Lipid A from the nitrogen-fixing bacterium *Rhizobium leguminosarum* displays many structural differences compared with lipid A of *Escherichia coli*. *R. leguminosarum* lipid A lacks the usual 1- and 4′-phosphate groups but is derivatized with a galacturonic acid substituent at position 4′. *R. leguminosarum* lipid A often contains an aminogluconic acid moiety in place of the proximal glucosamine 1-phosphate unit. Striking differences also exist in the secondary acyl chains attached to *E. coli* versus *R. leguminosarum* lipid A, specifically the presence of 27-hydroxystearoacylanoate and the absence of laurate and myristate in *R. leguminosarum*. Recently, we have found that lipid A isolated by pH 4.5 hydrolysis of *R. leguminosarum* cells is more heterogeneous than previously reported (Que, N. L. S., Basu, S. S., White, K. A., and Raetz, C. R. H. (1998) FASEB J. 12, A1284 (abstr.)). Lipid A species lacking the 3-O-linked β-hydroxymyristoyl residue on the proximal unit contribute to this heterogeneity. We now describe a membrane-bound deacetylase from *R. leguminosarum* that removes a single ester-linked β-hydroxymyristoyl moiety from some lipid A precursors, including lipid X, lipid IV A, and (3-deoxy-D-manno-octulosonic acid)2-lipid IV A. The enzyme does not cleave *E. coli* lipid A or lipid A precursors containing an acyloxyacyl moiety on the distal glucosamine unit. The enzyme is not present in extracts of *E. coli* or *Rhizobium meliloti*, but it is readily demonstrable in membranes of *Pseudomonas aeruginosa*, which also contains a significant proportion of 3-O-deacetylated lipid A species. Optimal reaction rates are seen between pH 5.5 and 6.5. The enzyme requires a nonionic detergent and divalent metal ions for activity. It cleaves the monosaccharide lipid X at about 5% of the rate of lipid IV A and (3-deoxy-D-manno-octulosonic acid)2-lipid IV A. 1H NMR spectroscopy of the deacetylase reaction product, generated with lipid IV A as the substrate, confirms unequivocally that the enzyme cleaves only the ester-linked β-hydroxymyristoyl residue at the 3-position of the glucosamine disaccharide.

Lipopolysaccharide, a macromolecular glycolipid found in the outer membranes of Gram-negative bacteria (1–6), is anchored to the outer leaflet of the outer membrane by its lipid A moiety (Fig. 1). The biosynthesis of the lipid A portion of *Escherichia coli* lipopolysaccharide is required for cell viability (3, 7, 8). Additionally, lipid A (endotoxin) causes extreme stimulation of the innate immune system of animals, resulting in the overproduction of diverse cytokines, which can cause the syndrome of Gram-negative sepsis (3, 4, 9, 10). Pharmacological studies have shown that both phosphate groups, the glucosamine disaccharide, and the correct number of fatty acyl chains (Fig. 1) are crucial for the cytokine-inducing activities of lipid A (3, 4, 9).

The structure of lipid A varies slightly among different Gram-negative bacterial pathogens (1, 11), such as *E. coli* versus *Pseudomonas aeruginosa* (Fig. 1), but most of the distinguishing structural features are conserved. However, the lipid A from the nitrogen-fixing bacterium *Rhizobium leguminosarum* differs strikingly from that of *E. coli* (Fig. 1) (12–15). Both phosphate groups are missing, a galacturonic acid residue is attached at the 4′-position, and the glucosamine 1-phosphate unit of *E. coli* lipid A is largely replaced with an aminoglucuronate moiety (Fig. 1) (12, 13). In the initial structural studies by Carlson and co-workers (12, 13), it was further suggested that *R. leguminosarum* lipid A does not possess any acyloxyacyl residues and that it contains a peculiar long fatty acid, 27-hydroxyoctacosanoic acid (Fig. 1) (16). *R. leguminosarum* lipid A, therefore, lacks many of the features thought to be necessary for stimulation of innate immunity in animals (1, 3, 4, 9). Conceivably, the unique structure of *R. leguminosarum* lipid A might be important for the establishment of successful symbiosis in plants (17, 18).

Despite the structural diversity of their lipid A moieties, both *E. coli* and *R. leguminosarum* employ the same seven enzymes to synthesize the key, phosphate-containing lipid A precursor, Kdo2-lipid IV A (19). A number of distinct *R. leguminosarum* enzymes are then required for the alternative processing of Kdo2-lipid IV A to generate *R. leguminosarum* lipid A. We have previously identified a 4′-phosphatase (20), a 1-phosphatase (21), a long chain acyl transferase (22), a mannosyl transferase (23, 24), a galactosyl transferase (21, 24), and a special Kdo transferase (24) that are involved in the unique metabolism of Kdo2-lipid IV A in extracts of *R. leguminosarum*. The biosynthetic origins of the galacturonic acid and the aminoglucuronate moieties are unknown.

