Purification and Characterization of a Specific 3-Deoxy-D-manno-Octulosonate 8-Phosphate Phosphatase from Escherichia coli B

PAUL H. RAY* AND CHARLES D. BENEDICT

Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

A phosphatase specific for the hydrolysis of 3-deoxy-D-manno-octulosonate (KDO)-8-phosphate was purified approximately 400-fold from crude extracts of Escherichia coli B. The hydrolysis of KDO-8-phosphate to KDO and inorganic phosphate in crude extracts of E. coli B, grown in phosphate-containing minimal medium, could be accounted for by the enzymatic activity of this specific phosphatase. No other sugar phosphate tested was an alternate substrate or inhibitor of the purified enzyme. KDO-8-phosphate phosphatase was stimulated three- to fourfold by the addition of 1.0 mM Co⁺ or Mg⁺² and to a lesser extent by 1.0 mM Ba⁺², Zn⁺², and Mn⁺². The activity was inhibited by the addition of 1.0 mM ethylenediaminetetraacetic acid, Cu⁺², Ca⁺², Cd⁺², Hg⁺², and chloride ions (50% at 0.1 M). The pH optimum was determined to be 5.5 to 6.5 in both tris(hydroxymethyl)aminomethane-acetate and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer. This specific phosphatase had an isoelectric point of 4.7 to 4.8 and a molecular weight of 80,000 ± 6,000 as determined by molecular sieving and Ferguson analysis. The enzyme appeared to be composed of two identical subunits of 40,000 to 43,000 molecular weight. The apparent Kₘ for KDO-8-phosphate was determined to be 5.8 ± 0.9 × 10⁻⁸ M in the presence of 1.0 mM Co⁺², 9.1 ± 1 × 10⁻⁸ M in the presence of 1.0 mM Mg⁺², and 1.0 ± 0.2 × 10⁻⁴ M in the absence of added Co⁺² or Mg⁺².

3-Deoxy-D-manno-octulosonic acid (KDO) is a unique sugar that is an integral part of the lipopolysaccharide region of most gram-negative bacteria. This eight-carbon keto sugar is the direct link between lipid A and the growing polysaccharide chain (10, 23, 25, 26). Osborn and co-workers (16, 20, 24, 26) have isolated temperature-sensitive mutants defective in KDO biosynthesis and demonstrated the involvement of KDO in the maturation of lipid A.

The biosynthesis and utilization of KDO is known to involve at least five sequential reactions:

1. d-ribulose 5-phosphate ⇌ d-arabinose 5-phosphate
2. d-arabinose 5-phosphate + phosphoenolpyruvate (PEP) → KDO-8-phosphate + Pᵢ
3. KDO-8-phosphate → KDO + Pᵢ
4. KDO + CTP → CMP·KDO + PPᵢ
5. 2 CMP·KDO + lipid A precursor → KDO·CMP lipid a precursor + 2 CMP

These five reactions are catalyzed by d-arabinose 5-phosphate isomerase (18, 29), KDO-8-phosphate synthetase (17), KDO-8-phosphate phosphatase (4), CMP·KDO synthetase (9), and KDO-transferase(s) (20), respectively. A previous paper described the purification and characterization of KDO-8-phosphate synthetase (reaction 2) from Escherichia coli B (23). The enzyme catalyzes the stoichiometric condensation of 1 mol of PEP and 1 mol of d-arabinose 5-phosphate to form 1 mol of KDO-8-phosphate with the release of 1 mol of Pᵢ.

Previous work on this pathway by Ghalambor and Heath (9) suggested the existence of a phosphatase for the following reasons: (i) crude preparations of KDO-8-phosphate synthetase contained an active phosphatase that rapidly dephosphorylated KDO-8-phosphate, and (ii) KDO, not KDO-8-phosphate, was shown to be the substrate in the CMP·KDO synthetase reaction. It was not known whether this phosphatase was a specific KDO-8-phosphate phosphatase similar to the phosphatase described by Jourdian et al. (13) that was involved in the dephosphorylation of N-acetylneuraminic 9-phosphate or a general acid or alkaline phosphatase. Berger and Hammerschmid (4) reported the isolation of a specific phosphatase fraction from a DEAE-cellulose column that would hydrolyze KDO-8-phosphate but not d-arabinose 5-phosphate or p-nitropheno-lyphosphate. In this paper, we describe the purification and characterization of a specific phos-
phatase from *E. coli* B that catalyzes the hydrolysis of KDO-8-phosphate to KDO and Pi.

**MATERIALS AND METHODS**

**Bacterial strain.** *E. coli* B (ATCC 11303), grown to midlogarithmic phase in glucose minimal medium containing Pi, was purchased from Grain Processing Inc., Muscatine, Iowa, as a frozen cell paste and stored at −80°C until used.

