18 10-μm reversed phase column (250 × 6.5 mm), a Waters 6000A solvent delivery system, and a Waters 480 LC spectrophotometer. Elution of the kinase product was detected at 210 nm. Two solvent mixtures were employed: (a) acetonitrile:water (60:40, v/v) containing 5 mM tetrabutylammonium phosphate; and (b) isopropanol:water (85:15, v/v) containing 5 mM tetrabutylammonium phosphate. The partially purified product was eluted at 50% isocratic elution shortly after KDO (21, 23). Specifically, a portion of the lipid A molecules of *E. coli* bear a pyrophosphate residue at the reducing end (8, 25) (as indicated by the dashed bond), while in *S. typhimurium* additional polar substituents, including 4-amino-4-deoxy-L-arabinose and/or phosphoethanolamine, may be attached to lipid A (8, 25) (as indicated by the dashed bond). The "late acylations" by which the lauroyl and myristoyl residues (1, 9, 26) are incorporated have not been studied, but the presence of these residues is correlated with extreme toxicity in animals (27, 28). The final structure shown is the minimal unit thought to be required for growth and outer membrane biogenesis in *E. coli* (1, 2). All other core sugars and O-antigens of lipopolysaccharides are essential for cell growth under laboratory conditions, and mutants lacking these are not temperature-sensitive (1, 29).
purified kinase product (~10 mg) was dissolved in 3.5 ml of solvent A (solvent B (2:1, v/v)). Five individual injections (0.7 ml each) were required to purify all of the available material. After each injection, the column was eluted at a flow rate of 2.3 ml/min with a linear gradient, starting with solvent A (solvent B (2:1, v/v)) and ending with solvent B. Each chromatography was completed in 60 min, and in each case the kinase product emerged at min 39, as did polar lipid IVα (determined in a separate experiment).

The peaks of the product recovered from the five individual injections were pooled, and the volume (~60 ml) was reduced to ~4 ml by rotary evaporation. Next, 60 ml of CHCl₃:methanol:water (2:3:1, v/v) was added, and the sample was applied to a second column (40-ml bed volume) of DEAE-cellulose in the same solvent (solvent B) to remove the tetrabutylammonium phosphate. After washing the column with 120 ml of CHCl₃:methanol:water (2:3:1, v/v), the kinase product was eluted with 100 ml of CHCl₃, methanol, 480 mM ammonium acetate (2:2:1, v/v), the kinase product was adjusted by adding appropriate amounts of CHCl₃, water, and concentrated HCl to generate a two-phase, acidic Bligh-Dyer system (11, 22). The lower phase was dried, and the residue was weighed. Approximately 3.5 mg of highly purified kinase product was recovered. Since the lipid is obtained in the free acid form under these conditions, it is redissolved immediately in 0.4 ml of CDCl₃:CD₃OD (2:1, v/v) for NMR analysis. Prolonged storage of the kinase product (or lipid X), as the free acid, results in the gradual loss of the anomeric phosphate residue.

RESULTS

Identification and Properties of a Novel Kinase in Extracts of E. coli Specific for Lipid A Precursors—When tetraacyldisaccharide 1-phosphate is incubated with a crude extract or with membranes of E. coli in the presence of [γ-³²P]ATP and Mg⁴⁺, as described in Fig. 2, a slowly migrating ³²P-labeled lipid product is formed, having an Rₐ upon thin layer chromatography that is identical to that of the tetraacyldisaccharide-1,4'-bisphosphate (Fig. 1, also designated precursor IVα), isolated from KDO-deficient mutants (22, 23). Little or no product is generated in the absence of the tetraacyldisaccharide 1-phosphate substrate (Fig. 2, lanes 1 and 3) or in the absence of enzyme (lane 5). Membranes from wild-type cells (lanes 1 and 2) also incorporate ³²P into the glycerophospholipids (the more rapidly migrating components), independently of added tetraacyldisaccharide 1-phosphate. Presumably, the latter process is mediated by diglyceride kinase, since membranes prepared from E. coli mutant (30) lacking the diglyceride kinase (lanes 3 and 4) do not incorporate much ³²P into the glycerophospholipid fraction. Further, the incorporation of ³²P into the lipid A precursor is unaffected by the dgk-6 mutation (Fig. 2), demonstrating that the lipid A kinase is a distinct enzyme.