We have recently discovered that lipid A of *R. leguminosarum* can be separated into five related molecular species (14, 15), two of which are shown in Fig. 1A (dashed bond at position 3). Structural studies have revealed that some of this heterogeneity can be attributed to lipid A variants lacking the equivalent of the ester-linked β-hydroxymyristoyl moiety that is usually attached to the 3-position of lipid A disaccharides (Fig. 1) (14, 15). Unexpectedly, our reevaluation of the structure of *R. leguminosarum* lipid A also indicates the presence of...
A lipase in *R. leguminosarum* That Attacks Lipid A Precursors

Institute, Norwich, United Kingdom), and mutant 24AR of *P. aeruginosa* were stored as aqueous dispersions at Yale University. *E. coli* viciae *R. leguminosarum* *R. meliloti* Curie Sklodowska University, Lubin, Poland) (28).

contains 5 g/liter tryptone, 3 g/liter yeast extract, 10 mM CaCl2, and 20 m

*E. coli* from S. Long (Stanford University). All other strains of sonicator.

substrates were dispersed by sonic irradiation for 1 min in a bath pH 7.8, containing 1 mM EDTA and 1 mM EGTA. Prior to use, all lipid sonicator.

addition, 200 g/ml streptomycin was also added to the medium for the growth of CE3.

membranes that selectively removes a single acyloxyacyl moiety in all five molecular species (14, 15), as illustrated in Fig. 1A for two of the subtypes.

We now describe a divalent cation-dependent deacylase from *R. leguminosarum* membranes that selectively removes a single 3-O-linked β-hydroxyacyl chain from certain precursors of lipid A that are common to both *E. coli* and *R. leguminosarum*. The enzyme removes only the ester-linked β-hydroxyacylmoiety residue that is attached to the 3-position of the proximal glucosamine unit (3) of precursors like lipid IVX (Fig. 1B) and Kdo2-lipid IVX. It is also capable of cleaving the monosaccharide precursor lipid X at a slow rate. A similar deacylase is found in membranes of *P. aeruginosa*, in which the presence of 3-O-deacylated lipid A species is well established (Fig. 1A) (25, 26). *E. coli* K-12 and *Rhizobium melliloti* do not contain the deacylase. The enzyme may therefore account for the presence of the 3-O-deacylated lipid A subtypes found in *R. leguminosarum* (Fig. 1A), and it may be a useful reagent for the preparation of novel endotoxin analogs with which to study innate immunity.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials—**[γ-32P]ATP and [32P] were obtained from NEN Life Science Products. Silica gel 60 (0.25-mm thin layer plates were purchased from EM Separation Technologies. DEAE-cellulose (DE52) was obtained from Whatman. BAKERBOND octadecyl (C18) reverse phase resin was from J. T. Baker, and Silica Gel Davissil (grade 638, 100–200 mesh, 60 Å) was from Aldrich. Triton X-100 and bicinchoninic acid were from Pierce. Yeast extract and tryptone were purchased from Aldrich. Protein concentrations were determined with bicinchoninic acid (37), using bovine serum albumin as the standard.

**Deacylase Assay Conditions and Thin Layer Chromatography—**Optimized standard assay conditions for the deacylase were as follows. The reaction mixture (10–20 μl) contained 50 mM MES, pH 6.25, 1.0% Triton X-100, 2 mM dithiothreitol, 2 mM EDTA, 20 mM calcium chloride, and 10 μM [4-32P]lipid IVX (20,000 cpm/nmol). The reactions were incubated at 30 °C for 30 min or as indicated. The reactions were terminated by spotting 2–5-μl samples onto silica gel 60 thin layer chromatography plates. The spots were allowed to dry, and the plates were developed in the solvent chloroform/pyridine/88% formic acid/water (50:50:16.5, v/v/v/v). When substrates other than lipid IVX or tetracyclidsaccharide 4-phosphate were used, a different solvent system was employed, consisting of chloroform/pyridine/68% formic acid/water (50:70:16.5, v/v/v/v).

**Preparation of Cell-free Extracts and Membranes—**Two liters of mid-logarithmic phase cells (A550 = 0.6–0.8) were harvested by centrifugation (7,000 × g for 15 min at 4 °C) and were resuspended in 50 mM Heps, pH 7.5, to give a final protein concentration of 5–10 mg/ml. Cells were broken by passage through a French pressure cell at 18,000 p.s.i. Remaining intact cells and large debris were removed by centrifugation at 7000 × g for 15 min. Membranes were prepared by ultracentrifugation at 149,000 × g for 60 min. Membrane pellets were resuspended in 50 mM Heps, pH 7.5, at a protein concentration of ~10 mg/ml. All preparations were carried out at 4 °C, and samples were stored frozen in large concentrations at −80 °C. Protein concentrations were determined with a Molecular Dynamics PhosphorImager 425S, equipped with ImageQuant software. The percentage of conversion of unreacted [32P]labeled substrates to enzymatic products was calculated for each reaction tube and could be converted to specific activity (nmol/min/mg) based on the chemical concentration of the substrate in the assay.

