A Mono-functional 3-Deoxy-d-manno-octulosonic Acid (Kdo) Transferase and a Kdo Kinase in Extracts of Haemophilus influenzae*

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Lipopolysaccharide of Haemophilus influenzae contains a single 3-deoxy-d-manno-octulosonic acid (Kdo) residue, linked to the 6′ position of lipid A. In Escherichia coli and related organisms, a Kdo disaccharide is attached to lipid A. In previous studies, we cloned the gene (kdtA) encoding the E. coli Kdo transferase and demonstrated that homogeneous preparations of KdtA polypeptide catalyzed the attachment of both Kdo groups to the precursor, lipid IV A. E. coli KdtA produced only traces of mono-glycosylated product. We now show that a single Kdo is transferred to lipid IV α in extracts of H. influenzae. The mono-functional Kdo transferase of H. influenzae is membrane-bound, and the reaction is dependent upon a CMP-Kdo-generating system, as in E. coli. The specific activity of Kdo transfer to lipid IV α is 0.5–1 nmol/min/mg in H. influenzae membranes. Utilizing solubilized H. influenzae membranes, milligram quantities of Kdo-lipid IV α were prepared for analysis. Matrix-assisted laser desorption/ionization mass spectrometry revealed a parent ion (M + H) + at m/z 1626.0, consistent with the addition of a single Kdo moiety. Like lipid IV A, Kdo-lipid IV α was an excellent substrate for the bi-functional Kdo transferase of E. coli. In membranes of H. influenzae, but not E. coli, Kdo-lipid IV α was further phosphorylated in the presence of ATP, yielding a mono-phosphorylated Kdo-lipid IV α, with a parent ion (M + H) + at m/z 1703.9. The identification of the mono-functional H. influenzae Kdo transferase, which is encoded by a KdtA homologue that displays 50% identity to its E. coli counterpart, should facilitate the mechanistic dissection of more complex multi-functional Kdo transferases, like those of E. coli and Chlamydia trachomatis.

Haemophilus influenzae is a non-enteric pathogenic Gram-negative bacterium found in the human respiratory tract. Certain strains of H. influenzae are known to cause diseases such as bacterial meningitis, otitis media, and upper respiratory infections. One critical factor modulating the pathogenicity of H. influenzae is the lipopolysaccharide (LPS) of its outer membrane (1). LPS is a major outer membrane glycolipid found in almost all Gram-negative bacteria (2–4), and portions of the LPS molecule are required for viability and virulence. LPS is anchored into the outer membrane by the lipid A moiety, a unique acylated disaccharide of glucosamine (Fig. 1). The eight-carbon sugar 3-deoxy-d-manno-octulosonic acid (Kdo), another conserved component of LPS, is linked to the 6′ position of lipid A (Fig. 1). The 5-OH of the inner Kdo is the point of attachment of additional core sugars (data not shown) in wild-type cells. In Escherichia coli and most other Gram-negative bacteria, Kdo and lipid A are both required for growth under laboratory conditions (Fig. 1) (3–6), whereas the many other sugars of the core domain and the O-antigen are not. The key roles of Kdo and lipid A for bacterial viability are supported by the observation that inhibitors of Kdo and lipid A biosynthesis possess anti-bacterial activity (7–9). Furthermore, mutants defective in Kdo and lipid A formation must be isolated as conditional lethal mutants (10–12).

The enzyme responsible for Kdo transfer in E. coli is an unusual bi-functional glycosyl transferase capable of adding two Kdo residues with distinct glycosidic linkages to lipid IV α, a key precursor of lipid A (Fig. 2). The gene encoding the Kdo transferase (kdtA) of E. coli has been cloned (13). The KdtA protein has been purified to homogeneity and utilized to confirm that the addition of the two Kdo moieties is catalyzed by a single polypeptide of 425 amino acids (14). Chlamydia trachomatis possesses a more complex Kdo transferase, encoded by the gseA gene (15). The chlamydial transferase is capable of catalyzing at least three Kdo additions (15). GseA consists of 401 amino acids. Sequence comparisons of E. coli and C. trachomatis demonstrate that the C. trachomatis enzyme is 23% identical and 66% similar to the E. coli enzyme (15). Given the lengths of the polypeptides, the presence of additional catalytic domains cannot account for the multiple glycosylations catalyzed by the C. trachomatis Kdo transferase. Interestingly, the gseA gene can support the growth of an E. coli mutant in which kdtA is disrupted (16).

