Escherichia coli YrbI is 3-Deoxy-\textit{d-manno}-octulosonate 8-phosphate Phosphatase

Jing Wu and Ronald W. Woodard‡

Department of Medicinal Chemistry and Department of Chemistry
University of Michigan, Ann Arbor, Michigan 48109-1065, USA.

‡ To whom correspondence should be addressed: College of Pharmacy, 428 Church St., Ann Arbor, MI 48109-1065. Tel.: 734-764-7366; Fax: 734-763-2022; E-mail: rww@umich.edu

1 The abbreviations used are: KDO, 3-Deoxy-\textit{d-manno}-octulosonate; KDO 8-P, 3-Deoxy-\textit{d-manno}-octulosonate 8-phosphate; Tris, Tris-(hydroxymethyl)aminomethane; HEPES, N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]; PNPass, purine nucleoside phosphorylase; EDTA, (ethylenedinitrilo)tetraacetic acid; 2D gel electrophoresis, two dimensional gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; orf, open reading frame; MW, molecular weight; HAD, haloacid dehalogenase; NCBI, National Center for Biotechnology Information.

Running Title: YrbI is KDO 8-P phosphatase
SUMMARY

3-Deoxy-\(\text{-d-manno}\)-octulosonate 8-phosphate (KDO 8-P) phosphatase, which catalyzes the hydrolysis of KDO 8-P to KDO and inorganic phosphate, is the last enzyme in the KDO biosynthetic pathway for which the gene has not been identified. Wild-type KDO 8-P phosphatase was purified from \textit{Escherichia coli} B and the N-terminal amino acid sequence matched a hypothetical protein encoded by the \textit{E. coli} open reading frame, \textit{yrbI}. The \textit{yrbI} gene, which encodes for a protein of 188 amino acids, was cloned and the gene product was overexpressed in \textit{E. coli}. The recombinant enzyme is a tetramer and requires a divalent metal cofactor for activity. Optimal enzymatic activity is observed at pH 5.5. The enzyme is highly specific for KDO 8-P with an apparent \(K_m\) of 75 \(\mu\)M and a \(k_{cat}\) of 175 s\(^{-1}\) in the presence of 1 mM Mg\(^{2+}\). Amino acid sequence analysis indicates that KDO 8-P phosphatase is a member of the haloacid dehalogenase hydrolase superfamily.

INTRODUCTION

3-Deoxy-\(\text{-d-manno}\)-octulosonate (KDO)\(^1\) is an eight-carbon sugar that links the lipid A and polysaccharide moieties of the lipopolysaccharide region in Gram-negative bacteria (1,2). It has been demonstrated that an interruption in the biosynthesis of KDO leads to the accumulation of lipid A precursors, and subsequent arrest in cell growth (3-5). Thus, enzymes involved in KDO biosynthesis and/or its incorporation into lipid A are considered attractive targets for the design of novel antibiotics.

The biosynthesis and utilization of KDO involves five sequential enzymatic reactions that are catalyzed by d-arabinose 5-phosphate isomerase, 3-deoxy-\(\text{-d-manno}\)-octulosonate 8-
phosphate (KDO 8-P) synthase, KDO 8-P phosphatase, cytidine 5’-monophosphate-KDO synthetase, and KDO transferase (Figure 1) (2). During the past two decades, the genes responsible for the expression of KDO 8-P synthase (6-9), cytidine 5’-monophosphate-KDO synthetase (10-12), and KDO transferase (13,14) have been identified and their respective enzymes have been studied extensively. More recently, the gene encoding the d-arabinose 5-phosphate isomerase (KpsF) from *Neisseria meningitides* was identified by Tzeng et al. (15). KDO 8-P phosphatase, therefore, remains the last enzyme in the lipid A-KDO pathway for which a gene has not been assigned.

KDO 8-P phosphatase catalyzes the hydrolysis of KDO 8-P to KDO and inorganic phosphate. Gahlambor and Heath (16) first suggested the existence of a phosphatase for KDO 8-P in 1966. In 1975, Berger and Hammerschmid (17) reported the isolation of a specific phosphatase fraction from a DEAE-cellulose column that would hydrolyze KDO 8-P but not d-arabinose 5-phosphate or p-nitrophenylphosphate. Ray and Benedict (18) first purified and characterized the KDO 8-P phosphatase from *Escherichia coli* in 1980 but did not identify the encoding gene.

In the present work, for the first time, the gene for KDO 8-P phosphatase is identified and cloned into an overexpression vector. A wild-type phosphatase that specifically hydrolyzes KDO 8-P to KDO and inorganic phosphate was isolated and N-terminally sequenced. A BLAST search of the *E. coli* K12 genome database at National Center for Biotechnology Information (NCBI) web site with this N-terminal sequence revealed a hypothetical protein encoded by the open reading frame (orf) *yrbI*. The *yrbI* gene was cloned and the gene product was overexpressed in *E. coli*. The recombinant protein was purified to homogeneity and its
characteristics were consistent with those properties reported for the wild-type KDO 8-P phosphatase.