**Base Hydrolysis of the Deacylase Product Generated from [4-32P]Lipid IVX—**Two incubations (designated 1 and 2) were set up using the standard optimized assay conditions for the deacylase with 10 μM [4-32P]lipid IVX as the substrate. Incubation 1 contained no enzyme, while incubation 2 contained 1 mg/ml *R. leguminosarum* (8401) membranes. Both incubations 1 and 2 were incubated for 16 h at 30 °C. Under these conditions, the conversion of [4-32P]lipid IVX to the slower migrating deacylation product was almost complete in incubation 2, whereas no change was seen in incubation 1. For mild base hydrolysis of the products, a 2-μl portion of each incubation was mixed with 3 μl of triethylamine (TEA) and 5 μl of H2O, and the material was then incubated for various times at 37 °C. After 0, 2, 5, and 120 min, 2-μl samples of the TEA hydrolysis mixture were added to 2 μl of H2O and spotted onto a silica gel 60 thin layer plate. For strong base hydrolysis of the products, 2-μl portions of 1 or 2 were mixed with 18 μl of chloroform/methanol/water/acetic acid (25:15:4:2, v/v/v/v) and were spotted onto a silica gel 60 thin layer plate. The spots were allowed to dry, and the plates were developed in the solvent chloroform/pyridine/88% formic acid/water (50:50:16.5, v/v/v/v) and were run in a parallel fashion with nonradioactive standards.

**Large Scale Isolation of the Deacylase Product—** *R. leguminosarum* 8401 membranes, prepared by ultracentrifugation as described above, were enriched for the deacylase activity before being used for the large scale preparation of the reaction product. Membrane preparations (25 ml, 10 mg/ml protein) were mixed at 4 °C with 2.5% Triton X-100 for 90 min, followed by ultracentrifugation at 149,000 × g for 60 min. The
pellet, containing the deacylase activity, was resuspended in 10 ml of 50 mM Hepes buffer (pH 7.5) to a protein concentration of 7.0 mg/ml. The detergent extraction process was repeated another time with 1% Triton X-100. In the pellet (resuspended in 10 ml of 50 mM Hepes buffer, pH 7.5; 5.5 mg/ml protein), more than 90% of the deacylase activity was recovered. The activity was enriched 2-fold (final specific activity of 0.1 nmol/mg/min), while most of the interfering 49-phosphatase activity (>80%) was solubilized by the detergent. The procedure was also effective in removing about half of the membrane lipids.

Three 10-ml deacylase reaction mixtures were prepared using 50 mM lipid IVA substrate and 2 mg/ml of Triton X-100-extracted membrane protein under conditions otherwise similar to the standard assay. The reaction mixtures were initially incubated at 30 °C for 24 h. Then additional membranes were added to yield a final protein concentration of 3 mg/ml, and the reactions were continued for another 24 h. The progress of the reaction was monitored by thin layer chromatography, as described above for the assays, but the deacylase product was detected by charring the plates after spraying with 10% sulfuric acid in ethanol. More than 90% of the substrate was deacylated under these conditions. Prior to product isolation, the reaction mixtures were stored at −20 °C. After thawing, the reactions were distributed equally into two 150-ml Corex bottles. The reactions were diluted with water to yield a final volume of 20 ml/bottle. The proteins were precipitated by adding 1.25 ml of CHCl3, 2.5 ml of methanol, and 0.04 ml of concentrated HCl per ml of the diluted reaction mixtures. The samples were thoroughly mixed, and then centrifuged at 3,000 × g for 20 min at room temperature. The supernatant was decanted, and it was converted to a two-phase system by adding 0.263 ml of CHCl3 and 0.263 ml of water per ml of supernatant. After mixing, the phases were separated by centrifugation, as above. The CHCl3-rich lower phase was removed, and the upper phase was washed twice with fresh, preequilibrated lower phase (i.e. a lower phase generated by mixing chloroform, methanol, and 0.1 M HCl in a ratio of 2:2:1.8, v/v/v). The lower phases were pooled, 0.5 ml of pyridine was added to neutralize residual HCl, and the solvent was removed by rotary evaporation.

The residue was redissolved in ~15 ml of chloroform/pyridine/88% formic acid/water (70:60:16:3, v/v/v/v) and was loaded onto a 9.5-ml silicic acid column, equilibrated in the same solvent. The column was washed with another 20 ml of the same solvent, followed by 60 ml of chloroform/methanol (95:5, v/v). The lipid IVα-derived deacylase product was then eluted with ~20 ml of an acidic single phase Bligh and Dyer mixture, consisting of chloroform, methanol, and 0.1 M HCl (1:2:0.8, v/v/v) (38). The fractions containing the desired product were identified by thin layer chromatography, followed by charring as described.

