A Phosphotransferase That Generates Phosphatidylinositol 4-Phosphate (PtdIns-4-P) from Phosphatidylinositol and Lipid A in Rhizobium leguminosarum

A MEMBRANE-BOUND ENZYME LINKING LIPID A AND PtdIns-4-P BIOSYNTHESIS*

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Membranes of Rhizobium leguminosarum contain a 3-deoxy-d-manno-octulosonic acid (Kdo)-activated lipid A 4'-phosphatase required for generating the unusual phosphate-deficient lipid A found in this organism. The enzyme has been solubilized with Triton X-100 and purified 80-fold. As shown by co-purification and thermal inactivation studies, the 4'-phosphatase catalyzes not only the hydrolysis of (Kdo)2-4'-32P]lipid IVα but also the transfer of the 4'-phosphate of Kdo2-4'-32P]lipid IVα to the inositol headgroup of phosphatidylinositol (PtdIns) to generate PtdIns-4-P. Like the 4'-phosphatase, the phosphotransferase activity is not present in Escherichia coli, Rhizobium meliloti, or the nodulation-defective mutant 24AR of R. leguminosarum. The specific activity for the phosphotransferase reaction is about 2 times higher than that of the 4'-phosphatase. The phosphotransferase assay conditions are similar to those used for PtdIns kinases, except that ATP and Mg2+ are omitted. The apparent Km for PtdIns is ~500 μM versus 20–100 μM for most PtdIns kinases, but the phosphotransferase specific activity in crude cell extracts is higher than that of most PtdIns kinases. The phosphotransferase is absolutely specific for the 4-position of PtdIns and is highly selective for PtdIns as the acceptor. The 4'-phosphatase/phosphotransferase can be eluted from heparin- or Cibacron blue-agarose with PtdIns. A phosphoenzyme intermediate may account for the dual function of this enzyme, since a single 32P-labeled protein species (Mw ~88,000) can be trapped and visualized by SDS gel electrophoresis of enzyme preparations incubated with Kdo2-4'-32P]lipid IVα. Although PtdIns is not detected in cultures of R. leguminosarum/elli (CE3), PtdIns may be synthesized during nodulation or supplied by plant membranes, given that soybean PtdIns is an excellent phosphate acceptor. A bacterial enzyme for generating PtdIns-4-P and a direct link between lipid A and PtdIns-4-P biosynthesis have not been reported previously.

Lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria (1–5). The lipid A moiety of LPS makes up much of the outer monolayer of the outer membrane (1–5). LPS acts as barrier to antibiotics (6, 7) and helps bacterial cells resist complement-mediated lysis (8). The lipid A portion of LPS is essential for bacterial viability (9–11), and it is also the active component of LPS responsible for many of the pathophysiological effects associated with Gram-negative infections in animals, including septic shock (3, 4, 11, 12).

In certain plant systems LPS is required for the establishment of symbiosis between nitrogen-fixing strains of Rhizobium and their hosts (13, 14). Rhizobium leguminosarum mutants with truncated LPS structures lacking O-antigens are defective in generating functional nodules within root cells (15–19). Changes in LPS structure are also associated with the physiological adaptation to the symbiotic microenvironment (20, 21). For instance, developmentally regulated expression of distinct LPS epitopes can be demonstrated in planta by immunostaining (22). Whether or not the lipid A moiety of R. leguminosarum LPS plays a role in infection and nodulation is unknown, since defined mutants in the lipid A pathway are not yet available. A complete understanding of the structure, biosynthesis, and molecular genetics of lipid A in R. leguminosarum is a prerequisite for defining its functions during symbiosis.

The structure of lipid A in R. leguminosarum is strikingly different from that of E. coli (23–26) (Fig. 1A). R. leguminosarum lipid A lacks the 1- and 4'-phosphate groups found in the lipid A of most other Gram-negative bacteria (23, 24). A galacturonic acid residue is incorporated in place of the 4'-phosphate, and the proximal glucosamine 1-phosphate unit of Escherichia coli lipid A may be replaced with an aminoglucosamine moiety (23, 24) (Fig. 1A). R. leguminosarum lipid A also lacks the laurate and myristate residues present in E. coli lipid A (3, 27) but is acylated with an unusual 28-carbon chain (23, 24, 28). Despite these differences, both E. coli and R. leguminosarum employ the same seven enzymes to generate the conserved, phosphate-containing precursor, Kdo2-lipid IVα (Fig. 1B) (29). Several distinct enzymes must therefore exist in R. leguminosarum that catalyze the conversion of Kdo2-lipid IVα to R. leguminosarum lipid A. We have recently discovered a 4'-phosphatase (30), a 1-phosphatase (31), a long chain acyltransferase (32), and a mannosyl transferase (31, 33, 34) that are involved in the processing of Kdo2-lipid IVα in extracts of R. leguminosarum but not of E. coli. Many additional enzymes.

PtdGro, phosphatidylglycerol; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-3-P, phosphatidylinositol 3-phosphate; MES, 4-morpholinolethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DS-1-P, tetraacyldisaccharide 1-phosphate; HPLC, high pressure liquid chromatography.
unique to *R. leguminosarum* remain to be found.