The Kdo region of H. influenzae LPS is distinct from that of most other characterized Gram-negative bacteria. Structural studies by several laboratories have established that the LPS of H. influenzae contains only a single Kdo (17–19). In addition, the sole Kdo of H. influenzae is phosphorylated at the 4-OH position that is occupied by the second Kdo in E. coli LPS (Fig. 1) (18). These observations raise the interesting question of whether a minimal LPS, consisting of lipid A and a single Kdo, would be sufficient to support the growth of all Gram-negative bacteria. Alternatively, a single Kdo substituted with a phosphate might be necessary, given that the negative charge of the phosphate would be in approximately the same place as that of the carboxylate of the outer Kdo normally present in
Kdo-lipid IV A was incubated with functional Kdo transferases of a mono-functional Kdo transferase, distinct from the multi-functionality of the Kdo region of H. influenzae evidence for the unique enzymes that participate in the assembly shown in Fig. 2. Our studies provide the first direct evidence for the unique enzymes that participate in the assembly shown in Fig. 2. Our studies provide the first direct evidence for the assembly of lipid A and Kdo in E. coli (6) are also present in H. influenzae (39). A myristate residue in H. influenzae substitutes for the laurate found in E. coli (19, 40). X represents partial substitution with a phosphate, whereas Y indicates partial substitution with phosphoethanolamine (38). The enzymes that incorporate X and Y are not known. The enzymes responsible for the formation of the distinct phosphorylated Kdo moiety of H. influenzae are described in the present study.

**E. coli LPS**

The implication of the structural analysis of H. influenzae LPS is that this bacterium should contain a mono-functional Kdo transferase. In addition, the presence of phosphate on the 4-OH position of the Kdo in H. influenzae LPS suggests the presence of a novel Kdo kinase (Fig. 2). The enzymatic transfer of Kdo to lipid A precursors and the possibility of Kdo phosphorylation have not been investigated previously in H. influenzae. The structural characterizations of H. influenzae LPS, although convincing, have been performed using degradation products of isolated LPS, not with intact natural products or enzymatically synthesized precursors.

In the present work, we have used extracts of non-typeable strain 722 of H. influenzae (20) to demonstrate the existence of a mono-functional Kdo transferase, distinct from the multifunctional Kdo transferases of E. coli or C. trachomatis. When Kdo-lipid IV A was incubated with H. influenzae membranes and ATP, an additional novel mono-phosphorylated derivative was generated. Mass spectrometry of both Kdo-lipid IV A and its mono-phosphorylated derivative support the proposed reaction scheme shown in Fig. 2. Our studies provide the first direct evidence for the unique enzymes that participate in the assembly of the Kdo region of H. influenzae LPS.
extract was further fractionated into cytosolic and membrane components by centrifugation at 150,000 $\times g$ for 60 min at 4 °C. The supernatant (cytosol) was centrifuged again to remove any remaining membrane contaminants. The membrane pellet was resuspended in ~25 ml of 30 mM HEPES, pH 7.5, with 1 mM EDTA and 1 mM EGTA, and also centrifuged at 100,000 $\times g$ to produce a washed membrane fraction. Both membrane fractions were prepared in the same manner. All membrane suspensions were stored frozen in aliquots at ~8 °C.

Protein concentrations were determined using the bicinchoninic assay (Fierce) with bovine serum albumin as the standard (24).

**Preparation and Isolation of Substrates—**Large scale preparations of lipid IV A (10–100 mg quantities) were performed as described previously (25). Prior to use in the large scale product isolations, the lipid IV A was subjected to reverse phase chromatography as described by Hampton et al. (26). [4-32P]Lipid IV A was prepared enzymatically using *E. coli* BR7 membranes as the source of 4'-kinase, [3-32P]ATP, and tetraacyl disaccharide 1-phosphate as the substrate, as described by Brozek et al. (22). All lipid substrates were dispersed by sonic irradiation for approximately 1 min in a bath prior to use. Recombinant *E. coli* CMP-Kdo synthase was partially purified as described by Brozek et al. (27) from JM103/pTJB201.2, kindly provided by Dr. R. Goldman of Abbott Laboratories. Recombinant *E. coli* CMP-Kdo transferase was purified as described previously (14).