Fig. 1. Structures of lipid A from three diverse Gram-negative bacteria and their relationship to the conserved precursor lipid IVα. A, predominant species of lipid A found in E. coli K-12 (3), R. leguminosarum (12), and P. aeruginosa (25, 26). The presence of an acyloxyacyl moiety involving the C28 acyl chain and the 3-O-deacylated forms of R. leguminosarum lipid A was discovered recently in our laboratory based on new isolation techniques (14, 15). Molecular species of R. leguminosarum and P. aeruginosa lipid A may differ by the presence or absence of a hydroxyacyl chain at position 3, as indicated by the dashed bond. B, proposed reaction catalyzed by the 3-O-deacylase of R. leguminosarum with lipid IVα as the substrate. Key hydrogen atoms used to assign the structure of the product by 1H NMR spectroscopy are labeled in this representation.
The pertinent fractions were pooled and converted to a two-phase system by the addition of 0.263 ml of CHCl₃ and 0.263 ml of water per ml. The solution was mixed, and the phases were separated by centrifugation. The lower phase was collected, and the upper phase was washed twice with preequilibrated acidic lower phase (see above). The lower phases were pooled, 5–10 ml of high pressure liquid chromatography grade pyridine was added, and the solvents were removed by rotary evaporation.

A small amount of contaminating lipid IVₐ was removed from the deacylase product by the reverse phase chromatography procedure described by Hampton et al. with minor modifications (39). The method utilizes a two-solvent system for the resolution of lipid IVₐ derivatives with octadecysilane silica gel (C18 silica). Solvent A was 50% (v/v) acetonitrile in water, and solvent B was 85% isopropyl alcohol in water. Both solvents contained 5 mM tetrabutylammonium phosphate. The dried compound was redissolved in 3 ml of a 1:1 (v/v) solvent mixture of A and B. The compound was loaded onto a 0.5-ml C18 silica column equilibrated in the same solvent ratio. The column was washed with 3 ml of the 1:1 (v/v) solvent ratio. The column was then washed with 1.5 ml of solvent consisting of a 1:2 (v/v) ratio of A to B. Finally, the column was washed with 1.5 ml of solvent consisting of 1:4 (v/v) ratio of A to B. During the chromatography, 0.5-ml fractions were collected. Fractions containing the deacylase product were identified by charring. The relevant fractions were pooled and diluted 1:1 with chloroform/methanol/water (2:3:1, v/v/v). The diluted pool was loaded directly onto a 1-ml DE52 column equilibrated in chloroform/methanol/water (2:3:1, v/v/v) to remove the tetrabutyl-ammonium phosphate. The column was then washed with 1.5 ml of solvent consisting of a 1:2 (v/v) ratio of A to B. Finally, the column was washed with 1.5 ml of solvent consisting of 1:4 (v/v) ratio of A to B. During the chromatography, 0.5-ml fractions were collected. Fractions containing the deacylase product were identified by charring. The relevant fractions were pooled and diluted 1:1 with chloroform/methanol/water (2:3:1, v/v/v). The compound was then eluted with ~10 ml of chloroform, methanol, and 480 mM ammonium acetate (2:3:1, v/v/v). The compound-containing fractions were again identified by charring and were pooled. The pooled fractions were converted to a two-phase Bligh and Dyer system by the addition of 0.167 ml of CHCl₃ and 0.283 ml of water per ml of pool. The solution was mixed, and the phases were separated by centrifugation. The lower phase was collected, and the upper phase was washed twice with preequilibrated acidic lower phase (see above). The lower phases were pooled, and the solvents were removed by rotary evaporation. The isolated deacylase product was stored at −20 °C prior to further

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**FIG. 2.** Deacylation of [4-³²P]lipid IVₐ in membranes of *R. leguminosarum* and *P. aeruginosa* but not of *E. coli* or *R. meliloti*. Membranes of the indicated strains were assayed for deacylase activity using the standard conditions. The protein concentration was 1.0 mg/ml, and the incubations were carried out for 60 min at 30 °C. The products generated from [4-³²P]lipid IVₐ were separated by thin layer chromatography and detected with a PhosphorImager. Lanes 1, no membrane control; lane 2, *E. coli* W3110; lane 3, *R. leguminosarum* biovar phaseoli CE3; lane 4, *R. leguminosarum* biovar vicieae 8401; lane 5, *R. leguminosarum* biovar trifolii 24AR; lane 6, *R. meliloti* 1021; lane 7, *P. aeruginosa* PAO1; lane 8, *P. aeruginosa* 27853.

**FIG. 3.** Deacylase activity is associated with the membrane fraction of *R. leguminosarum*. Deacylase was assayed under standard conditions with 10 μM [4-³²P]lipid IVₐ and crude extract, cytosol, or membranes as the enzyme source. Reactions were analyzed after the indicated times by thin layer chromatography and PhosphorImager analysis, as described under "Experimental Procedures".