**Materials.** Buffers, phosphorylated compounds, and molecular weight markers for Sephadex G-200 column chromatography were purchased from Sigma Chemical Co., St. Louis, Mo. If required, phosphorylated compounds were further purified by Bio-Gel P-2 column chromatography (Bio-Rad Laboratories, Richmond, Calif.) to remove contaminating Pi. All other chemicals were of reagent grade.

DEAE-Sephadex A-50, Sephadex G-150, Sephadex G-100, and carrier ampholytes (Pharmalytes, pH 4.5 to 6.5) for isoelectric focusing, and molecular weight markers for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoreses were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Hydroxylapatite Bio-Gel HT, Dowex AG-1 (Cl−), and chemicals for polyacrylamide gel electrophoreses were purchased from Bio-Rad Laboratories. Hydroxylapatite containing 25% CF-1 cellulose (Whatman, Clifton, N.J.) was prepared in 5 mM potassium phosphate buffer, pH 7.2, and stored at 4°C. Just before use, the gel was washed thoroughly in 0.5 M ammonium sulfate, pH 7.1, in order to remove Pi, and was suspended in 10 mM ammonium sulfate.

**Assays.** Protein was measured by the method of Lowry et al. (19), with bovine serum albumin as the standard. KDO was assayed by the method of Weissbach and Hurwitz (30) as described previously (23). Organic and inorganic phosphate were determined by the method of Ames (1) at 725 nm; KH₂PO₄ was used as the standard. Alkaline phosphatase (Sigma) activity was determined by the method of Garen and Levintal (8), using p-nitrophenylphosphate as the substrate, or by the release of Pi. KDO-8-phosphate phosphatase was measured by the release of Pi. A typical reaction mixture contained, in a final volume of 1.0 ml: 100 μmol of buffer, 3 μmol of KDO-8-phosphate, and enzyme. At specific times, portions were withdrawn, diluted with an equal volume of cold 10% trichloroacetic acid, and centrifuged at 10,000 × *g* for 5 min in an Eppendorf microcentrifuge to remove precipitated protein. Pi concentrations were determined by adding 2.4 ml of the phosphate reagent to 0.1-ml portions of the supernatants of the above sample and incubating them at 37°C for 60 min. Under these conditions, 1.0 μmol of Pi gave a change in optical density of 5.0 at 725 nm. The stoichiometry of the enzymatic hydrolysis of KDO-8-phosphate to KDO and Pi was measured by the release of Pi, and the release of KDO, using [1-14C]KDO-8-phosphate. [14C]KDO-8-phosphate was prepared enzymatically, as described below, using [1-14C]PEP (Amersham Corp., Arlington Heights, Ill.; 14 mCi/ml). KDO and KDO-8-phosphate were separated chromatographically, using polyethyleneimine-cellulose (Brinkmann Instruments Co., Westbury, N.Y.) plates by a solvent system containing 0.1 M ammonium bicarbonate in 0.25 M LiCl. The *R*ₜ values of KDO and KDO-8-phosphate in this system are 0.8 and 0.25, respectively. One unit of enzyme activity equals 1 μmol of Pi, released per min. Specific activities are expressed as units per milligram of protein.

**Preparation of substrate.** KDO-8-phosphate was prepared enzymatically, using KDO-8-phosphate synthetase purified 300-fold as described elsewhere (23). The reaction was followed to completion by monitoring the release of Pi, from PEP and the formation of KDO. After completion, the reaction mixture was diluted with ice and immediately loaded onto a Dowex AG-1 column. The products and substrates were separated by using a linear gradient of LiCl (0 to 0.4 M). Fractions containing KDO-8-phosphate were lyophilized and desalted by Bio-Gel P-2 column chromatography.

**Separation of E. coli phosphatases by DEAE-Sephadex column chromatography.** A crude extract was prepared from 454 g of cells and acid precipitated as described below. After extensive dialysis in 0.025 M Tris buffer, pH 7.2, containing 0.025 M KCl, the crude extract was loaded onto a DEAE-Sephadex column (5 by 60 cm) at a flow rate of 2.0 ml/min. The column was washed at a flow rate of 1.5 ml/min initially with a concave 5-liter gradient containing 3.0 liters of 0.025 M KCl and 2.0 liters of 0.2 M KCl in 0.025 M Tris buffer, pH 7.2. At tube 210, a linear gradient was then used containing 1.5 liters of 0.2 M KCl and 1.5 liters of 0.4 M KCl in 0.025 M Tris buffer (see Fig. 1).

**Enzyme purification.** All buffers used during the enzyme purification consisted of 2 × 10⁻⁴ M dithiothreitol, were adjusted to the designated pH at 23°C, and were equilibrated at 4°C before use. The purification procedure described yields the highest-specific-activity enzyme of various procedures attempted.