We now report a novel, chemically specific and highly active phosphotransferase reaction associated with the 4'-phosphotransferase of *R. leguminosarum* (CE3) (Fig. 1B). In the presence of phosphatidylinositol (PtdIns), the 4'-phosphotransferase can transfer the 4'-phosphate of Kdo2-[4-32P]lipid IV, and related metabolites including *E. coli* lipid A to the inositol moiety of PtdIns, generating exclusively PtdIns-4-P (Fig. 1B). The phosphotransferase and the previously described 4'-phosphotransferase activity (30) appear to be catalyzed by the same enzyme, given their identical behavior during purification and thermal inactivation. Although the apparent 

**FIG. 1. Structures of *E. coli* and *R. leguminosarum* lipid A and of Kdo2-[4-32P]lipid IV, A, the 1-, 3-, and 4'-positions of each lipid A structure are indicated. Evidence for the presence of an acyloxyacyl residue and partially deacylated species has recently been presented by Que et al. (25, 26).**

**Experimental Procedures**

**Chemicals and Materials—[γ-32P]ATP was obtained from NEN Life Science Products. Ptd[2-3H]Ins-4-P and [1,2-3H]Ins were purchased from American Radiolabeled Chemicals, Inc. PtdIns-3-P, digalactosyl diglyceride and monogalactosyl-diglyceride were obtained from Matreya, Inc. Silica gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technology, E. Merck. Triton X-100 and bicinechonic acid were from Pierce. Yeast extract and tryptone were obtained from Difco. Other chemicals were from Sigma or Mallinckrodt.**

**Bacterial Strains and Growth Conditions—** *R. leguminosarum* biovar *viciae* 4801, *Rhizobium meliloti* 1021, and *E. coli* W3110 were described in previous studies (29–31, 38). All other strains of *Rhizobium* were purchased from the American Type Culture Collection. All *Rhizobium* strains were grown at 30 °C on TY medium, which contains 5 g/liter yeast extract, 10 mM CaCl2, and 20 μg/ml nalidixic acid. For the growth of strains CE3, 24AR, 1021, and 8401, streptomycin (200 μg/ml) was also added to the medium. *E. coli* W3110 was grown on LB broth (39) at 30 °C.

**Preparation of Radiolabeled Substrates—** The substrate [4-32P]lipid IV was generated using [γ-32P]ATP, tetraacyldisaccharide 1-phosphate acceptor (DS-1-P) (40), and membranes of *E. coli* BLR(DE3)/pLysS/pJK2, which contain large amounts of the 4'-kinase (41). Kdo2-[4-32P]lipid IV could then be prepared from the [4-32P]lipid IV using the purified Kdo transferase (38, 42). Alternatively, Kdo2-[4-32P]lipid IV was also prepared directly from DS-1-P and [γ-32P]ATP without purification of the [4-32P]lipid IV intermediate. Briefly, a 100-μCi portion of [γ-32P]ATP (3000 Ci/mmol) was dried in a 1.5-ml polypropylene microcentrifuge tube under a stream of N2. Next, the following components were added: (a) 25 μl of a mixture of DS-1-P and cardiolipin in 1 ml of water, followed by ultrasonic dispersion; (b) 5 μl of 10% Nonidet P-40, a nonionic detergent; and (c) 10 μl of 25 mM MgCl2. The 4'-kinase reaction was started by adding 5 μl of membranes (500 μg/ml in 50 mM Hepes, pH 7.5) of *E. coli* strain BLR(DE3)pLysS/pJK2 and was held at room temperature. After 10 min, a second 5-μl portion of the enzyme stock was added, and the incubation was continued for another 10 min. The Kdo transferase reaction was then carried out in the same tube by adding the following: 2 μl of 5 mM lipid IV, 25 μl of 2% Triton X-100, 50 μl of 100 mM CTP in 0.5 M Hepes, pH 7.5, 50 μl of 40 mM Kdo, 50 μl of 100 mM MgCl2, 50 μl of purified CMP-Kdo-phosphotransferase (0.03 total units) (42, 43), and 25 μl of purified Kdo transferase (38) (from a stock solution at 80 μg/ml). The final reaction volume was 500 μl. After incubation at 30 °C for 30 min, 80–90% of the [γ-32P]ATP was incorporated into the
Kdo₄[4,9-³²P]lipid IVₐ, as judged by analytical thin layer chromatography and autoradiography.

Next, the reaction mixture was carefully spotted across the origin of a 20 x 20-cm silica gel 60 thin layer plate. The plate was dried under a cold air stream and developed in the solvent chloroform/pyridine (88% formic acid/water, 90:10, v/v) for 30 min. The plate was again air-dried under a cold air stream and exposed to x-ray film for 30–60 s to locate the Kdo₄[4,9-³²P]lipid IVₐ band. The appropriate region was scraped and transferred to a 30-ml sintered glass filter funnel. The silica powder was washed with 10 ml of chloroform, and the ³²P-labeled product was then transferred to a 30-ml sintered glass filter funnel. The silica powder was washed with 10 ml of chloroform, and the ³²P-labeled product was then transferred to a 30-ml sintered glass filter funnel. The silica powder was washed with 10 ml of chloroform, and the ³²P-labeled product was then transferred to a 30-ml sintered glass filter funnel. The silica powder was washed with 10 ml of chloroform, and the ³²P-labeled product was then transferred to a 30-ml sintered glass filter funnel.