**Assay for Kdo Transferase to Lipid IV A**—The transfer of Kdo to the acceptor was measured by the method of Nierhaus et al. (28) with minor modifications. Reaction mixtures (10–20 μl) contained 50 mM Hepes, pH 7.5, 2 mM Kdo, 0.1% Triton X-100, 100 μM [4-32P]Lipid IV A, (3,000–6,000 cpm/nmol), 5 mM CTP, 10 mM MgCl2, and 1.8 milliunits of partially purified CMP-Kdo synthase. Standard assays were initiated by addition of enzyme, usually *H. influenzae* extracts or membranes, as indicated, and incubated at 30 °C. The reactions were terminated by spotting 5 μl of the mixtures onto a thin layer silica plate. The plate was then air-dried and developed in chloroform/pyridine/88% formic acid/H2O (30:70:16:10, v/v). The solvent was evaporated with a hot air stream, and the plate was exposed to a PhosphorImager screen for 12–16 h. The extent of conversion of [4-32P]lipid IV A (typically 2 ml of a 15 mg/ml suspension) were thawed and solubilized immediately as the enzyme source for the large scale product preparation (13, 14).

**Membrane Solubilization Conditions—** *H. influenzae* extracts was monitored by centrifugation at 150,000 $\times g$ for 20 min at room temperature to remove protease inhibitors. The supernatant was transferred to one or more fresh Corex tubes. Next, 0.263 ml of CHCl3 and 0.263 ml of H2O were added per ml to convert the sample to a two-phase system. The phases were separated by centrifugation, as described above. The CHCl3 lower phase was removed, and the upper phase was washed twice with 10 ml of a fresh, pre-equilibrated neutral lower phase (i.e. a lower phase generated by mixing chloroform/methanol/H2O, 2:2:1.8, v/v) (28). The lower phases containing the lipid product (~170 ml) were pooled, 0.5 ml pyridine was added, and the solvents were removed by rotary evaporation. The residue was redissolved in ~20 ml of the solvent mixture chloroform/pyridine/58% formic acid/H2O/methanol (50:60:15:3:2.5, v/v), and loaded onto a 9-ml silicic acid column equilibrated in the same solvent. The column was washed with 25 ml of the same solvent mixture used to load the column, followed by 60 ml of chloroform/methanol (95:5, v/v). The desired compound was eluted with ~30 ml of neutral Bligh-Dyer single phase (chloroform/methanol/H2O, 2:1:0.8, v/v) (28). Fractions (~19 ml) containing Kdo-lipid IV A were identified by spotting 5 μl of each fraction onto a silicic acid thin layer chromatography plate, which was developed using the same solvent system as described above for the Kdo transferase assay. The presence of the Kdo-lipid IV A was confirmed by washing with chloroform/methanol, ether, and methanol, and staining with 0.1% toluidine blue O. The lower phases were pooled and divided into two-phase Bligh-Dyer system by the addition of 0.263 ml of CHCl3 and 0.263 ml of H2O/ml of pool. The mixture was agitated vigorously and centrifuged at 1,000 $\times g$ for 20 min at room temperature. The CHCl3-rich lower phase was removed, and the upper phase was washed twice with neutral lower phase (prepared in Bligh-Dyer proportions with H2O). The lower phases were pooled and 5–10 μl of HPLC-grade pyridine was added. The solvents were removed by rotary evaporation, and the Kdo-lipid IV A was further dried by lyophilization.

The reaction mixture for generating phosphorylated Kdo-lipid IV A (metabolite B) contained the same components as that for making Kdo-lipid IV A with the addition of 5 mM ATP. The reaction was incubated at 37 °C for 30 min. Metabolite B was isolated exactly as described above for the Kdo-lipid IV A.

**Mass Spectrometry—** Matrix-assisted laser desorption/ionization (MALDI) mass spectra were acquired on a Kompact III time-of-flight (TOF) mass spectrometer (Kraus Analytical, Manchester, UK) in a linear mode. Gentisic acid (Aldrich) was used as a matrix. Analyte ions were generated from the matrix by irradiation with a 297-nm pulsed nitrogen laser. Each spectrum was an average of 50 scans.