The formation of the putative tetraacyldisaccharide-1,4'-bisphosphate was also demonstrable by incubation of tetraacyldisaccharide 1-[³²P]phosphate with unlabelled ATP (data not shown). CTP, UTP, and GTP could be substituted, but ATP supported approximately twice the rate of the others. As shown in Table I, cardiolipin, Nonidet P-40, and Mg⁴⁺ all stimulated the reaction rate. Efficient conversion of the lipid substrate to product at a linear rate was observed only if cardiolipin was included in the reaction mixture, together with an ATP regenerating system (Fig. 3 and Table I). Other phospholipids, including phosphatidylethanolamine, phosphatidylycerine, phosphatidylcholine, and phosphatidylglycerol, were not as effective as cardiolipin. The inclusion of a phospholipid "cofactor" and a nonionic detergent in the reaction mixture was essential, because of the intrinsic insolubility of the tetraacyldisaccharide 1-phosphate in water, especially in the presence of divalent cations. The conditions for the preparation of stable, squalous emulsions of disaccharide/cardiolipin mixtures are described in the legend to Fig. 3.

Other Properties of the Kinase—In contrast to the other enzymes shown in the upper half of Fig. 1 (16, 17), most of the lipid A kinase sedimented with the membrane pellet when centrifuged at 150,000 × gₑₑ for 1 h. Fig. 4 gives the results of a semiquantitative assay of the lipid A kinase in crude cell-free extracts or in membrane preparations. Fig. 4A shows the protein dependency of the activity. Although crude extracts and membrane preparations had approximately the same specific activities, Fig. 4B shows that the assay was linear for a much longer time (and consequently more reliable) with membrane preparations. Typically, the specific activity of a crude extract is 0.3-0.5 nmol/min/mg or about one-half of the specific activity of the lipid A disaccharide-1-phosphate.

TABLE I

| Requirement for the phosphorylation of the tetraacyldisaccharide 1-phosphate by membrane preparations of E. coli | % control |
|---------------------------------------------------------------|----------|
| Incubation conditions                                      | Relative amount of product formation |
| Complete system                             | 100      |
| Omit enzyme                                    | <2       |
| Enzyme heated for 10 min at 60 °C                   | <2       |
| Omit MgCl₂                                    | 2.5      |
| Omit cardiolipin                               | 15       |
| Omit Nonidet P-40                              | 5.7      |
| Omit P-enolpyruvate                             | 44       |
| Omit pyruvate kinase                           | 43       |

FIG. 2. A novel kinase specific for lipid A precursors. The complete reaction mixture (50 µl) contained 0.25 µg/ml tetraacyldisaccharide 1-phosphate, 2 mg/ml cardiolipin, 50 mM HEPES, pH 7.4, 1% Nonidet P-40, 5 mM MgCl₂, 4 mM [γ-³²P]ATP (10⁶ cpm/nmol), and E. coli membranes (1.7 µg/mg) that were used to start the reaction. After 20 min at 37 °C, the lipids in the reaction mixture were extracted under acidic Bligh-Dyer conditions (11), and a portion of each CHCl₃ phase was spotted on a Silica Gel 60 thin layer plate that was developed in CHCl₃:pyridine, 88% formic acid, water (40:60:16.5, v/v). The plate was subjected to autoradiography, as shown. Lanes 2 and 4, complete system with membranes of R477 (dgk*) and RZ60 (dgk-6), respectively; lanes 1 and 3, complete system minus tetraacyldisaccharide 1-phosphate with membranes of R477 and RZ60, respectively; lane 5, complete system minus enzyme. Ori, origin; DS 1, 4'-P₂, tetraacyldisaccharide-1,4'-bisphosphate; S.F., solvent front.
than at EPPS at pH 8.5, together with unlabeled ATP and an ATP regenerating system, consisting of 10 mM P-enolpyruvate and 100 units/ml. 