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Fig. 2. Presence of a PtdIns-dependent phosphotransferase in membranes of R. leguminosarum. Membranes of different strains were assayed for phosphotransferase activity using the standard assay. A protein concentration of 0.2 mg/ml was used, and the incubation was carried out for 20 min at 30 °C. The thin layer chromatographic analysis of the reaction products generated from Kdo₄[4,9-³²P]lipid IVₐ and PtdIns are shown. Lane 1, no membranes; lane 2, E. coli W3110; lane 3, R. leguminosarum biovar etli CE3; lane 4, R. leguminosarum biovar trifoli CE3; lane 5, R. leguminosarum biovar vicie 8401; lane 6, R. leguminosarum biovar trifoli ATCC 14479; lane 7, R. meliloti 1021. No PtdIns-4-P was formed in the absence of added PtdIns.

The final concentrated active fractions (15 ml) equilibrated with Buffer C. Next, the column was washed with Buffer C containing no detergent. The cycle was repeated three times. Active fractions were pooled into the same 15-ml Corex tube used for the initial lower phases, and the resulting solution was again dried under a stream of N₂. The final dried Kdo₄[4,9-³²P]lipid IVₐ, the [4,9-³²P]lipid IVₐ, and [4,9-³²P]lipid IVₐ were each suspended by sonic irradiation for 1 min in a bath sonicator.

Assays of the 4'-Phosphatase and the Phosphotransferase Reactions—Standard assay conditions for the 4'-phosphatase were as follows. The reaction mixture (10–20 μl) contained 50 mM MES, pH 6.5, 0.1% Triton X-100, 2 mM dithiothreitol, 2 mM EDTA, 10 mM potassium phosphate, and 10 μM Kdo₂[4,9-³²P]lipid IVₐ (20,000 cpm/nmol). PtdIns (usually 1 mg/ml or at the indicated concentrations) was added as the acceptor substrate in the phosphotransferase reactions. When PtdIns was included as the phosphate acceptor, a 1-μl solution of PtdIns in chloroform/methanol (4:1, v/v) was transferred to each reaction tube, and the solvent was removed with a stream of N₂. Next, the Triton X-100 and the other reaction components were added. The reaction mixture was subjected to sonic irradiation to disperse the phospholipids into the detergent-containing buffer. Reactions were initiated with enzyme. The reaction mixtures were incubated at 30 °C for 15 min as indicated. Reactions were terminated by spotting 2-μl samples onto a silica gel 60 thin layer chromatography plate, which was developed in the solvent chloroform/pyridine (88% formic acid/water, 90:10, v/v) for 30 min. The plate was exposed to x-ray film for 30–60 s to locate the Kdo₂[4,9-³²P]lipid IVₐ band. The appropriate region was scraped and transferred to a 30-ml sintered glass filter funnel.

Partial Purification of the 4'-Phosphatase and Its Associated Phosphotransferase Activity—All enzyme preparations were carried out at 0–4 °C. Protein was determined by the bichinchoninic acid method (46), using bovine serum albumin as a standard. R. leguminosarum CE3 cells in late logarithmic phase (A₅₅₀ = 1.2–1.4) were harvested from 5 liters of culture by centrifugation (8000 × g, 15 min) and resuspended in 50 mM Hepes, pH 7.5, to give a final protein concentration of 8–10 mg/ml. Cells were broken by two passages through a French pressure cell at 18,000 p.s.i., and the debris was removed by centrifugation at 8000 × g for 15 min. Membranes were prepared by ultracentrifugation at 149,000 × g for 60 min. The membrane pellet was resuspended in Buffer A (60 mM Hepes, pH 7.5, 3% glycerol, and 10 mM potassium phosphate) at 4–5 mg/ml. Solubilization was carried out by the addition of 10% Triton X-100 (reduced) to yield a protein:detergent ratio of 1:2 (0.9% final detergent concentration), followed by stirring for 2 h, and centrifugation at 149,000 × g for 60 min. The supernatant, which contained over 95% of both activities, was collected and stored at −80 °C.

Next, a Q-Sepharose (Amersham Pharmacia Biotech) column (30 ml) was equilibrated with Buffer B (20 mM Tris-HCl, pH 7.8, 0.15% Triton X-100, 2 mM EDTA, 3% glycerol, and 10 mM potassium phosphate). Solubilized membranes (30 ml, 3.1 mg/ml protein) were loaded onto the column. Unbound proteins were washed out with Buffer C (50 mM MES, pH 6.5, 0.15% reduced Triton X-100, 2 mM EDTA, 3% glycerol, and 10 mM potassium phosphate) at 4–5 mg/ml. Solubilization was carried out by the addition of 10% Triton X-100 to bring the protein:ligand ratio to 1:0.1. Elution was done with a 400-ml linear gradient from 0.1 to 1.0 M NaCl in Buffer C. The column fractions (8 ml) were assayed for the 4'-phosphatase and 4'-phosphotransferase. The active fractions (~80 ml total volume) were pooled, desalted, and concentrated (~3-fold) by ultrafiltration through an Amicon (30K) filter. The concentrated material was then diluted to the original volume with Buffer C containing no detergent. The cycle was repeated three times. The final concentrated active fractions (~25 ml) from the Q-Sepharose column were then loaded onto a heparin-agarose Type 1 (Sigma) column (15 ml) equilibrated with Buffer C. Next, the column was washed with equilibration buffer containing 100 mM NaCl. The 4'-phosphotransferase/phosphatase activities were eluted with a 200-ml linear gradient from 0.1 to 1.5 M NaCl in Buffer C. The column fractions (5 ml) were assayed for both the phosphotransferase and the 4'-phosphatase. The
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RESULTS

A Phosphotransferase in Membranes of R. leguminosarum That Uses Kdo₂-[4'-32P]lipid IVₐ to Phosphorylate PtdIns—The enzyme (2 μg purified through the heparin-agarose step or 6 μg purified through the Q-Sepharose step as indicated) was mixed with (Kdo₂-[4'-32P]lipid IVₐ (25,000 cpm at 8 Ci/nmol) in a reaction buffer containing 0.5 M CAPS (pH 10), containing 12% SDS, 60% glycerol, and bromphenol blue (reduced), and 10 ml of Buffer C. Finally, the elution of the 4'-phosphatase/phosphotransferase was carried out with 35 ml of Buffer C containing 0.5% Triton X-100 and 0.5 mM NaCl. The column fractions (2.5 ml) were assayed for both the phosphotransferase and the 4'-phosphatase activities.