Liquid secondary ion mass spectra (LSIMS) were acquired on a Concept II (Kraus Analytical, Manchester, UK) two-sector (BE geometry) mass spectrometer at a resolution of 1000, 1-μl aliquot of sample solution in methanol/chloroform (1:2) was mixed with the mono-thioglycerol or triethanolamine (Aldrich) on the tip of the probe. Analyte ions were desorbed from the matrix by an 8-kV Ca secondary ion beam. Mass spectra were acquired by scanning the magnet in the 100–2500-atomic mass unit range at a scan rate of 10 s/decade. Normally 10–20 scans were signal-averaged for each spectrum.

**RESULTS**

**Identification of a Kdo Transferase and a Putative Kdo Kinase in Extracts of *H. influenzae*—** The presence of a Kdo transferase activity in *H. influenzae* was determined by assaying extracts under conditions previously identified to identify the *E. coli* Kdo transferase (13, 14, 27). Under these conditions, CMP-Kdo is generated in situ and the transfer of Kdo to [4-32P]Lipid IV A is detected by thin layer chromatography and PhosphorImager analysis. As shown in Fig. 3, the *H. influenzae* extracts are capable of converting [4-32P]Lipid IV A to two new, more slowly migrating metabolites. Metabolite B is the predominant product (5.3% conversion after 20 min in Fig. 3), and, as shown below, it corresponds to the addition of a single Kdo residue to
Transfer of a Single Kdo to Lipid A Precursors in Haemophilus

**Fig. 3.** Conversion of [4'-32P]lipid IVₐ to two more hydrophilic metabolites by *H. influenzae* extracts. Each reaction was initiated by the addition of 0.5 mg/ml (final concentration) of *H. influenzae* or *E. coli* BR7 crude extract. The conditions were those described for the Kdo transferase assay under “Experimental Procedures.” The reaction was terminated after 20 min by spotting a 5-µl portion of the reaction mixture onto a thin layer chromatography plate. Lane 1 is a no enzyme control. Lanes 2 and 4 show the products formed in the complete reaction mixture containing all the components needed for a Kdo transferase. Lanes 3 and 5 show the effects of the inclusion of 1 mM ATP in the reaction mixture.

lipid IVₐ. Metabolite A, which is not generated in significant amounts in *E. coli* extracts, migrates faster than the Kdo₂-lipid IVₐ formed by the *E. coli* Kdo transferase under the same conditions (Fig. 3, lane 4), supporting the view that the Kdo transferase of *H. influenzae* may be mono-functional, or at least displays significantly different kinetics for each Kdo transferase than does the *E. coli* enzyme.

A small amount of a second hydrophilic derivative of [4'-32P]lipid IVₐ, designated metabolite B, was also formed (Fig. 3) in *H. influenzae* extracts. Metabolite B migrates with Kdo₂-lipid IVₐ and *a priori*, it could correspond to the formation of a doubly glycosylated [4'-32P]lipid IVₐ. Given previous characterizations of the structure of the Kdo region of *H. influenzae* LPS (17–19), however, a more plausible explanation for metabolite B would be the incorporation of a phosphate moiety, which might have a similar effect on the Rf as the addition of a second Kdo residue. When ATP was included in the reaction mixture (Fig. 3, lane 3), a significant increase in the formation of metabolite B was observed. The formation of a some metabolite B without the addition of exogenous ATP could be attributed to residual ATP present in the crude extracts employed as the source of enzyme or to the CTP-dependent conversion of ADP to ATP by nucleoside diphosphate kinase.

**Fig. 4.** Subcellular fractionation of the Kdo transferase and the putative Kdo kinase of *H. influenzae*. The *H. influenzae* enzymes were localized to either the cytosolic fraction or the membranes. Lanes 1–3 demonstrate the localization of the Kdo transferase. The assays were performed under the conditions described for the Kdo transferase. Each reaction mixture contained 100 µM [4'-32P]lipid IVₐ (6,000 cpm/nmol) and was incubated for 15 min at 30 °C. Lane 1 is a no enzyme control. The reaction in lane 2 contains membranes (0.5 mg/ml), and the reaction in lane 3 contains cytosol (0.5 mg/ml). Lanes 4–6 demonstrate the localization of the putative Kdo kinase. These assays were performed under the conditions described for the assay of the kinase activity. Each reaction contained 100 µM Kdo-[4'-32P]lipid IVₐ (3,000 cpm/nmol) and was incubated for 15 min at 30 °C. Lane 4 shows a control containing no enzyme. Lane 5 demonstrates the kinase activity associated with the membranes (0.05 mg/ml), and lane 6 shows the activity of the cytosol (0.05 mg/ml).