**Step 1: cell disruption.** Frozen *E. coli* B cells (454 g) were thawed at 37°C in 0.1 M Tris-chloride, pH 7.4, in a final volume of 600 ml. The cell suspension was cooled to 4°C, and 10 mg each of DNase and RNase was added in the presence of 10 mM MgCl₂. After mixing, the cell suspension was disrupted by sonication for 30 s (10 times) at maximal output in a Branson W350 Sonifier while maintaining the temperature below 6°C. The sonicated suspension was centrifuged at 23,000 × *g* for 60 min to remove unbroken cells and cell debris. The supernatant was decanted and saved, and the pellets were resuspended in 100 ml of 0.1 M Tris-chloride, pH 7.4, mixed thoroughly, and sonicated again for 2.5 min at 30-s intervals as before. The suspension was centrifuged as before, and the supernatant was combined with the first. The combined supernatants were centrifuged at 48,000 × *g* for 30 min to remove cellular debris, yielding a final crude extract volume of 430 ml.

**Step 2: protamine sulfate.** Nucleic acids and some protein were removed from the combined supernatants by the subsurface addition of 2.2% protamine sulfate (pH 7.0), at a flow rate of 2.0 ml/min, to give a final concentration of 0.267%. After stirring at 4°C for 15 min, the precipitated material was removed by centrifugation at 25,000 × *g* for 30 min, and the supernatant (475 ml) was retained.

**Step 3: acid precipitations.** Cold 5.0 N acetic acid was added to the previous supernatant, at a flow rate
62 Ray and Benedict

of 1.0 ml/min, to a final pH of 4.5. The precipitated material was removed, after stirring for 10 min, by centrifugation at 24,000 X g for 15 min. The supernatant was adjusted to pH 7.0 by the addition of cold 5.0 N KOH at a flow rate of 1.0 ml/min. The resulting small precipitate was removed as before. The pH of the neutralized supernatant was adjusted to 5.0 as before. The supernatant was dialyzed overnight against 20 liters of 0.025 M Tris-chloride containing 0.075 M KCl, pH 7.2. The resulting precipitate was removed as before, and the pH of the supernatant (460 ml) was adjusted to 7.2 by the addition of 2.0 M Tris base.

Step 4: DEAE-Sephadex. The protein solution was adsorbed onto a column (5.0 by 60 cm) of DEAE-Sephadex that had been equilibrated with 0.025 M Tris-chloride, pH 7.2, containing 0.075 M KCl. After loading, the column was washed with 500 ml of starting buffer, and the protein was eluted with a 6-liter linear gradient of 0.075 to 0.4 M KCl in 0.025 M Tris-chloride buffer, pH 7.2. The column was eluted at a flow rate of 1.5 ml/min, and 25-ml fractions were collected. Fractions containing KDO-8-phosphate phosphatase activity (0.27 to 0.32 M KCl) were pooled (506 ml) and concentrated by Amicon ultrafiltration, using a PM-10 filter, to a volume of 120 ml. The concentrated protein solution was dialyzed overnight four times against 4 liters of 20 mM Tris-acetate, pH 7.2.

Step 5: hydroxylapate. The dialyzed protein solution was adsorbed onto a column (2.5 by 20 cm) of hydroxyapatite equilibrated with 0.01 M (NH4)2SO4, pH 7.1, at a flow rate of 1.0 ml/min. Protein was eluted from the column by a 1.5-liter linear gradient of 0.01 M to 0.5 M (NH4)2SO4. The column was eluted at a flow rate of 0.5 ml/min, and 14-ml fractions were collected. Hydrolysis of KDO-8-phosphate was determined after correction for Pi eluted from the gel. Fractions 69 through 81, containing maximal activity (0.4 M (NH4)2SO4), were pooled (250 ml) and dialyzed overnight three times against 4 liters of 20 mM Tris-acetate, pH 7.2. After dialysis, the volume was reduced to 30 ml by Amicon ultrafiltration (PM-10).

Step 6: isoelectric focusing. The concentrated enzyme solution was dialyzed for 1 h against 2 liters of distilled water and mixed in a total volume of 50 ml containing 5% sucrose and 2.8% ampholytes (pH 4.0 to 6.5). The enzyme was distributed throughout a linear 5 to 55% sucrose gradient containing 2.8% ampholytes in a 110-ml LKB isoelectric focusing column. The upper electrode (anode) contained 1% H3PO4, and the lower electrode (cathode) contained 1% NaOH in 57% sucrose. The gradient was prerun at 4.0 mA for 1 h, and the proteins were focused at 500 V for 6 h. After completion, the gradient was removed from the bottom of the column at the rate of 1.0 ml/min, and 30-drop fractions were collected. Fractions were assayed for protein at 280 nm, pH, and hydrolysis of KDO-8-phosphate. The fractions with maximal phosphatase activity (46 through 51, pI 4.7) were pooled, dialyzed against 0.05 M Tris-chloride containing 0.025 M KCl, pH 7.2, and reduced in volume to 3 ml by Amicon ultrafiltration (PM-10).