Separation of Inner and Outer Membranes—R. leguminosarum (CE3) membranes were separated by isopycnic sucrose gradient centrifugation as described previously (33). The turbidity (A₆₀₀) of each fraction was determined to confirm the presence of membrane fragments, and each fraction was assayed for NADH oxidase as the outer membrane marker (33).

The protein content of each fraction was determined by the bicinchoninic acid assay (46) using bovine serum albumin as the standard. Last, each fraction was assayed for both phosphotransferase and 4'-phosphatase activities under the standard assay conditions described above.

Deacylation and HPLC Analysis of the [32P]-Labeled PtdIns Derivative Generated from Kdo₂-[4'-32P]lipid IVₐ—Prior to HPLC analysis, the product of the phosphotransferase reaction and the appropriate lipid standards were deacylated. This was accomplished by treating the compounds with methylamine reagent (47). Briefly, a 0.5-ml portion of methylamine reagent, prepared by bubbling methylamine (Fluka, Buchs, Switzerland) at 0 °C through a mixture in which it is very soluble (6.2 ml of methanol, 4.6 ml of deionized water, and 1.5 ml of 1-butanol) to give a final volume of 20 ml was added to the dried lipids (see Fig. 10 legend). The mixture was incubated at 53 °C for 40 min. Cold 1-propanol (0.2 ml) was added to the sample. After mixing, the sample was dried and resuspended in 0.2 ml of water. This aqueous dispersion was extracted three times with 0.5-ml portions of butanol/petroleum ether/ethyl formate (20:41, v/v/v). The lower aqueous phase was dried, and deacylation products were redissolved in 200 μl of deionized water for HPLC analysis. Anion exchange HPLC was performed using a Partisil SAX column (Whatman) (4.6 x 250 mm) (47).

The deacylation products were eluted (flow rate of 1 ml/min) with a linear gradient from 10 to 510 mM NH₄H₂PO₄ (pH 3.8) in 50 min. Effluent from the HPLC column flowed directly into a Betaram™ in-line continuous flow scintillation detector (INUS, Tus, FL).

Fig. 3. Inner membrane localization of the 4'-phosphatase and the PtdIns-dependent phosphotransferase of R. leguminosarum. The inner and outer membranes of strain CE3 were separated by isopycnic sucrose density gradient centrifugation, and -0.4-ml fractions were collected. NADH oxidase and phospholipase A activities were assayed to locate inner and outer membrane fragments, respectively. A, NADH oxidase and phospholipase A activities in each fraction are expressed as a percentage of the total activity across the entire gradient. B, the 4'-phosphatase and the phosphotransferase activities were assayed in each fraction. (30). In the presence of phosphatidylinositol, however, which was initially tested as a stabilizing agent, the same membranes that dephosphorylate Kdo₂-[4'-32P]lipid IVₐ also catalyze transfer of [32P] from Kdo₂-[4'-32P]lipid IVₐ onto PtdIns (Fig. 2, lanes 3, 5, and 6). This phosphotransferase activity is not detected in membranes of the nodulation-deficient mutant 24AR of R. leguminosarum, which lacks the 4'-phosphatase (30) and possesses a 4'-phosphate moiety on its lipid A (48). As with the 4'-phosphatase (30), no phosphotransferase activity is seen in the cytosol.

Fractionation of R. leguminosarum CE3 membranes by sucrose density gradient centrifugation (33) revealed that both the 4'-phosphatase and the phosphotransferase are localized in the inner membrane (Fig. 3). The distribution of NADH oxidase activity served as the inner membrane marker in the same gradient fractions. Phospholipase A indicated the presence of outer membrane fragments (Fig. 3).

The Solubilized 4'-Phosphatase and Phosphotransferase Activities Co-purify—The possibility that the same enzyme might be catalyzing the 4'-phosphatase and the phosphotransferase reactions was investigated by partially purifying the 4'-phosphatase. Isolates of membranes as the first step resulted in a 2.8-fold increase in 4'-phosphatase-specific activity (Table 1). Quantitative solubilization of both activities was achieved with Triton X-100 (reduced) at a detergent:protein ratio of 2:1, as described under “Experimental Procedures.” In Table I, the -fold purification and yields were calculated based on the 4'-phosphatase activity, but the pattern was the same if the

active fractions were pooled, concentrated (3 fold), and desalted as described above.