The reaction was initiated by the addition of E. coli membranes (1 mg/ml, final concentration) from strain RZ60 (dgk-6). The rate of the reaction was about 2-fold greater at pH 8.5 than at 7.4. To quantitate product formation, 5 μl of the reaction mixture was spotted on a thin layer silica gel plate (Silica Gel 60), and the plate was developed in chloroform, pyridine, 88% formic acid, and water (40:60:16:5, v/v). Next, they are dried under a stream of N2, and Fig. 3, except that the volume of the reaction mixture was 0.5 ml and the substrate concentration was varied. Under the standard assay conditions (Fig. 4) the tetraacyldisaccharide 1-phosphate is employed at a concentration of approximately 200 μM.

Although product formation was linear for 1 h in the presence of detergent and an ATP regenerating system containing P-enolpyruvate (Figs. 3 and 4), the enzyme was rapidly inactivated upon preincubation with detergent alone, even at 0 °C (Table II). Inclusion of Mg2+ (Table II) had no effect. When P-enolpyruvate was present during preincubation, the enzyme was stabilized significantly (Table II). Glycerol (25%, w/v) also greatly stabilized the kinase (data not shown).

When the tetraacyldisaccharide 1-phosphate substrate was subjected to mild alkaline hydrolysis in the presence of triethylamine (14) to remove the ester-linked fatty acids, the resulting diacyldisaccharide 1-phosphate was still a substrate for the lipid A kinase, but the rate of phosphorylation was about two times slower (data not shown). The enzyme did not phosphorylate 2,3-diacylglycerol 1-phosphate (lipid X) under the conditions shown in Fig. 2.

**Fig. 3.** Efficient product formation in the presence of an ATP regenerating system and cardiolipin. The conditions were essentially as described in Fig. 2, except that 32P-labeled tetraacyldisaccharide 1-phosphate (10⁶ cpm/nmol) was employed in 50 mM EPPS at pH 8.5, together with unlabeled ATP and an ATP regenerating system, consisting of 10 mM P-enolpyruvate and 100 units/ml of pyruvate kinase. The reaction was initiated by the addition of E. coli membranes (1 mg/ml, final concentration) from strain RZ60 (dgk-6). The rate of the reaction was about 2-fold greater at pH 8.5 than at 7.4. To quantitate product formation, 5 μl of the reaction mixture was spotted on a thin layer silica gel plate (Silica Gel 60), and the plate was developed in chloroform, pyridine, 88% formic acid, water (40:60:16:5, v/v). Next, they are dried under a stream of N2, and Fig. 3, except that the volume of the reaction mixture was 0.5 ml and the substrate concentration was varied. Under the standard assay conditions (Fig. 4) the tetraacyldisaccharide 1-phosphate is employed at a concentration of approximately 200 μM.

**Fig. 4.** An assay for the lipid A 4'-kinase in crude extracts and in membrane preparations of E. coli. Panel A shows the dependency of product formation on protein concentration. The incubation time was 15 min at 37 °C under conditions otherwise like those of Fig. 3. Membrane preparations and crude extracts have approximately the same specific activity, but the quantitation is much better with membranes, probably because of inhibitors or interfering activities in the cytoplasm. Panel B shows the dependency of product formation on the time of incubation. The final protein concentration was 1.6 mg/ml. O—O, E. coli membranes; ●—●, E. coli crude extract.

**Fig. 5.** Rate of product formation as a function of tetraacyldisaccharide 1-phosphate concentration. The amount of product formed by the kinase was determined after 20 min at 37 °C under conditions identical to those of Fig. 3, except that the volume of the reaction mixture was 0.5 ml and the substrate concentration was varied. Under the standard assay conditions (Fig. 4) the tetraacyldisaccharide 1-phosphate is employed at a concentration of approximately 200 μM.