Step 7: Sephadex G-150. To remove the ampholytes, the concentrated enzyme solution was made 5% with respect to glycerol and loaded onto a column (1.5 by 150 cm) of Sephadex G-150 equilibrated with 0.05 M Tris-chloride containing 0.025 M KCl, pH 7.2. Fractions were collected and assayed for protein and KDO-8-phosphate hydrolysis, as before; those containing the highest enzyme activity were pooled and frozen at -90°C until used.

Estimation of molecular weight. The molecular weight of KDO-8-phosphate phosphatase was estimated by the method of Andrews (3), using a column (1.5 by 120 cm) containing Sephadex G-200 equilibrated in 0.1 M Tris-chloride buffer, pH 7.4. The molecular weight was also estimated by Ferguson analysis (7) as described by Rodbard and Chrambach (27) by measuring the relative mobilities in gels containing different acrylamide concentrations. The buffers (5), gel concentrations, and conditions have been described previously (23). After electrophoresis, the distance of the dye migration was measured and the tube gels (0.5 by 10 cm) were cut into 1.0-mm slices, using a Haerfer electric gel slicer. Alkaline phosphatase activity was determined by incubating each slice in 1.0 ml of 1.0 M Tris-chloride buffer, pH 8.0, containing 2.0 mM p-nitrophenylphosphate. KDO-8-phosphate phosphatase activity was determined after extraction of the gel slices overnight in 0.2 ml of HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer at 4°C.

Enzyme purity was estimated by slab gel electrophoresis, using a 7.5% polyacrylamide running gel (0.15 by 12 cm) prepared according to the method of Laemmli (14) without the addition of SDS. Samples of less than 0.1 ml containing 0.062 M Tris-chloride (pH 6.8), 10% glycerol, 5% mercaptoethanol, and 0.01% bromophenol blue were stacked at 50 V and electrophoresed at 100 V until the dye had migrated approximately 9 cm. Electrophoresis was conducted at 4°C. Proteins were stained by the method of Fairbanks et al. (6), and enzyme activity was determined as above.

SDS-polyacrylamide slab gel electrophoresis was done according to the method of Ames (2), using 12.5% polyacrylamide containing 0.1% SDS in all solutions. Samples were heated at 100°C for 7 min in a solution containing 0.062 M Tris-chloride (pH 6.8), 10% glycerol, 5% mercaptoethanol, 2.0% SDS, and bromophenol blue. The samples were stacked at 70 V and electrophoresed at 120 V. Proteins were stained according to the method of Fairbanks et al. (6). Protein bands were recorded by scanning 1.0-cm longitudinal slices of the gel at 540 nm with a Gilford 240 spectrophotometer equipped with a linear gel transport. Denaturometer tracings were made with a Hewlett-Packard 7015B X-Y recorder connected to a microcomputer as described by Woodward and Reilley (31). The low-molecular-weight protein standard kit was purchased from Pharmacia and treated in the same manner as the samples.

RESULTS

A specific KDO-8-phosphate phosphatase was isolated and purified from E. coli B cells grown in minimal medium supplemented with high P; in order to repress the synthesis of alkaline phosphatase. This specific KDO-8-phosphate phosphatase was separated from enzymes, present in crude extracts, having phosphatase activ-
ity on other phosphorylated compounds by column chromatography on DEAE-Sephadex. After elution of the column with a nonlinear KCl gradient (Fig. 1), fractions were assayed for the hydrolysis of five phosphorylated substrates: (i) D-glucose 6-phosphate; (ii) D-arabinose 5-phosphate; (iii) D-ribose 5-phosphate; (iv) p-nitrophenylphosphate; and (v) KDO-8-phosphate. Three distinct peaks of phosphatase activity were detected (Fig. 1). Fractions from each peak were pooled and assayed for the hydrolysis of all five compounds. Peak A possessed phosphatase activity for p-nitrophenylphosphate, D-arabinose 5-phosphate, D-ribose 5-phosphate, and D-glucose 6-phosphate; peak B had activity for D-arabinose 5-phosphate, D-ribose 5-phosphate, and D-glucose 6-phosphate; and peak C, which was well separated from the other two peaks, could only hydrolyze KDO-8-phosphate. KDO-8-phosphate was not hydrolyzed by phosphatases present in peaks A and B.

Having detected a specific KDO-8-phosphate phosphatase, we devised a procedure for purifying this enzyme. The procedure used conventional methods (Table 1) and produced a 380-fold purification with an 8% recovery of total enzyme units. The enzyme was stable for up to 2 months in dilute buffer (0.02 M Tris-acetate) when frozen at −90°C; the enzyme solution lost activity upon repeated freezing and thawing. During enzyme purification, the enzyme solution was maintained at 4°C for 2 weeks without loss of activity.