Cibacron Blue Column Chromatography and Substrate Elution—A Cibacron blue 3GA Type 300 (Sigma) column (3 ml) was equilibrated with Buffer C (see above). Partially purified enzyme from the heparin-agarose step (0.3 mg/ml protein) was loaded onto the column and was recirculated through the column two more times. The column was then washed successively with 5 ml of Buffer C, 15 ml of Buffer C containing 0.5 mM NaCl, 15 ml of Buffer C containing 0.5% Triton X-100 (reduced), and 10 ml of Buffer C. Finally, the elution of the 4'-phosphatase/phosphotransferase was carried out with 35 ml of Buffer C containing 0.5% Triton X-100 and 0.5 mM NaCl. The column fractions (2.5 ml) were assayed for both the phosphotransferase and the 4'-phosphatase activities.

tratove of the lipid A disaccharide precursor Kdo₂-[4'-32P]lipid IVₐ,
The activities were assayed with $50 \mu M$ Kdo-$_2$-[4-$^32$P]lipid IV$_A$, either with or without 1 mg/ml PtdIns. The $\times$-fold purification and yield after each step were calculated using the 4'-phosphatase activity data. Within the experimental error of the assays, the ratio of the phosphotransferase specific activity divided by the 4'-phosphatase specific activity was the same after each step following solubilization.

| Purification step | Total volume | Protein | 4'-Phosphatase specific activity | Phosphotransferase specific activity | Ratio | Purification | Yield |
|-------------------|--------------|---------|---------------------------------|-------------------------------------|-------|--------------|-------|
| Crude extract     | 60           | 8.2     | 0.65                            | 1.0                                 | 1.5   | 1            | 100   |
| Membranes         | 40           | 4.4     | 1.82                            | 3.27                                | 1.8   | 2.8          | 120   |
| Solubilized membranes | 30     | 3.1     | 3.52                            | 7.74                                | 2.2   | 5.4          | 120   |
| Q-Sepharose       | 25           | 0.46    | 21.4                            | 51.36                               | 2.4   | 32.9         | 77    |
| Heparin-agarose   | 15           | 0.3     | 53.2                            | 117.04                              | 2.2   | 81.8         | 75    |

**Table I**

Solubilization and partial purification of the 4'-phosphatase/phosphotransferase

Two ion exchange resins were surveyed for their ability to bind the solubilized 4'-phosphatase. The enzyme displayed strong, reversible affinity for Q-Sepharose and heparin-agarose. About 80% of both the 4'-phosphatase and the phosphotransferase bound to Q-Sepharose, and both were quantitatively eluted from the column at $0.3–0.4 M$ NaCl (Fig. 4A). The pooled peak fractions were purified about 33-fold over the cell extract based on the specific activity of the 4'-phosphate (Table I). Next, column chromatography on heparin-agarose (Fig. 4B) resulted in an additional 2.5-fold purification of the 4'-phosphatase with an overall yield of about 75% (Table I). Importantly, the peaks of 4'-phosphatase and the phosphotransferase activities in both of these chromatograms (Fig. 4, A and B) overlapped each other within experimental error. Following solubilization of the membranes, the ratio of the specific activity of the phosphotransferase to the 4'-phosphatase remained constant at each step of the purification (2.2–2.4) (Table I). The identical chromatographic behaviors of both activities (Fig. 4) and the constant ratios of specific activities (Table I) indicate that a single enzyme species probably catalyzes both the 4'-phosphatase and the phosphotransferase reactions. However, the heparin-agarose fractions are yet not homogeneous, as judged by SDS gel electrophoresis (not shown).

Following solubilization and purification, both the 4'-phosphatase and the phosphotransferase activities are linear with time for up to 20 min at 30 °C (Fig. 5).

**Fig. 4.** Identical chromatographic behaviors of the 4'-phosphatase and the phosphotransferase. A, co-elution of 4'-phosphatase and phosphotransferase activities from Q-Sepharose. B, co-elution of 4'-phosphatase and phosphotransferase activities from heparin-agarose.

**Fig. 5.** Kinetic and Substrate Specificity of the 4'-Phosphatase and the Phosphotransferase—In crude extracts, the 4'-phosphatase functions optimally when a Kdo disaccharide is part of the substrate, as in Kdo$_2$-lipid IV$_A$ (30). We confirmed the Kdo dependence of both the 4'-phosphatase and the phosphotransferase with the 80-fold purified enzyme (heparin-agarose step). The apparent $K_m$ for lipid IV$_A$ was about 5 times higher than that for Kdo$_2$-lipid IV$_A$ (Table II) with both activities, but Kdo-[4-$^32$P]lipid IV$_A$ prepared with the monofunctional Kdo transferase of H. influenzae was a good substrate as well (Table II). Either one or two Kdo moiety was also increased the $V_{\text{max}}$ by 2–3-fold in comparison with lipid IV$_A$ in each case (Table II). The $V_{\text{max}}$ for the phosphotransferase, using PtdIns as the acceptor, was 3 times higher than that of the 4'-phosphatase with the partially purified enzyme, using Kdo$_2$-lipid IV$_A$ as the substrate (Table II).

The ability of the phosphotransferase to tolerate structural modifications of the donor substrate was investigated. Kdo$_2$-[4-$^32$P]1-dephospholipid IV$_A$ (38), galactose-mannose-Kdo$_2$-[4-$^32$P]lipid IV$_A$ (34), mannose-Kdo$_2$-[4-$^32$P]lipid IV$_A$ (34), lauroyl-Kdo$_2$-[4-$^32$P]lipid IV$_A$ (49), and [4-$^32$P]lipid A (41) were tested in both assays (not shown). These substances were all utilized efficiently. Hexa-acylated [4-$^32$P]lipid A was utilized at about
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**FIG. 6.** Thermal inactivation of the 4'-phosphatase and the phosphotransferase. Samples of the partially purified enzyme (heparin-agarose step) were preincubated at various temperatures (30, 37, or 45 °C) for the indicated times. A portion of the incubation was then used to assay for remaining 4'-phosphatase and phosphotransferase activities. The percentage of the activity remaining is normalized to a control sample of the enzyme held on ice.