The positive mode FAB mass spectrum of the in vitro product, shown in Fig. 6, revealed a strong peak at m/z 1406, attributed to (M + H)+, as expected for a tetraacyldisaccharide bisphosphate product (22). The molecular formula is C₄₀H₆₅N₂O₇S₂P₂ with a predicted molecular weight of 1405.72. Other major peaks are observed: at m/z 1309, corresponding...
**TABLE II**

*Effect of preincubation of *E. coli* membranes with detergent on lipid A 4'-kinase activity*

*E. coli* membranes (3 mg/ml) were preincubated at 0 °C with 0.5% (w/v) Nonidet P-40 for the times indicated. Assay conditions are described in the legend to Fig. 3, except that the final concentrations of tetraacyldisaccharide 1-phosphate and cardiolipin were 0.13 and 1 mg/ml, respectively.

| Additions       | Kinase activity remaining* | 30 min | 60 min |
|-----------------|----------------------------|--------|--------|
| None*           | 43.2 ± 1.7                 | 27.6 ± 4.3 |
| Mg²⁺**          | 38.1 ± 2.3                 | 22.0 ± 4.3 |
| P-enolpyruvate† | 70.0 ± 3.1                 | 61.1 ± 2.8 |

*Enzyme was assayed for 20 min after either 30 or 60 min of preincubation at 0 °C. Values are given as the percent activity remaining in comparison to no preincubation ± S.E.

†Preincubation included *E. coli* membranes, 3 mg/ml protein; Nonidet P-40, 0.5% (w/v); and 40 mM EPPS, pH 8.5.

‡Preincubation also included 5 mM MgCl₂.

§Preincubation included 10 mM P-enolpyruvate but no MgCl₂.

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**Fig. 6.** Positive ion fast atom bombardment mass spectrum of the kinase product.

The mass spectrum of the tetramethyl derivative of lipid IVₐ in CDCl₃:CD₃OD (10:1, v/v) (w/v) Nonidet P-40 for the times indicated. The best resolution (data not shown) is obtained if the sample is dissolved in CDCl₃:CD₃OD (2:1, v/v), but then the HDO peak is situated in the region of interest at ~4.5 ppm.

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**Fig. 7.** ¹H NMR spectra at 270 MHz of the kinase product and the lipid IVₐ precursor, IVₐ, isolated from a KDO-deficient mutant of *S. typhimurium*. Peaks were assigned by comparison with the ¹H NMR spectrum of the tetramethyl derivative of lipid IVₐ reported previously (23) and were verified by proton decoupling experiments (data not shown). Chemical shifts are referenced to tetramethylsilane. Lipid samples (3–5 mg) were prepared as the underderivatized free acid forms by acidic Bligh-Dyer partitioning (11) and were dissolved in CDCl₃:CD₃OD (10:1, v/v). Spectra were recorded immediately at 270 MHz at 30 °C. The best resolution (data not shown) is obtained if the sample is dissolved in CDCl₃:CD₃OD (2:1, v/v), but then the HDO peak is situated in the region of interest at ~4.5 ppm.
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![Image of NMR spectra]

**Fig. 8.** A partial $^1\text{H}$ NMR spectrum at 500 MHz of the kinase product with selective decouplings to verify the assignment of H-4'. The sample, purified by HPLC, was prepared as described in the legend to Fig. 7. The insets are the relevant peaks observed when the resonances, indicated by the arrows, are selectively irradiated. The assignments are explained in the text. The chemical shift, $\delta$, is expressed as parts/million relative to tetramethylsilane.

**Table III**

| Kinase product | $^3\text{P}$ | $^1\text{H}$ |
|---------------|-------------|-------------|
| $^3\text{P}$  | -1.6        | -0.6       |
| $^1\text{H}$  | 6.6         | 10.2       |
| $^3\text{P}$  | -1.6        | -0.10      |
| $^1\text{H}$  | 6.6         | 10.0       |
| $^3\text{P}$  | 2.7         |           |
| $^1\text{H}$  |            |            |

**DISCUSSION**

The kinase described in this article appears to be a novel enzyme that incorporates the 4'-monophosphate moiety of lipid A (Fig. 1). The existence of this kinase in extracts of *E. coli* supports the proposed biosynthetic scheme for lipid A shown in Fig. 1 and is consistent with the view that the tetraacyldisaccharide 1-phosphate intermediate generated by the lipid A disaccharide synthase (pxB gene product) is a true biosynthetic precursor (Fig. 1). The lipid A 4'-kinase is distinct from diglyceride kinase (30), since mutants lacking diglyceride kinase activity (such as RZ60) have normal levels of the 4'-kinase (Fig. 2).