Properties of KDO-8-phosphate phosphatase. KDO-8-phosphate phosphatase catalyzed the hydrolysis of KDO-8-phosphate to

![Fig. 1. Separation of KDO-8-phosphate phosphatase activity from other phosphatases by DEAE-Sephadex column chromatography. A crude extract was prepared as described in Materials and Methods. After elution by the gradient described, every fifth fraction was analyzed for protein (absorbance at 280 nm), KCl (conductivity), and phosphatase activity. Phosphatase activity (P, release), using glucose 6-phosphate, ribose 5-phosphate, D-arabinose 5-phosphate, and KDO-8-phosphate as substrates, was assayed in a final volume of 0.125 ml containing 0.05 ml of each fraction, 10 μmol of HEPES buffer (7.0), and 10 μmol of each substrate. P, was determined as described. The hydrolysis of 1.0 mM p-nitrophenylphosphate in 1.0 M Tris-chloride (pH 8.0) was assayed in a final volume of 1.0 ml containing 0.1 ml of enzyme for 80 min.

Table 1. Purification of KDO-8-phosphate phosphatase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total vol (ml)</th>
<th>Total protein (g)</th>
<th>Total units</th>
<th>Sp act (U/mg)*</th>
<th>Recovery (%)</th>
<th>Fold purification</th>
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<tr>
<td>Cells</td>
<td>600</td>
<td>30.6</td>
<td>1,226</td>
<td>0.04</td>
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<td></td>
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<tr>
<td>Crude extract</td>
<td>430</td>
<td>15.0</td>
<td>1,045</td>
<td>0.07</td>
<td>85.2</td>
<td>1.8</td>
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<tr>
<td>Protamine sulfate</td>
<td>475</td>
<td>14.4</td>
<td>1,045</td>
<td>0.07</td>
<td>85.2</td>
<td>1.8</td>
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<tr>
<td>Acid precipitation</td>
<td>460</td>
<td>4.96</td>
<td>1,030</td>
<td>0.21</td>
<td>84.0</td>
<td>5.2</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>506</td>
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<td>971</td>
<td>1.45</td>
<td>79.2</td>
<td>36</td>
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<tr>
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<td>360</td>
<td>5.54</td>
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<td>138</td>
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<tr>
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<td>ND*</td>
<td>127</td>
<td>ND</td>
<td>10.4</td>
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<tr>
<td>Sephardex G-150</td>
<td>24</td>
<td>0.0062</td>
<td>97</td>
<td>15.6</td>
<td>7.9</td>
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</tr>
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</table>

* Enzyme activities and protein concentrations were determined after each step after dialysis against 0.025 M Tris-acetate, pH 7.2, containing 2 × 10⁻⁴ M dithiothreitol.

* Specific activity of cells (step 1) was determined after sonication as described in Materials and Methods.

* ND, Not determined. Total protein and specific activity were not determined due to ampholyte interference.
KDO with the stoichiometric release of P i (data not shown). By measuring the release of P i, KDO-8-phosphate hydrolysis was linear with both time and protein concentration over a 20-fold range. A number of buffers were tested at 0.1 M to determine the optimal pH for enzyme activity and stability. The pH optimum for activity in both 0.1 M Tris-acetate and 0.1 M HEPES buffer, the two best buffers, was between 5.5 and 6.5 (data not shown). For the routine assay of enzyme activity, 0.1 M HEPES buffer at pH 7.0 was used due to the increased stability of enzyme activity. Other buffers tested included succinate (pH 4.5 to 5.5), imidazole (pH 6.0 to 7.0), Tris-chloride (pH 7.2 to 8.5), and glycylglycine (pH 7.0 to 9.0), all of which decreased the activity compared with HEPES or Tris-acetate. Buffers containing chloride ions strongly inhibited the reaction. Both sodium and potassium chloride (0.1 M) inhibited the reaction by 50%, whereas sodium and potassium sulfate at the same concentration did not inhibit the enzyme activity. Enzyme activity in 0.1 M Tris-chloride (pH 6.5) was only 50% that in 0.1 M Tris-acetate (pH 6.5).

The activity of KDO-8-phosphate phosphatase as a function of temperature is shown in Fig. 2. The enzyme activity appeared to be fairly thermostable, showing a linear rate of activity at 50°C for 2 min. After 2 min the linearity of the reaction decreased, even at 40°C. However, the enzyme activity could be recovered after heating for 20 min at 55°C if the activity was assayed at 35°C. Routine assays were conducted at 35°C. The energy of activation calculated according to the Arrhenius equation (Fig. 2) was 13,700 ± 800 cal/mol (ca. 57,348 ± 3,348 J) which is similar to the energy of activation determined for the formation of KDO-8-phosphate from PEP and D-ribose 5-phosphate by KDO-8-phosphate synthetase as shown previously (23).