**FIG. 5.** Linearity of the partially purified 4'-phosphatase and phosphotransferase reactions with time. Partially purified enzyme (heparin-agarose step) was assayed for the 4'-phosphatase activity in the absence of PtdIns (open circles and squares) or for the phosphotransferase activity in the presence of PtdIns (closed circles and squares). Protein concentrations of 0.6 μg/ml (circles) or 1.2 μg/ml (squares) were used, and the assays were incubated at 30 °C for the indicated times under standard conditions (30 μl final volume). At every time point indicated, a 2-μl portion of the reaction mixture was withdrawn and analyzed by thin layer chromatography and PhosphorImager analysis, as described under "Experimental Procedures."

The 4'-phosphatase activity is expressed as the amount of inorganic phosphate released per ml of reaction mixture in absence of PtdIns, while the phosphotransferase activity is expressed as the amount of PtdIns-4-P formed in the presence of PtdIns.

**TABLE II**

| Donor substrate | Acceptor substrate | Apparent $K_m$ (μM) | $V_{max}$ (nmol/min/mg) |
|-----------------|--------------------|----------------------|------------------------|
| Kdo$_2$-lipid IV$_A$ | Water              | 30.4                 | 73.9                   |
| Kdo$_2$-lipid IV$_A$ | PtdIns             | 32.1                 | 170.0                  |
| Kdo$_2$-lipid IV$_A$ | Water              | 40.5                 | 55.6                   |
| Kdo$_2$-lipid IV$_A$ | PtdIns             | 38.2                 | 122.3                  |
| Lipid IV$_A$      | Water              | 144.6                | 30.0                   |
| Lipid IV$_A$      | PtdIns             | 150.4                | 57.0                   |

Other membrane lipids (Fig. 8, A and B). The phosphotransferase exhibits a very high degree of selectivity toward PtdIns. PtdIns from bovine liver (containing mainly stearate and arachidonate) and PtdIns from soybean (containing mostly palmitate and linoleate) were equally active as acceptors (Fig. 8A). No other inositol-containing compound served as an acceptor (Fig. 8A), including PtdIns-4-P, which is actually an inhibitor (not shown), PtdIns-3-P, lysophosphatidylinositol, or myo-inositol. Among the other membrane lipids tested, inefficient transfer of the 4'-phosphate of Kdo$_2$-[4-$^32$P]lipid IV$_A$ to phosphatidyglycerol (PtdGro) was detected (Fig. 8B, lane 4). However, the rate of transfer of $^{32}$P to PtdGro was 50 times slower than to PtdIns (Fig. 9) over a wide range of acceptor concentrations.

Since glycosylated diacylglycerols are major components of *R. leguminosarum* membranes, commercially available digalactosyl diacylglycerol and monogalactosyl diacylglycerol were tested. However, neither compound was active as a phosphate acceptor (not shown). As shown in Fig. 9, the apparent $K_m$ for PtdIns is approximately 500 μM. This is 10-fold higher than what is typically seen with eucaryotic PtdIns kinases (35–37), but the specific activity of the phosphotransferase in membranes is higher than that of many eucaryotic PtdIns kinases assayed under comparable conditions. The relatively high apparent $K_m$ for PtdIns therefore does not eliminate the possibility of a physiological role for the phosphotransferase. Indeed, many key enzymes of glycerophospholipid and lipid A biosynthesis display even higher apparent $K_m$ values for their physiological substrates (40, 50).
Selective Transfer of the 4'-Phosphate Group of Kdo$_2$-Lipid IV$_A$ to the Inositol 4-Position of PtdIns—To determine the site of phosphorylation on PtdIns, the $^{32}$P-labeled phosphotransferase product was characterized by chromatography and by chemical degradation. Migration of the $^{32}$P-labeled PtdIns-dependent product with authentic PtdIns-4-P was demonstrated in the presence of excess of Kdo$_2$-[4-32P]lipid IV$_A$ (50 μM). Heparin-agarose-purified enzyme (10 μg/ml) was used, and the incubation was carried out for 10 min at 30 °C.

Evidence for a Phosphoenzyme Intermediate—When either 6 μg of Q-Sepharose or 2 μg of heparin-agarose 4'-phosphatase/phosphotransferase (Table I) was incubated with Kdo$_2$-[4'-$^{32}$P]lipid IV$_A$ for 5 min, followed by the addition of buffer containing SDS at pH 10, a single $^{32}$P-labeled 68-kDa protein was detected by SDS-gel electrophoresis and PhosphorImager analysis (Fig. 11A, lanes 2 and 3). The intensity of the radiolabeled band increased with increasing protein concentrations (not shown). Heat inactivation of the enzyme preparation prior to incubation with the Kdo$_2$-[4'-$^{32}$P]lipid IV$_A$ abolished the incorporation of $^{32}$P (Fig. 11A, lane 1). The formation of the phosphoprotein was transient, since maximum labeling was observed after only 2–5 min of incubation with Kdo$_2$-[4'-$^{32}$P]lipid IV$_A$ (not shown). Thereafter, the intensity of the band steadily decreased until it was virtually undetectable after 90 min (not shown).