**FIG. 4.** Time course and protein concentration dependence of the *R. leguminosarum* deacylase. A, membranes of *R. leguminosarum* 8401 were used at 1 mg/ml. The deacylase reaction was performed under standard conditions in 30 μl. At each time point, a 2-μl portion was withdrawn and analyzed by thin layer chromatography and PhosphorImager analysis. B, the deacylase reaction was performed under standard conditions in 10 μl. Membranes of *R. leguminosarum* 8401 were used as the enzyme source at the indicated protein concentrations. Reaction mixtures were incubated for 20 or 40 min at 30 °C.
analysis.

"H NMR Analysis of the Deacylase Reaction Product—The purified enzymatic reaction product (2 mg) was dissolved in 0.6 ml of CDCl₃/CD₂OD/CD₃OD (2:3:1, v/v/v). Its "H NMR spectrum was recorded on a Varian 600 Unity spectrometer using a 277.29-Hz spectral window with the 5-mm probe at 30 °C. Chemical shifts were referenced to the methyl protons of internal tetramethylsilane (0.00 ppm). A line broadening of 0.2 Hz before Fourier transformation was used to process the data. The water signal at 4.6 ppm was suppressed by presaturation (satpower = 0).

Two-dimensional "H correlation (COSY) spectra were recorded in the absolute value mode over the same spectral region used in the one-dimensional "H NMR spectrum. Four hundred time increments were collected and zero-filled to 2048 points with sine-bell weighting along both f₁ and f₂ dimensions. Three hundred twenty scans were collected per increment, and the relaxation delay was 1 s. Presaturation of the water line was also included in the pulse sequence.

RESULTS

A Novel Deacylase in Membranes of R. leguminosarum and P. aeruginosa—As shown in Fig. 2, membranes of R. leguminosarum 8401 and R. leguminosarum etli CE3 can convert ["³²P]lipid IVₐ to a more hydrophilic metabolite in the presence of 1% Triton X-100 and 20 mM CaCl₂. As will be demonstrated below, this product, designated metabolite A in Fig. 2, corresponds to a deacylated derivative of lipid IVₐ specifically lacking the 3-O-linked β-hydroxymyristoyl residue (Fig. 1B). Membranes of the nodulation-deficient mutant 24AR of R. leguminosarum biovar trifolii, which were previously shown to lack the 4'- and 1-phosphatases of the R. leguminosarum lipid A pathway (20), do contain this deacylase. A similar deacylase is also detected in cell extracts and membranes (Fig. 2) of two wild type strains of P. aeruginosa (PA10 and 27853). However, deacylation of ["³²P]lipid IVₐ is not observed in extracts or membranes (Fig. 2) of either E. coli or R. meliloti, as judged by comparison with the no enzyme control.

R. leguminosarum biovar viciae 8401 was used as the source of the enzyme in all subsequent experiments. Strain 8401 (20) lacks the pSym plasmid that carries many of the nodulation genes. All of the deacylase activity in R. leguminosarum 8401 extracts is membrane-associated (Fig. 3). Similarly, in P. aeruginosa PA01 and 27853, all of the deacylase also is membrane-bound (not shown).

Assay and Catalytic Properties of the R. leguminosarum Deacylase—Deacylation of ["³²P]lipid IVₐ by R. leguminosarum membranes proceeds in a linear fashion for up to 4 h at 30 °C with 1.0 mg/ml protein (Fig. 4A). After prolonged incubation, deacylation is nearly complete. Deacylation activity also increases with increasing membrane protein concentrations, but the effect is not linear above 0.5 mg/ml (Fig. 4B), perhaps reflecting the presence of inhibitors in R. leguminosarum membranes. Optimal deacylase activity is observed between pH 5.5 and 6.5 (not shown).

The deacylation reaction is absolutely dependent upon the presence of a nonionic detergent. Maximal activity is seen in the presence of 1% Triton X-100 at membrane protein concentrations below 2 mg/ml. Nonidet P-40 (1% also) supports deacylase activity, but Tween 20, deoxycholate, CHAPS, and dodecylmannoside are inhibitory (not shown).

The presence of EDTA or EGTA at 2 mM completely inhibits the deacylase activity, suggesting a divalent metal requirement. Accordingly, the deacylase was assayed in the presence of varying concentrations of calcium, magnesium, or manganese ions (Fig. 5). In this experiment, the concentration of EDTA was held constant at 2 mM. Among these three divalent metal ions, calcium is the most effective. Enzymatic activity increases with increasing concentrations of calcium ions up to 20 mM. Partial stimulation of the activity is also seen with magnesium or manganese chloride (Fig. 5). However, 5 mM ferrous and zinc ions completely inhibit the activity (not shown), and monovalent cations (like sodium and potassium) have no effect.