Crucial to the identification of metabolite A as the product of a Kdo transferase was the determination of the dependence of the reaction on various assay components. The effect of removing the substrates necessary for the generation of CMP-Kdo (Kdo and CTP) was examined by determining the amount of Kdo-lipid IVₐ (metabolite A) formed from [4'-32P]lipid IVₐ, and the reaction in lane 3 contains cytosol (0.5 mg/ml).

**Isolation of Metabolite A and Demonstration of Its Competence as a Substrate for the E. coli Kdo Transferase**—The isolation of metabolite A (presumably Kdo-[4'-32P]lipid IVₐ) by thin layer chromatography facilitated its further characterization (see “Experimental Procedures”). If metabolite A did indeed correspond to Kdo-lipid IVₐ, it should be chemically competent as substrate for the purified *E. coli* Kdo transferase (14). As shown in Fig. 7 (lane 7), the conversion of metabolite A to a substance migrating with Kdo₂-[4'-32P]lipid IVₐ by the purified *E. coli* Kdo transferase was rapid and efficient. Alternatively, when optimum of 7.5 in Heps buffer (data not shown). Using these conditions, the formation of the putative Kdo-lipid IVₐ (metabolite A) by membranes was linearly dependent upon protein concentration (Fig. 5, panel A) and time (Fig. 5, panel B). Furthermore, the conversion of the substrate (100 µM [4'-32P]lipid IVₐ) to metabolite A was very efficient (85%) with the addition of excess enzyme (data not shown). The specific activity of the Kdo transferase in washed membranes under these assay conditions is 0.5–1.0 nmol/min/mg.
isolated metabolite A (Kdo-[4'-'32P]lipid IV$_A$) was incubated under the same Kdo transferase conditions with _H. influenzae_ membranes (Fig. 7, lane 2), no significant further products were formed, supporting the view that the Kdo transferase present in _H. influenzae_ membranes is monofunctional. If ATP was also added under these conditions, however, extensive conversion of metabolite A to metabolite B was catalyzed by the _H. influenzae_ membranes (Fig. 7, lane 3), as observed when [4'-32P]lipid IV$_A$ was utilized as the substrate (Fig. 7, lane 6). The dependence of metabolite B formation upon ATP addition (Fig. 7, lanes 3 and 6) by the _H. influenzae_ system supports the view that this product corresponds to a phosphorylated Kdo-[4'-32P]lipid IV$_A$ derivative, not to Kdo$_2$-[4'-32P]lipid IV$_A$. As expected (see below), the CMP-Kdo-generating system is not required for the conversion of metabolite A to metabolite B.

**Mass Spectrometry of Kdo-Lipid IV$_A$—** Positive identification of metabolite A as Kdo-lipid IV$_A$ was accomplished by mass spectral analysis with MALDI-TOF mass spectrometry and LSIMS. The MALDI-TOF mass spectrum in the negative mode (Fig. 8, panel A) revealed a prominent peak at m/z 1626.0 (calculated molecular weight for (M–H)– anion of Kdo-lipid IV$_A$ is 1624.9 atomic mass units). Higher mass peaks at m/z 1648.4 and 1667.6 are interpreted as (M–2H + Na)$^–$ (calculated molecular weight 1646.9 atomic mass units) and (M–3H + 2Na)$^–$ (calculated molecular weight 1668.9 atomic mass units), respectively. Additional smaller peaks at m/z 1405 and 1427 probably correspond to the loss of a Kdo unit (Y$_2$– fragment ions, following the nomenclature by Costella and Vath (Ref. 29)). Desorption by LSIMS results in more facile fragmentation of analyte ions (Fig. 8, panel B), thus providing more structural information and reinforcing the conclusion that metabolite A is indeed Kdo-lipid IV$_A$. The major high mass fragment ions are those at m/z 1404.2 and 1425.8, representing the lipid IV$_A$ moiety of Kdo-lipid IV$_A$ (calculated molecular masses for Y$_2$– and (Y$_2$–H + Na)$^–$ fragments of Kdo-lipid IV$_A$ are 1403.8 and 1425.8, respectively). De-O-acylation of these fragment ions gives rise to peaks at m/z 1177.9 and 1200.0 (calculated molecular masses of (Y$_2$–C$_{14}$OH)$^-$ and (Y$_2$–C$_{14}$OH–H + Na)$^-$ are 1177.7 and 1199.7, respectively). The reducing end unit of lipid A or its disaccharide precursors is usually identified by a characteristic series of negative fragment ions 1,5X$_1$–, Y$_1$–, Y$_1$–H$_2$, and Z$_1$– (30). These fragment ion peaks are very prominent in the LSIMS spectra of metabolite A, and their masses match perfectly those calculated for 1,5X$_1$–, Y$_1$–, Y$_1$–H$_2$, and Z$_1$– fragments of Kdo-lipid IV$_A$ (m/z 738.4, 710.4, 708.4, and 692.4, respectively). The mass spectrometry data strongly support the conclusion that metabolite A has the composition of Kdo-lipid IV$_A$.