The addition of PtdIns to the enzyme preparation together with Kdo$_2$-[4'-$^{32}$P]lipid IV$_A$ resulted in decreased labeling of the 68-kDa protein (Fig. 11B, lane 2). Faster turnover of the proposed phosphoenzyme intermediate in the presence of the 4'-phosphate of Kdo$_2$-[4'-32P]lipid IV$_A$ either in the presence (Fig. 10, B and C) or absence of PtdIns (Fig. 10A) elutes 3–4 min before glycerophosphoinositol 3-phosphate. The HPLC analysis of the phosphotransferase reaction product therefore provides unequivocal evidence that the 4'-phosphate of Kdo$_2$-lipid IV$_A$ is transferred only to the 4-position of the inositol moiety of PtdIns. This remarkable specificity shows that the enzyme cannot transfer the 4'-phosphate of Kdo$_2$-[4'-$^{32}$P]lipid IV$_A$ to any other hydroxyl group present on the surface of the Triton X-100/PtdIns mixed micelle, since alternative phosphorylated products, like PtdIns-3-$^{32}$P or $^{32}$P-Triton X-100, are not detected.
Enzymatic Synthesis of PtdIns-4-P from PtdIns and Lipid A

The Kdo-dependent 4'-phosphatase of *R. leguminosarum* catalyzes a key reaction in generating a phosphate-deficient variant of lipid A (30). Here we show that the same enzyme preparations can also transfer the 4'-phosphate group from lipid A and certain lipid A precursors to PtdIns. An enzymatic reaction that directly links lipid A biosynthesis with PtdIns-4-P production has not been described previously. This unique *R. leguminosarum* phosphotransferase is the first example of pro-caryotic enzyme capable of generating PtdIns-4-P, a molecule that plays a central role in eucaryotic signal transduction (53, 54).

The current studies strongly suggest that one enzyme catalyzes both the 4'-phosphatase and the phosphotransferase reactions. Like the 4'-phosphatase (30), the phosphotransferase

verse reaction (Fig. 1) with excess unlabeled PtdIns-4-P, would explain this effect.

A pulse-chase experiment (Fig. 12) confirmed that the loss of 32P label from the 68-kDa phosphoprotein was dependent both upon time and the concentration of the unlabeled Kdo2-lipid IVα used to dilute the specific radioactivity. The formation and turnover of the 32P-labeled phosphoprotein are consistent with the proposed mechanism involving a phosphoenzyme intermediate (Fig. 1).

The chemical stability of the 32P-labeled protein species visualized in Figs. 11 and 12 was explored to determine the nature of the covalent linkage that might be involved. Treatment with 8 M urea or 200 mM dithiothreitol did not reduce the intensity of the labeled band (data not shown). The 32P-labeled protein was very labile under acidic conditions, but it was stable under mild basic or at neutral conditions in the presence of SDS. The stability of the protein-bound 32P actually increased under mild alkaline conditions. This pattern is consistent with a phosphohistidine residue (51, 52). Isolation and analysis of the active site peptide labeled with 32P will be necessary to confirm this proposal.

**DISCUSSION**

The Kdo-dependent 4'-phosphatase of *R. leguminosarum* catalyzes a key reaction in generating a phosphate-deficient variant of lipid A (30). Here we show that the same enzyme preparations can also transfer the 4'-phosphate group from lipid A and certain lipid A precursors to PtdIns. An enzymatic reaction that directly links lipid A biosynthesis with PtdIns-4-P production has not been described previously. This unique *R. leguminosarum* phosphotransferase is the first example of pro-caryotic enzyme capable of generating PtdIns-4-P, a molecule that plays a central role in eucaryotic signal transduction (53, 54).

The current studies strongly suggest that one enzyme catalyzes both the 4'-phosphatase and the phosphotransferase reactions. Like the 4'-phosphatase (30), the phosphotransferase
is present in wild type strains of *R. leguminosarum* but not in *E. coli*, *R. leguminosarum* mutant 24AR, or *R. meliloti*. In *R. leguminosarum* (CE3), both activities are localized in the inner membrane (Fig. 3). Both activities display similar chromatographic properties on Q-Sepharose (Fig. 4), heparin-agarose (Fig. 4) and Cibacron blue-agarose (Fig. 7), and their thermal inactivation profiles are identical (Fig. 6). Furthermore, both activities co-purify (Table I) and show the same donor substrate specificity (Table II).

The ability of one enzyme to catalyze both the 4'-phosphatase and the phosphotransferase reaction could be explained by a mechanism involving a covalent phosphoenzyme intermediate (Fig. 1B). The intermediate would be generated by the initial transfer of the 4'-phosphate moiety of Kdo2-lipid IVA to the enzyme. The intermediate could transfer the phosphate group either to water or to the 4-OH of PtdIns. The direct demonstration (Figs. 11 and 12) of a 32P-labeled phosphoprotein, obtained by incubation of the 80-fold purified enzyme with Kdo2-[4'-32P]lipid IVA and chased by nonradioactive (Kdo)2-lipid IVA, respectively, of the percentage of the 32P-labeled phosphoprotein remaining at various times after the start of the chase (indicated by the arrow) is shown in D.