Under standardized assay conditions with R. leguminosarum 8401 membranes, partial dephosphorylation of the 4'-phosphate of lipid IVₐ by the 4'-phosphatase is observed in parallel with deacylation as judged by the appearance of ["³²P] (Fig. 2, lane 4), but the 4'-phosphatase is partially inhibited by the presence of 20 mM CaCl₂ and 1% Triton X-100. Since Kdo₂-lipid IVₐ is strongly preferred over lipid IVₐ by the 4'-phosphatase (20), lipid IVₐ was generally employed for the deacylase assay. The 1-phosphatase of the R. leguminosarum lipid A pathway (21) was inhibited completely by 20 mM CaCl₂; therefore, its activity was not apparent under the assay conditions used for the deacylase. Furthermore, the lipid product that is generated by the 4'-phosphatase (20) is not seen under these assay conditions, since it is not radioactive after the removal of the 4'-phosphate residue.

With 25 μM ["³²P]lipid IVₐ under optimized conditions, the specific activity of the deacylase in crude extracts and membrane preparations of R. leguminosarum 8401 is 0.019 and 0.045 nmol/min/mg protein, respectively. The apparent Kₘ for lipid IVₐ in this mixed micelle system (40) is estimated at 17.9 μM (Fig. 6).

Substrate Specificity of the Deacylase—As shown in Fig. 7, the deacylase utilizes lipid IVₐ and Kdo₂-lipid IVₐ at about the same rate. Unlike the 4'-phosphatase, the deacylase is not dependent upon the Kdo domain. However, the rate of deacylation of the monosaccharide precursor lipid X is about 20 times slower than that of lipid IVₐ (Fig. 7), indicating that the distal diacylglycosamine unit of lipid IVₐ somehow enhances catalytic efficiency. The biosynthetic precursor tetraacyldisaccharide-1-"³²P (3) and the analog tetraacyldisaccharide-["³²P] (prepared by acid hydrolysis of ["³²P]lipid IVₐ) are both deacylated efficiently (data not shown), indicating that both phosphates are not required for efficient turnover. Extra core sugars (mannose and galactose) (21, 24) attached to Kdo₂-lipid IVₐ do not interfere with deacylation. However, the presence of an acyloxyacyl group on the distal glycosamine residue of Kdo₂-lipid IVₐ as in lauroyl-Kdo₂-lipid IVₐ generated by HtrB (35) or in lipid A generated from compound 505 by the 4'-kinase (30), prevent deacylation (data not shown).

Chromatographic Characterization of the Deacylase Product Generated from Lipid IVₐ—To characterize the structure of the

![Fig. 5. Dependence of the deacylase reaction on divalent metal ions.](image-url)
material generated by the enzymatic deacylation of lipid IVₐ, the reaction product was subjected to mild alkaline hydrolysis. The substrate [4⁹⁻³²P]lipid IVA (not treated with enzyme) (Fig. 8) was processed in parallel (see “Experimental Procedures”). Treatment with TEA removes both ester-linked β-hydroxyacyl chains from lipid IVA. When carried out at 30 °C, TEA hydrolysis of [4⁹⁻³²P]lipid IVA proceeds via two distinct and separable intermediates (designated intermediates 1 and 2 in lanes 3 and 5 of Fig. 8). These eventually collapse to form the same limiting alkaline hydrolysis product, which lacks both ester-linked β-hydroxyacyl chains (Fig. 8, lane 7). Intermediates 1 and 2 arise by the loss of either the 3- or the 3⁹-β-hydroxyacyl chains of lipid IVA. Stronger base (NaOH) hydrolysis of lipid IVA (Fig. 8, lane 9) rapidly removes both O-linked β-hydroxyacyl chains without accumulation of intermediates 1 and 2 under the conditions employed.

The more rapidly migrating intermediate 1 derived by TEA treatment of [4⁹⁻³²P]lipid IVA (Fig. 8, lanes 3 and 5) is the same as the product made by the R. leguminosarum deacylase (Fig. 8, lane 2, metabolite A). Consequently, treatment of the deacylase reaction product with TEA (Fig. 8, lanes 4, 6, and 8) or with NaOH (Fig. 8, lane 10) results in the direct conversion of the deacylase reaction product to a compound that migrates with the limiting NaOH hydrolysis product of [4⁹⁻³²P]lipid IVₐ. This finding demonstrates that the deacylase does not remove an N-linked β-hydroxyacyl chain from lipid IVₐ, and it also shows that the deacylase removes only one specific O-linked β-hydroxyacyl chain from [4⁹⁻³²P]lipid IVₐ. Last, it can be inferred that the glucosamine disaccharide backbone structure of the deacylase reaction product is likely to be the same as that of lipid IVₐ.

¹H NMR Spectroscopy of the Deacylase Reaction Product Generated from Lipid IVₐ—A definitive assignment of the position (3 or 3⁹) that is attacked by the deacylase in lipid IVₐ cannot be made by thin layer chromatography analysis alone. Accordingly, a 2-mg sample of the deacylase reaction product was isolated and subjected to ¹H NMR spectroscopy under conditions reported previously (41).