After extensive dialysis of KDO-8-phosphate phosphatase, no metal addition was required for catalytic activity even though 1.0 mM EDTA completely inhibited the reaction. This indicates that a metal required for activity was bound to the enzyme. The effect of the addition of divalent cations to the reaction mixture on enzyme activity is shown in Table 2. The metals were added as the chloride salt, but at concentrations of 1 and 10 mM no inhibition of activity was detected due to the chloride ion. The addition of 1.0 mM Co 2+ or Mg 2+ stimulated the hydrolysis of KDO-8-phosphate three to fourfold. Less stimulation of the phosphatase activity was seen with the addition of Ba 2+, Zn 2+, and Mn 2+ at the same concentration. The addition of 10 mM cations stimulated the activity to a lesser extent than did 1.0 mM, and the addition of 1.0 mM Ca 2+, Cd 2+, and Cu 2+ inhibited the reaction. Hg 2+ at a concentration of 1.0 mM inhibited the phosphatase reaction by 60%. This inhibition was completely reversible by the addition of 2 mM diethiothreitol, indicating the possibility of a sulfhydryl group involved in either enzyme activity or subunit association. The inhibition by EDTA could be reversed by the addition of either Co 2+ or Mg 2+. The optimal concentration of Co 2+ and Mg 2+ at a fixed substrate concentration was 1.0 mM.

![Fig. 2. Effect of incubation temperature on the rate of KDO-8-phosphate hydrolysis by KDO-8-phosphate phosphatase. The reaction mixtures contained, in a volume of 1.0 ml: 100 μmol of HEPES buffer, pH 7.0, 1 μmol of Mg 2+, 0.05 ml of enzyme, and 3.0 μmol of KDO-8-phosphate. After preincubation of the reaction mixture at the designated temperature, the reaction was initiated by the addition of enzyme. The insert is a replot of part of the data according to the Arrhenius equation whereby the log of the enzyme activity is plotted versus the reciprocal of the absolute temperature. The slope was calculated by regression analysis.](http://jb.asm.org/)
shown to be 1.0 mM for both divalent cations (Fig. 3). Stimulation by the addition of Co$^{2+}$ and Mg$^{2+}$ to the reaction mixture was reflected by a change in both the apparent $K_m$ of the enzyme for the substrate and the velocity of the reaction. In the absence of added metal, the apparent $K_m$ for KDO-8-phosphate was 1.8 ± 0.2 × 10$^{-4}$ M, with a maximal velocity of 1.32 ± 0.1 × 10$^{-4}$ mol/min per mg of protein. In the presence of 1.0 mM Mg$^{2+}$ and Co$^{2+}$, the apparent $K_m$ and maximal velocity values for KDO-8-phosphate were 9.1 ± 1 × 10$^{-5}$ M and 4.8 ± 0.2 × 10$^{-4}$ mol/min per mg of protein with Mg$^{2+}$ and 5.8 ± 0.9 × 10$^{-5}$ M and 5.1 ± 0.2 × 10$^{-4}$ mol/min per mg of protein with Co$^{2+}$.

Physical properties of KDO-8-phosphate phosphatase. Isoelectric focusing was used to purify KDO-8-phosphate phosphatase (Table 1). Electrophoresis of a partially purified enzyme for 65 h at 400 V gave a single peak of enzyme activity with an isoelectric point of 4.7 to 4.8. Refocusing with less protein and the same units of activity for 60 h at 500 V gave identical results, showing only one peak of phosphatase activity with a pI of 4.7 to 4.8.

The molecular weight of KDO-8-phosphate phosphatase was estimated to be 80,000 ± 6,000 by molecular sieving through Sephadex G-200 according to the method of Andrews (3). In an independent method, the molecular weight was estimated by electrophoresis in acrylamide gels of varying concentrations, and the $K_r$ values (slope) were calculated by the method of Ferguson (7). Using alkaline phosphatase as a standard, molecular weight 86,000 (15), the relative mobilities of both enzymes as a function of acrylamide concentration were directly compared (data not shown). By linear regression of the data, the $K_r$ values were calculated to be −0.067 ± 0.005 for alkaline phosphatase and −0.063 ± 0.004 for KDO-8-phosphate phosphatase, indicating the molecular weight of KDO-8-phosphate phosphatase to be approximately 86,000. Also, when the $K_m$ value of KDO-8-phosphate phosphatase was compared with a standard curve plotting $K_r$ values as a function of known molecular weight standards, the $K_m$ value corresponded to this molecular weight.