FIG. 12. Turnover of the labeled phosphoprotein following a chase with unlabeled Kdo2-lipid IVα. The enzyme preparation (heparin-agarose step) was labeled for 5 min with Kdo2-[4'-32P]lipid IVA as described under “Experimental Procedures.” A, B, and C are the images of the SDS-polyacrylamide gels used to resolve the remaining 32P-labeled phosphoprotein present at different times after being chased with 0, 10, or 50 μM nonradioactive (Kdo)2-lipid IVA, respectively. The percentage of the 32P-labeled phosphoprotein remaining at various times after the start of the chase (indicated by the arrow) is shown in D.

Our studies show that the phosphotransferase, like the 4'-phosphatase (30), functions more efficiently in the presence of at least one Kdo residue on the donor substrate (Table II). The enzyme is less sensitive to other structural modifications of Kdo2-[4'-32P]lipid IVA, such as removal of the 1-phosphate, the addition of extra sugars, or further acylation. Such a dependence on Kdo may be necessary, given that the Kdo transferase requires the presence of the 4'-phosphate residue for catalysis (38, 42). Premature removal of the 4'-phosphate might inhibit LPS maturation. Although the 4'-phosphatase/phosphotransferase is associated with the inner membrane, its active site might face the periplasm, further limiting access to LPS precursors lacking Kdo to the 4'-phosphatase (3).

A 1-phosphatase, distinct from the 4'-phosphatase and capable of cleaving several precursors of lipid A including Kdo2-lipid IVA, is present in membranes of *R. leguminosarum* (31). Using Kdo2-[1-32P]lipid IVA (31) as the donor, however, transfer of the 1-phosphate to PtdIns was not observed in *R. leguminosarum* membranes (data not shown). Thus, phosphotransferase activity appears to be associated only with the 4'-phosphatase of *R. leguminosarum*. In the presence of [γ-32P]ATP and Mg2+, no transfer of 32P to PtdIns was observed when either *R. leguminosarum* CE3 membranes or partially purified heparin-agarose fractions were employed (data not shown). Hence, the *R. leguminosarum* phosphotransferase reaction is not attributable to a PtdIns kinase (35–37).

Although the phosphotransferase exhibits a high degree of selectivity toward PtdIns, the latter could not be demonstrated among the membrane lipids of *R. leguminosarum* (CE3) by
labeling cells with either $^{32}$P, or [1,2-$^{3}$H]inositol. A search for an alternative endogenous lipid acceptor among the total membrane lipids of *R. leguminosarum* proved unsuccessful. Gerson and Patel (55) reported PtdIns in *Rhizobium loti*, but other studies (56) of different strains of *R. leguminosarum* failed to demonstrate PtdIns. One intriguing explanation for this anomaly might be that the synthesis of PtdIns is induced in the bacteria by root cell exudates or during symbiosis. A recent report (57) showed the presence of PtdIns in membranes of free-living *Bradyrhizobium japonicum* grown at low oxygen concentrations, one of the environmental factors known to induce conversion of bacteria to bacteroids in root nodules (58).

An alternative possibility is that plant membranes supply PtdIns or a related compound to *R. leguminosarum* during nodulation. PtdIns is available in the peribacteroid membranes of soybean root nodules (58), and soybean PtdIns is an excellent acceptor substrate for the phosphotransferase (Fig. 8). In addition, Perotto *et al.* (59) have demonstrated presence of other inositol-containing phosphoglycolipids in pea nodules. The close association of the bacteroid outer membrane with the plant-derived peribacteroid membrane is well documented by immunostaining and electron microscopy (60–62). Inside the symbiosome, the outer membrane of the bacteroid may act as a specific transport protein which is not yet known to exist in plants and could serve as PtdIns carriers (63).

In heptose-deficient mutants of *Salmonella typhimurium*, small quantities of exogenous glycerophospholipids can fuse with the outer membrane and move to the inner membrane (64, 65). In the case of exogenous phosphatidylserine, the incorporated lipid is metabolized to form phosphatidylethanolamine within the inner membrane (64, 65). While retrograde transport of exogenous lipids is not sufficiently rapid in Gram-negative bacteria to enable the isolation of phospholipid auxotrophs, it is nevertheless established that such transport occurs with a variety of lipids (64, 65). In the case of minor lipids like PtdIns-4-P, which function at low concentrations in specific signal transduction pathways, the possibility that the plant provides the 4′-phosphatase/phosphotransferase with PtdIns deserves serious consideration. The PtdIns-4-P made by *Salmonella* in plants have not been fully established, evidence is accumulating that signal transduction mediated by the turn-over of inositol phospholipids may indeed occur (68). For instance, Ehrhardt *et al.* (69) observed calcium spiking, as is typically associated with inositol trisphosphate signaling in animal cells, when alfalfa root hairs are exposed to the *R. meliloti* nod factor. In animal cells, phosphatidylinositol kinases play additional important roles in membrane biogenesis, secretion, vesicle trafficking, and regulation of the actin cytoskeleton (53, 54, 70, 71). All of these processes accompany bacterial invasion of plant root hair cells and nodule formation (58). Since the inositol phospholipid cycle begins with the phosphorylation of PtdIns to generate PtdIns-4-P, the *R. leguminosarum* phosphotransferase could play a key role in any of the above processes during symbiosis. Cloning of the gene encoding the 4′-phosphatase/phosphotransferase and isolation of mutants specifically defective in the enzyme should reveal the significance of the phosphate-deficient lipid A that is found in *R. leguminosarum* and the biological role of the phosphotransferase activity associated with the 4′-phosphatase.

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