Characterization of the purified reaction product by COSY spectroscopy established unequivocally that lipid IVₐ is deacylated by the enzyme exclusively at the 3-position. While the ¹H NMR spectrum of the substrate, lipid IVₐ (not shown), displays two downfield overlapping triplets at 5.18 ppm, which are attributed to H-3 and H-3′ of the acylated glucosamine disaccharide (42, 43), the deacylase product retains only one of these two triplets at 5.17 ppm (Fig. 9). Based on the COSY spectrum of the deacylase product, this remaining downfield triplet is assigned to an axial H-3′ (Fig. 9), indicating that the 3′-position is still acylated in the product (33, 45). However, a
the proximal glucosamine unit of lipid IV A.

The NMR spectrum also revealed the ester-linked D-glucosamine H-3 of the proximal glucosamine residue in the product composition of Kdo 2-lipid IVA as well as from the precursors lipid IVA and lipid X (Figs. 1, 7, and 9). This membrane-associated deacylase specific to E. coli extracts that convert Kdo 2-lipid IVA (20–24), was observed in the product (Fig. 2). We have now identified a novel deacylase specific to R. leguminosarum (Fig. 1). We have now identified a novel deacylase specific to R. leguminosarum membranes that selectively removes the O-linked β-hydroxyacyl chain from the 3-position of lipid IV A, as well as from the precursors lipid IV A and lipid X (Figs. 1, 7, and 9). This membrane-associated deacylase is present in extracts of all R. leguminosarum strains tested so far, but not of E. coli or R. meliloti (Fig. 2).

Although the deacylase is not dependent upon the Kdo domain of Kdo 2-lipid IV A for activity, it displays a strong kinetic preference for substrates containing a distal diacylglycerol glucosamine unit (Fig. 7). However, the enzyme selectively cleaves only the β-hydroxyacylmyristoyl moiety attached to position 3 of the proximal glucosamine moiety (Figs. 1 and 9). 1H NMR spectroscopy (Fig. 9) provided unequivocal evidence for the specificity of the enzyme, as demonstrated by the 1.5-ppm upfield shift of the H-3 of the proximal glucosamine residue in the product compared with the substrate. The NMR spectrum also revealed that the proton chemical shifts of the distal glucosamine unit of the product were virtually unchanged relative to those of lipid IV A (43).

The deacylase does not require the presence of a phosphate group at either the 1- or the 4-position for activity, and the attachment of the inner core sugars (mannose and galactose) to Kdo 2-lipid IV A does not interfere with the deacylation reaction (data not shown). Interestingly, the enzyme does not deacylate lauroyl-Kdo 2-lipid IV A (in which an acyloxyacyl group is present on the N-linked hydroxymyristate group of the distal glucosamine unit) (35). These findings suggest that the deacylase functions after lipid A disaccharide formation but before the Kdo-dependent acylation of the distal unit to generate an acyloxyacyl residue (3). The observation that the deacylase strongly prefers glucosamine disaccharides as substrates (Fig. 7) is reasonable given that the disaccharide synthase of E. coli (34) is highly specific for diacylated monosaccharide precursors, like lipid X. If the deacylase were to catalyze rapid cleavage of these monosaccharide precursors, it would interfere with the functioning of the disaccharide synthase.

Like many of the lipases that deacylate glycerophospholipids (46), the R. leguminosarum deacylase requires Triton X-100 and divalent metal ions for activity (Fig. 5). Whether or not the R. leguminosarum deacylase also utilizes glycerophospholipids as substrates can only be assessed once the enzyme is purified on the relevant carbohydrate proton resonances is shown along the edges. Proton cross-peak assignments are indicated, and the atom labeling of the glucosamine disaccharide protons follows the numbering scheme shown in Fig. 1B. The distal glucosamine H-3 chemical shifts of the deacylase reaction product are nearly the same as those in the lipid IV A substrate (43). The most pronounced difference between lipid IV A and the deacylation reaction product is seen with H-3 of the proximal glucosamine residue, which is shifted upfield by 1.5 ppm relative to H-3 of lipid IV A (43) because of the loss of the acyl chain.

FIG. 8. Mild alkaline hydrolysis and thin layer analysis of [4-32P]lipid IV A and of the deacylase reaction product. As described under "Experimental Procedures," two incubations (designated 1 and 2) were set up using the standard optimized assay conditions with 10 μM [4-32P]lipid IV A as the substrate. Incubation 1 contained no enzyme, while incubation 2 contained 1 mg/ml R. leguminosarum 8401 membranes. After 16 h at 30 °C, portions of these incubations were treated with TEA or 0.1 M NaOH for the times indicated in the figure, after which a portion of each treated sample was analyzed by thin layer chromatography and PhosphorImager analysis.

FIG. 9. Partial COSY spectrum of the deacylase reaction product generated from lipid IV A. The one-dimensional spectrum in the region of the relevant carbohydrate proton resonances is shown along the edges. Proton cross-peak assignments are indicated, and the atom labeling of the glucosamine disaccharide protons follows the numbering scheme shown in Fig. 1B. The distal glucosamine H-3 chemical shifts of the deacylase reaction product are nearly the same as those in the lipid IV A substrate (43). The most pronounced difference between lipid IV A and the deacylase reaction product is seen with H-3 of the proximal glucosamine residue, which is shifted upfield by 1.5 ppm relative to H-3 of lipid IV A (43) because of the loss of the acyl chain.