In addition, we have recently confirmed the presence of a single Kdo in metabolite A by $^1$H NMR spectroscopy of the intact compound dissolved in chloroform/methanol (2:1, v/v).$^2$

**Subcellular Localization and Characterization of the Kdo-Lipid IV$_A$ Kinase of _H. influenzae_—** To obtain further evidence for an enzyme capable of phosphorylating Kdo-lipid IV$_A$ to form metabolite B in _H. influenzae_ extracts, several additional studies were carried out. Subcellular localization of the putative kinase, using Kdo-[4'-32P]lipid IV$_A$ as the substrate, showed that the activity was predominantly in the membrane fraction (75–89% of the total activity), as was the Kdo transferase (Fig. 4, lanes 4–6). Accordingly, washed membranes were used in

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$^2$G. Dotson, K. A. White, and C. R. H. Raetz, manuscript in preparation.
As with the Kdo transferase, the phosphorylation of Kdo-[4-32P]lipid IVₐ was dependent upon the presence of Triton X-100 (0.1%, w/v), and it had a pH optimum of 7.5 (data not shown). Fig. 9 shows that the formation of metabolite B from Kdo-[4-32P]lipid IVₐ was linearly dependent upon protein concentration (Fig. 9, panel A) and time (Fig. 9, panel B). The rate of Kdo-[4-32P]lipid IVₐ conversion to metabolite B (30 nmol/min/mg) was notably faster than was the transfer of Kdo to lipid IVₐ (27).

To provide additional evidence that metabolite B is a phosphorylated derivative of Kdo-[4-32P]lipid IVₐ and is not Kdo-lipid IVₐ, the dependence of metabolite B formation from Kdo-[4-32P]lipid IVₐ on the components of a Kdo-generating system and/or ATP was investigated (Fig. 10). As seen by comparison of lanes 2, 3, and 6 in Fig. 10, the formation of metabolite B is clearly independent of the Kdo-generating system but is supported by the addition of ATP alone. CTP appears to be a poor substitute for ATP (lanes 2 and 4 versus lanes 3 and 6).

Since the formation of the phosphorylated Kdo-[4-32P]lipid IVₐ by H. influenzae membranes was dependent upon the addition of ATP, the nucleotide specificity of the reaction was probed in more detail. The results are summarized in Table I. ATP is the preferred phosphate donor, with 23.7% of the Kdo-[4-32P]lipid IVₐ being converted to the phosphorylated product in 10 min under standard conditions. GTP can also support the reaction, but to a lesser extent (9.33% conversion of substrate). Only a small amount of metabolite B is formed with CTP (1.80%) or UTP (2.50%) as phosphate donors. The enzyme responsible for the conversion of Kdo-[4-32P]lipid IVₐ to metabolite B is unique to H. influenzae as evidenced by the fact that E. coli membranes (isolated from strain BR7) were incapable of catalyzing the reaction (Table I).

In a separate set of experiments (data not shown), nonradioactive Kdo-lipid IVₐ was used as the acceptor and [γ-32P]ATP was employed as the donor. In this manner, we demonstrated that the 32P of [γ-32P]ATP is indeed transferred to metabolite B in a manner that is absolutely dependent upon the addition of Kdo-lipid IVₐ to the system.