After purification as described in Table 1, the enzyme solution was electrophoresed on a preparative (3 mm) 7.5% polyacrylamide slab gel, the activity was determined, and the solution was eluted and again subjected to electrophoresis. Figure 4A illustrates a densitometer scan of KDO-8-phosphate phosphatase electrophoresed through a 7.5% non-SDS-acrylamide gel. The preparation was electrophoretically homogeneous, with 95% of the protein resolved into a single band at 4 to 5 cm from the origin. When a duplicate gel was sliced and assayed for hydrolysis of KDO-8-phosphate, a single peak of activity was detected coincidental with the major protein peak. When a portion of the protein sample from the non-SDS-gel was eluted, denatured by heating with SDS and mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis, only one major band and two minor bands were detected. When the $R_f$ values of these bands were used to calculate the molecular weight from a standard curve of known molecular weight standards (insert, Fig. 4B), bands 1, 2, and 3 corresponded to molecular weights of 78,000 to 84,000, 40,000 to 43,000, and 20,000 to 23,000, respectively. Band 2 was clearly the major protein, comprising 84% of the total. These data indicate that KDO-8-phosphate phosphatase (molecular weight 80,000 to 86,000) is composed of two equal-molecular-weight subunits (40,000 to 43,000). Band 1, molecular weight 80,000, can be attributed to contamination of the KDO-8-phosphate phosphatase peak shown in Fig. 4A or enzyme that was undisassociated under these conditions. Band 3, molecular weight 20,000 to 23,000, could either be a degradation product or a contaminant, since Fig. 4A showed that protein used for SDS-gel electrophoresis was virtually homogeneous.

Specificity of KDO-8-phosphate phos-
Fig. 4. Polyacrylamide gel electrophoresis of KDO-8-phosphate phosphatase. (A) 7.5% non-SDS-polyacrylamide gel densitometer scan. Approximately 32 µg of protein was treated as described in Materials and Methods, giving a final volume of 0.09 ml. Two equal portions were loaded onto the center wells of a ¼-inch (about 0.16-cm) slab gel and electrophoresed as described. After electrophoresis, one well was sliced horizontally, extracted as described in the text, and assayed for hydrolysis of KDO-8-phosphate. The remaining well was stained for protein and scanned as described in Materials and Methods. (B) 12.5% SDS-polyacrylamide gel densitometer scan. Approximately 32 µg of protein was treated as described in Materials and Methods. Portions of 0.015 and 0.03 ml (approximately 6 and 11 µg of protein, respectively) were loaded onto the center wells of a ¼-inch (about 0.16-cm) slab gel. Standards were included in 0.01- and 0.02-ml portions in separate cells. After electrophoresis, gels were stained and scanned as before. A plot of the molecular weights versus the relative mobilities of the standards is shown in the insert. Standards included: (A) phosphorylase b (94,000), (B) albumin (67,000), (C) ovalbumin (43,000), (D) carbonic anhydrase (30,000), (E) soybean trypsin inhibitor (20,000), and (F) alpha-lactalbumin (14,000). The relative mobilities of protein peaks located from the sample are indicated on the standard curve as: (1) \( R_i = 0.2 \), (2) \( R_i = 0.4 \), and (3) \( R_i = 0.72 \).

**DISCUSSION**

KDO is a site-specific constituent of the lipopolysaccharide of most gram-negative organisms, providing the link between lipid A and the growing polysaccharide chain. There are at least five sequential enzymes involved in the synthesis and utilization of KDO. A preceding phosphatase. Initially, a number of sugar phosphates were assayed as possible alternate substrates for this phosphatase due to the cost and time involved in the large-scale preparation and purification of KDO-8-phosphate. None of the phosphorylated sugars shown in Table 3 was acceptable as an alternate substrate for KDO-8-phosphate phosphatase. All of the phosphorylated sugars were substrates that could be dephosphorylated by alkaline phosphatase (Table 3). These substrates were also tested as inhibitors of the specific KDO-8-phosphate phosphatase, and none was an effective inhibitor of this reaction.

**Table 3. Specific activities of E. coli alkaline phosphatase and KDO-8-phosphate phosphatase on various phosphorylated compounds**