DISCUSSION

In previous studies, we described six enzymes unique to R. leguminosarum extracts that convert Kdo 2-lipid IV A (20–24), an intermediate made both by E. coli and R. leguminosarum, to novel compounds that are precursors of the unusual lipid A of R. leguminosarum (Fig. 1). We have now identified a novel deacylase specific to R. leguminosarum membranes that selectively removes the O-linked β-hydroxyacyl chain from the 3-position of Kdo 2-lipid IV A, as well as from the precursors lipid IV A and lipid X (Figs. 1, 7, and 9). This membrane-associated deacylase is present in extracts of all R. leguminosarum strains tested so far, but not of E. coli or R. meliloti (Fig. 2).

Although the deacylase is not dependent upon the Kdo domain of Kdo 2-lipid IV A for activity, it displays a strong kinetic preference for substrates containing a distal diacylglycerol glucosamine unit (Fig. 7). However, the enzyme selectively cleaves only the β-hydroxyacylmyristoyl moiety attached to position 3 of the proximal glucosamine moiety (Figs. 1 and 9). 1H NMR spectroscopy (Fig. 9) provided unequivocal evidence for the specificity of the enzyme, as demonstrated by the 1.5-ppm upfield shift of the H-3 of the proximal glucosamine residue in the product compared with the substrate. The NMR spectrum also revealed
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A-like molecules have been reported in eucaryotic systems, these appear to be distinct from the *R. leguminosarum* deacylase. For instance, the acyloxyacyl hydrolase of human leukocytes (47) removes the secondary acyl chains from the lipid A residue of intact lipopolysaccharide, thereby reducing the immunostimulatory activity and the toxicity of the lipid A moiety. Since the *R. leguminosarum* deacylase does not attack Kdo-

(lauroyl)-[4',32P]lipid IV A, it is obviously not an acyloxyacyl hydrolase (data not shown). Rosner et al. (48) and Verret et al. (49, 50) identified two distinct amidases in extracts of the slime mold, *Dictyostelium discoideum*, an organism that is likely to scavenge *E. coli* in nature. These enzymes remove the two amide-linked β-hydroxymyristoyl residues of lipid A, but only after complete O-deacylation by prior base treatment. Amidase I of *D. discoideum* is inhibited by chitobiose and N-acetylglucosamine 1-phosphate (49, 50). The *R. leguminosarum* deacylase does not cleave either of the amide-linked acyl chains (Fig. 8), and it is not affected by the above compounds up to 1.0 mM (data not shown). The specificity of our deacylase is thus completely different from those of the previously reported lipid A hydrolases (49, 50). Finally, Drozanski et al. (51) reported deacylation of lipopolysaccharide in *Dictyostelium discoideum* in a manner that is reminiscent of insects infected with bacteria or fungi (44). The unusual lipid A of *R. leguminosarum* might therefore help bacteroids evade the innate immune response of plants during symbiosis in root cells, while still allowing the plant to defend itself against Gram-negative pathogens containing the more typical, phosphorylated lipid A disaccharide. Isolation of *R. leguminosarum* mutants that specifically lack the 3-O-deacylase and the other unique enzymes recently identified in our laboratory (20, 21, 24) will be required to validate this hypothesis.

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21. R. leguminosarum* lipid A and its precursors isolated from diverse Gram-negative bacteria suggest that enzymatic cleavage of 3-O-linked β-hydroxyacyl chains may actually be a widespread phenomenon (52, 53).

Many workers have confirmed the importance of the structure and composition of the acyl chains attached to lipid A for biological activity in the stimulation of mammalian immune cells (1, 3, 4, 9). The deacylase of *R. leguminosarum* may provide a new tool for the selective modification and preparation of interesting lipid A analogs. Given the structure-activity relationships of known lipid A derivatives, one would expect that many of the unusual chemical features of *R. leguminosarum* lipid A (Fig. 1A), including the partial 3-O-deacylation, would reduce immune stimulation in animal systems (4, 9).

The distinctive structure of *R. leguminosarum* lipid A and its possible lack of immunostimulatory activity might also play a role the establishment of symbiosis in plants (17, 18). Although not yet characterized in terms of their ability to respond to lipid A, some plants have recently been shown to possess systems of innate immunity (54–56) and to synthesize antibacterial peptides in a manner that is reminiscent of insects infected with bacteria or fungi (44). The unusual lipid A of *R. leguminosarum* might therefore help bacteroids evade the innate immune response of plants during symbiosis in root cells, while still allowing the plant to defend itself against Gram-negative pathogens containing the more typical, phosphorylated lipid A disaccharide. Isolation of *R. leguminosarum* mutants that specifically lack the 3-O-deacylase and the other unique enzymes...
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