**Mass Spectrometry of Phosphorylated Kdo-Lipid IVₐ**—Further evidence that metabolite B is indeed phosphorylated Kdo-lipid IVₐ was provided by the mass spectral analysis (Fig. 11) of the compound. In the MALDI-TOF spectrum (panel A) of metabolite B, three prominent high mass molecular ions were observed. The peaks were located at m/z 1704.9, 1726.9, and 1748.9 atomic mass units, respectively. The presence of a rather abundant Y₁ fragment (29) ion peak at m/z 1405.9 in the MALDI-TOF spectrum of the compound indicates that the additional phosphate group in metabolite B is confined to the Kdo unit. Three high mass molecular anions were also observed in the LSIMS spectrum of metabolite B (Fig. 11, panel B). The peaks were located at m/z 1725.5, 1747.4, and 1769.4, corresponding to (M – H)⁻, (M – 2H + Na)⁻, and (M – 3H + 2Na)⁻ ions of phosphorylated Kdo-lipid IVₐ (calculated masses 1704.9, 1726.9, and 1748.9 atomic mass units, respectively). Since the formation of the phosphorylated Kdo-[4-32P]lipid IVₐ by H. influenzae membranes was dependent upon the addition of ATP, the nucleotide specificity of the reaction was probed in more detail. The results are summarized in Table I. ATP is the preferred phosphate donor, with 23.7% of the Kdo-[4-32P]lipid IVₐ being converted to the phosphorylated product in 10 min under standard conditions. GTP can also support the reaction, but to a lesser extent (9.33% conversion of substrate).
transferase, all of the substrate was converted to Kdo\textsubscript{2}-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} (plot 1) under the conditions employed. When an equal concentration of Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} was substituted in these assays (plot 12, lanes 3 and 4), the E. coli enzyme was also able to convert a significant portion of the Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} to Kdo\textsubscript{2}-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A}, although possibly at a slower rate than when [\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} was used. Under the same assay conditions, when phosphorylated Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} (plot 12, lanes 5 and 6) was incubated with the purified E. coli enzyme, no further products were obtained.

**DISCUSSION**

In the present study, we have demonstrated for the first time the existence of a Kdo transferase in H. influenzae that is strictly mono-functional, i.e. is capable of adding only a single Kdo residue to an acceptor lipid. All previously characterized Kdo transferases, such as those of Salmonella, E. coli, Rhizobium leguminosarum, and C. trachomatis, catalyze two or more Kdo additions (4, 14, 15, 31). The demonstration of the mono-functional activity of the Haemophilus Kdo transferase argues against any alteration or hydrolytic trimming of a more extensively glycosylated intermediate (such as Kdo\textsubscript{2}-lipid IV\textsubscript{A}) as the explanation for the presence of only one Kdo residue in the lipopolysaccharide of organisms like H. influenzae (17–19), Bordatella pertussis (32–34), Bacteroides (35), and Vibrio cholerae (33, 36, 37).

In addition to having a distinct Kdo transferase, extracts of H. influenzae possess a novel Kdo kinase, which is not detectable in E. coli extracts (Table I). The existence of a Kdo kinase was anticipated by previous structural studies of H. influenzae lipopolysaccharide, in which the single Kdo found in this material was shown to be phosphorylated at the 4-OH group (17, 18, 38). Indeed, the phosphate might serve a functional role analogous to the second Kdo residue of E. coli lipopolysaccharide, since the negative charge imparted by the phosphate could be located in approximately the same place as that of the carboxylate of the outer Kdo in E. coli.

The further characterization of both the Kdo transferase and the Kdo kinase of H. influenzae would be facilitated by the cloning and overexpression of the respective genes and the purification of both proteins. The gene encoding the kinase has not yet been identified. However, the completion of the Haemophilus genome project made possible the identification of a H. influenzae Kdo transferase homologue that is about 50% identical and 70% similar to the E. coli enzyme (39). We have utilized this information to clone and overexpress the H. influen-

**FIG. 10. Lack of dependence of metabolite B formation from Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} on the components of a CMP-Kdo-generating system.** In these assays, 10 \( \mu \)M Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} (–20,000 cpm/nmol, metabolite A) was used as the substrate, and the Kdo transferase conditions were utilized. The assays were initiated with H. influenzae membranes (0.5 mg/ml) and incubated for 5 min. Lane 1 is a no enzyme control. Lane 2 is a complete reaction mixture containing all the Kdo transferase components. Lane 3 is a complete assay mixture with the further addition of 1 mM ATP. Lanes 4–7 show the effects of the removal of some of the CMP-Kdo-generating system components: lane 4, no Kdo; lane 5, no CMP-Kdo-generating system components; lane 6, no CMP-Kdo-generating system components, but with 1 mM ATP added; lane 7, no CTP or ATP added.