Unlike the lipid A disaccharide synthase (16) or the enzymes responsible for the generation of UDP-2,3-diacylglycerol from UDP-GlcNAc (17), the 4'-kinase is predominantly membrane-bound. Its substrate, the tetraacyldisaccharide 1-phosphate, is extremely hydrophobic and insoluble in water (16). In the presence of Mg$^{2+}$, it forms a coarse precipitate in water that cannot be dispersed by sonic irradiation. Since the kinase requires magnesium for catalytic activity, however, it was necessary to develop conditions permitting the efficient dispersal of the tetraacyldisaccharide 1-phosphate in mixed micellar form in the presence of magnesium. This was achieved by the co-dispersion of the tetraacyldisaccharide 1-phosphate with cardiolipin and by the inclusion of a nonionic detergent, such as Nonidet P-40 or octyl-$\beta$-D-glucoside, in the reaction mixture.

While the inclusion of 1% Nonidet P-40 was required to obtain a maximal initial rate of 4' phosphorylation, the 4'-kinase was solubilized and rendered unstable by various detergents, particularly in the absence of the complete assay.
mixture and glycerol, as shown in Table II. Since phosphoenolpyruvate stabilized the enzyme significantly against inactivation, the best conversions of the tetraacyldisaccharide 1-phosphate substrate to the 1,4'-bisphosphate product were observed in the presence of phosphoenolpyruvate and an ATP regeneration system (Fig. 3). The reasons for the instability of the kinase in the presence of detergent and for the effects of P-enolpyruvate are not known, but the existence of an additional cofactor or labile phosphorlated intermediate cannot be excluded, given the crude enzyme system employed. In this regard, it is relevant that we have not yet established the stoichiometry of 4' phosphorylation and ADP formation because of the multiple fates of ATP in such cell-free extracts. Other nucleoside triphosphates, although less active than ATP, also support the 4' phosphorylation. However, the crude enzyme preparation employed does have an absolute requirement for a nucleoside triphosphate, since no product formation is observed when an excess of hexokinase and glucose are added (data not shown).

The specificity of the 4'-kinase for other lipid molecules will also require further investigation. In preliminary studies we have shown that the monosaccharide, lipid X, is not phosphorylated, suggesting that the enzyme is specific for disaccharides. When the ester-linked fatty acids are removed from the tetraacyldisaccharide 1-phosphate substrate by treatment with triethylamine (14), the resulting diacyldisaccharide 1-phosphate is still a relatively good substrate for the 4'-kinase. We believe that the 4'-kinase acts on a tetraacyldisaccharide 1-phosphate intermediate in vitro, however, since mutants of S. typhimurium deficient in KDO biosynthesis accumulate a tetraacyldisaccharide 1,4'-bisphosphate, having the structure shown in Fig. 1, that is identical to the 1,4'-bisphosphate generated in vitro by the 4'-kinase (Figs. 6 and 7) (22, 23). In addition, the disaccharide synthase (16) shows a 100-1000-fold kinetic selectivity for diacylated substrates relative to monoaoylated or triacylated substrates. The isolation of mutants deficient in the lipid A 4'-kinase would provide the strongest evidence that the tetraacyldisaccharide 1-phosphate is the true physiological substrate, since it would be expected to accumulate in such mutants. The effect of mutations in the 4'-kinase on lipopolysaccharide assembly and cell viability would also be of considerable interest.

Whatever the biological role of the 4'-kinase, we anticipate several novel uses for this enzyme in studies of lipid A physiology. We have developed conditions (data not shown) for generating the tetraacyldisaccharide 1,4'-bisphosphate product in vitro using [γ-32P]ATP of very high specific radioactivity. It may be possible to use these radiolabeled disaccharides as probes for the identification of lipid A receptors or other lipid A binding proteins in cell membranes. Probes of high specific radioactivity will also be useful for studying the metabolism of lipid A and lipid A precursors by bacterial and animal cells. If it is possible to clone the gene for the 4'-kinase and to purify the enzyme to homogeneity, the enzyme may be a useful reagent, in conjunction with chemical methods, for the synthesis of lipid A and lipid A-like molecules.

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