<table>
<thead>
<tr>
<th>Compound</th>
<th>KDO-8-phosphate phosphatase</th>
<th>Alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDO-8-phosphate</td>
<td>1.34</td>
<td>0.60</td>
</tr>
<tr>
<td>p-Nitrophenylphosphate</td>
<td>&lt;0.01</td>
<td>0.99</td>
</tr>
<tr>
<td>PEP</td>
<td>&lt;0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>d-Arabinose 5-phosphate</td>
<td>&lt;0.01</td>
<td>0.56</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>&lt;0.01</td>
<td>0.60</td>
</tr>
<tr>
<td>2-Deoxyglucose 6-phosphate</td>
<td>&lt;0.01</td>
<td>0.64</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>&lt;0.01</td>
<td>0.52</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>&lt;0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>Galactose 1-phosphate</td>
<td>&lt;0.01</td>
<td>0.57</td>
</tr>
<tr>
<td>Galactose 6-phosphate</td>
<td>&lt;0.01</td>
<td>0.53</td>
</tr>
<tr>
<td>2-Deoxyribose 5-phosphate</td>
<td>&lt;0.01</td>
<td>0.58</td>
</tr>
<tr>
<td>Ribulose 5-phosphate</td>
<td>&lt;0.01</td>
<td>0.60</td>
</tr>
<tr>
<td>Ribulose 1,5-diphosphoric acid</td>
<td>&lt;0.01</td>
<td>0.48</td>
</tr>
<tr>
<td>Mannose 1-phosphate</td>
<td>&lt;0.01</td>
<td>0.50</td>
</tr>
<tr>
<td>Mannose 6-phosphate</td>
<td>&lt;0.01</td>
<td>0.58</td>
</tr>
<tr>
<td>Maltose 1-phosphate</td>
<td>&lt;0.01</td>
<td>0.40</td>
</tr>
<tr>
<td>Erythrose 4-phosphate</td>
<td>&lt;0.01</td>
<td>0.46</td>
</tr>
<tr>
<td>Phosphoglyceric acid</td>
<td>&lt;0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>a-Glycerophosphate</td>
<td>&lt;0.01</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*a Reaction mixtures contained, in a volume of 0.5 ml: 50 µmol of HEPES, pH 7.0, 0.5 µmol of CoCl2, 45 µg of KDO-8-phosphate phosphatase or 5 µg of alkaline phosphatase, and 0.5 mg of the appropriate compound, incubated at 35°C. Data for both enzymes are expressed as specific activities under these conditions of incubation.*
paper (23) described the purification and characterization of KDO-8-phosphate synthetase from E. coli B. In this paper, we describe the purification and partial characterization of a cytoplasmic enzyme (no evidence of membrane attachment was indicated after using various techniques for cell disruption) that specifically dephosphorylates KDO-8-phosphate to KDO, the substrate for the next sequential enzyme, CMP-KDO synthetase. The various reactions involved in the synthesis and activation of KDO appear to be very similar to those described by Jourdan et al. (13) and Roseman (28) concerned with the synthesis and activation of N-acetyleneuraminic acid in mammalian cells.

KDO-8-phosphate phosphatase was purified approximately 400-fold by conventional methods. This phosphatase was KDO-8-phosphate substrate specific: no other sugar phosphates tested were hydrolyzed by this enzyme under the stated conditions. In crude soluble extracts, 90% of the hydrolysis of KDO-8-phosphate could be accounted for by the activity of this enzyme. When other soluble phosphatases from E. coli B, detected after DEAE-Sephadex column chromatography (Fig. 1), were assayed with KDO-8-phosphate as a substrate, less than 5% of the total activity was detected, indicating that no other cytoplasmic phosphatase can dephosphorylate KDO-8-phosphate to KDO, which is the substrate required in the next reaction (9). KDO-8-phosphate could be readily hydrolyzed by E. coli alkaline phosphatase (Table 3); however, E. coli B cells were grown to midlogarithmic phase in a phosphate-based minimal medium which repressed alkaline phosphatase synthesis (8, 12). It should be noted that alkaline phosphatase is a well-characterized periplasmic protein (11, 12) and KDO-8-phosphate synthesized by KDO-8-phosphate synthetase is a cytoplasmic constituent. KDO does not become membrane bound until it is transferred from CMP-KDO to the lipid A precursor, as shown by Munson et al. (20). We have seen no evidence in our work or that of others (9, 10, 17, 20) to indicate that D-arabinose 5-phosphate isomerase, KDO-8-phosphate synthetase, CMP-KDO synthetase, the enzymes involved in KDO synthesis and activation, are even loosely membrane bound. KDO transferase(s) is, however, tightly membrane bound (20). Even though KDO-8-phosphate phosphatase and E. coli alkaline phosphatase are similar in molecular weight (75,000 to 86,000), isoelectric point (pI 4.5 to 4.8), a divalent metal requirement, and KDO-8-phosphate hydrolysis, they differ substantially in pH optimum, \( K_m \) for KDO-8-phosphate, ability to hydrolyze multiple substrates, specific divalent cation requirements, and cellular location.

We are now purifying and characterizing D-arabinose 5-phosphate isomerase and CMP-KDO synthetase from E. coli B. The specific activities in crude extracts of the first four enzymes in the KDO pathway (see above) appear to be very similar and their \( K_m \) values are similar, which is to be expected in a sequential pathway. The controlling steps in this pathway are not obvious and may even include the regulation of D-glucuronic 6-phosphate dehydrogenase (21) by one of the intermediates of the KDO pathway. This enzyme produces a key intermediate in carbohydrate metabolism, D-ribulose 5-phosphate, which is utilized for the formation of both D-arabinose 5-phosphate and D-ribose 5-phosphate. It was previously shown (23) that KDO-8-phosphate is a weak end product inhibitor of the synthetase reaction, and we also have preliminary data suggesting that both KDO and P, are weak mixed-function inhibitors of the phosphatase reaction.

It should be noted that our data on the \( K_m \) for KDO-8-phosphate and the optimal pH for the phosphatase reaction differ from the data of Berger and Hammerschmid (4). We believe these differences can be explained by the method of assay; they measured their \( K_m \) and pH optimum by a combined assay using CMP-KDO synthetase, and we measured the reaction directly by the release of \( P_i \) from KDO-8-phosphate.

**LITERATURE CITED**