**TABLE I**

**Nucleotide specificity of the Kdo-lipid IV\textsubscript{A} kinase**

| Nucleotide added                          | Conversion to phosphorylated Kdo-lipid IV\textsubscript{A}%c |
|------------------------------------------|-------------------------------------------------------------|
| None                                     | 0.00                                                        |
| ATP                                      | 23.7                                                        |
| CTP                                      | 1.80                                                        |
| GTP                                      | 9.33                                                        |
| UTP                                      | 2.50                                                        |
| BR7 (E. coli) membranes with ATP         | 0.00                                                        |

**FIG. 9. Dependence of Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} conversion to metabolite B on time and protein concentration.**

Panel A shows the dependence of the formation of metabolite B on the concentration of H. influenzae membrane protein in the standard Kdo-kinase assay, utilizing 100 \( \mu \)M Kdo-[\textsuperscript{4'}\textsuperscript{32}P]lipid IV\textsubscript{A} (metabolite A) as the substrate for 15 min. Panel B demonstrates the linear dependence of metabolite B formation with time at 0.05 mg/ml H. influenzae membrane protein under the same assay conditions (see “Experimental Procedures”).
enzae Kdo transferase in a T7 polymerase system. The H. influenzae Kdo transferase is mono-functional even when over-expressed in E. coli, suggesting that E. coli does not have a factor or enzyme that modifies the transferase to render its activity bi-functional. By generating hybrid Kdo transferase genes derived from E. coli and H. influenzae sequences, it may be possible to identify key regions that determine the specificity of these enzymes. The availability of both a bi-functional and a mono-functional transferase will also allow comparative studies to dissect possible structural or kinetic factors that dictate the number of Kdo moieties added. The products generated in vitro by the transferase and the kinase require more detailed characterization. It is very likely that Kdo is attached to position 6’ of lipid IV A, as in E. coli, but the location of the additional phosphate moiety in phospho-Kdo-lipid IV A is uncertain. The fact that the E. coli Kdo transferase does not utilize phospho-Kdo-lipid IV A as a substrate (Fig. 11) suggests that the phosphate is at the 4-OH position of the Kdo. Conversely, the H. influenzae Kdo kinase does not phosphorylate Kdo-lipid IV A generated by the E. coli Kdo transferase (in which the 4-OH position of the inner Kdo is blocked by substitution with the second Kdo). Although these data are not conclusive because of the possibility of indirect effects, the evidence does support the view that the phosphorylation occurs at the 4-OH of Kdo in extracts of H. influenzae. A combination of mass spectrometry and 31P NMR correlation spectroscopy will be needed to establish the site(s) of phosphorylation in phospho-Kdo-lipid IV A unequivocally. Our findings now make it possible to generate milligram quantities of both Kdo-lipid IV A and phospho-Kdo-lipid IV A, allowing us to proceed with these critical structural analyses. The fact that Kdo-lipid IV A is a substrate for the second glycosylation catalyzed by the E. coli Kdo transferase will also enable the synthesis of Kdo2-lipid IV A analogs in which the inner or the outer Kdo moieties are different, either because of isotopic labeling or selective incorporation of Kdo analogs. The functions of Kdo and lipid A in bacterial cells are unknown. It is not certain why these substances are required for growth (2–4). The identification of a gene encoding a mono-functional Kdo transferase may provide a new opportunity to modify the structure of lipopolysaccharide in living cells of E. coli and to examine the effects of the modification on physiology. We intend to determine whether or not a mono-functional Kdo transferase is sufficient to support the growth of an E. coli strain in which the chromosomal bi-functional transferase gene has been disrupted. This approach has already been used to demonstrate that the tri-functional Kdo transferase of C. trachomatis can substitute for the bi-functional E. coli enzyme (16). If the Haemophilus mono-functional Kdo transferase does not support the growth of E. coli, it may indicate the necessity of substituting the 4-OH of the inner Kdo with a negatively charged moiety. The co-expression of the Kdo kinase would address this question. Whatever the outcome, new insights into the roles of Kdo in lipopolysaccharide assembly and function are certain to emerge.

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