EXPERIMENTAL PROCEDURES

Materials—The *E. coli* B cells, grown in glucose minimal medium supplemented with inorganic phosphate, were purchased from Grain Processing Inc., Muscatine, Iowa, as a frozen cell paste and stored at -80 °C. Genomic *E. coli* BL21 DNA was a generous gift from Dr. George A. Garcia (University of Michigan). The Promega Wizard DNA purification kit was utilized for plasmid isolation and purification. *E. coli* XL1-Blue chemically competent cells were obtained from Stratagene Cloning System. *E. coli* BL21(DE3) chemically competent cells were obtained from Novagen. The pCR®T7 TOPO®TA expression kit was purchased from Invitrogen. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Thermal cycling was performed using a MJ Research PTC-200 Peltier Thermal Cycler. DNA sequencing, N-terminal amino acid sequencing, two dimensional (2D) gel electrophoresis and DNA primer syntheses were performed by the University of Michigan Biomedical Resources Core Facility. Tris-(hydroxymethyl)aminomethane (Tris) and N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES) were purchased from Research Organics. Glycylglycine, phosphoenolpyruvate mono(cyclohexylammonium) salt, *p*-nitrophenylphosphate di(cyclohexylammonium) salt, D-ribose 5-phosphate disodium salt, D-arabinose 5-phosphate disodium salt, D-glucose 6-phosphate monosodium salt, D-mannose 6-phosphate monosodium salt, reagent grade CuCl2, CaCl2, ultra pure Trizma base, and Q-Sepharose resin were purchased from Sigma Chemical Company. The puratronic grade MgCl2, CoCl2, MnCl2, CdCl2, ZnSO4, and HCl (99.999%, metal basis) were purchased from Alfa Aesar. The
(ethylenedinitrilo)tetraacetic acid (EDTA) disodium salt and mercuric acetate were obtained from Mallinckrodt. The 2-(N-morpholino)ethanesulfonic acid was obtained from United States Biochemical Corporation. High grade Spectra/Por® 7 dialysis tubing (10,000 molecular weight cut-off and metal free) was obtained from VWR Scientific. The Centriprep YM-10 Concentrators were purchased from Millipore. AG-MPI and P2 resin were purchased from Bio-Rad. Mono Q (HR 5/5), Phenyl Superose (HR 10/10), and Superose 12 (HR 10/30) chromatography columns were purchased from Amersham Pharmacia Biotech.

**KDO 8-P Phosphatase Activity Assay**—The KDO 8-P phosphatase activity was determined by either a discontinuous colorimetric assay or a continuous spectrophotometric assay. One unit of enzyme activity is defined as the production of 1 μmol of inorganic phosphate per minute at 37 °C.

The standard discontinuous colorimetric assay was measured in a 50 μl reaction mixture containing 1 mM KDO 8-P, 1 mM Mg$^{2+}$ and 100 mM HEPES (pH 7.0) at 37 °C. The reaction was initiated by the addition of enzyme and quenched by the addition of 50 μl of 10% (w/v) ice-cold trichloroacetic acid. The amount of inorganic phosphate produced was quantitated by the malachite green assay (19) using KH$_2$PO$_4$ as the standard.

The continuous spectrophotometric assay was based on the purine nucleoside phosphorylase (PNPase)–coupled phosphate assay first reported by Webb (20) and later modified by Rieger and coworkers (21). The assay was performed in a 70 μl reaction mixture containing 1 mM KDO 8-P, 1 mM Mg$^{2+}$, 100 mM HEPES (pH 7.0), 200 μM 7-methylinosine, 2 μM recombinant bacterial PNPase, and 2 nM KDO 8-P phosphatase. The reaction mixture, including PNPase but excluding KDO 8-P phosphatase, was incubated at 37 °C for 5 min to allow the PNPase coupling system to remove any inorganic phosphate potentially present as a
contaminant in the substrates. The reaction was initiated by the addition of KDO 8-P phosphatase. In the coupled assays, the concentration of KDO 8-P phosphatase and PNPase was adjusted to ensure that the phosphatase activity was rate-limiting.

**Enzymatic Synthesis of KDO 8-P**—KDO 8-P was synthesized enzymatically (22) using KDO 8-P synthase purified as previously described (23). The *E. coli* KDO 8-P synthase (4 mg) was incubated with phosphoenolpyruvate mono(cyclohexylammonium) salt (24 mg) and D-arabinose 5-phosphate disodium salt (26 mg) in 100 mM Tris-HCl (pH 7.5) in a final volume of 4 ml at 37 °C for 2 h. The reaction mixture was quenched by the addition of 0.45 ml 50% (w/v) trichloroacetic acid and centrifuged to remove precipitated protein. The pH of the supernatant was adjusted to 7.0 by 1 N sodium hydroxide. The resulting solution was loaded onto an AG-MPI anion exchange column (chloride form, 2.5 × 30 cm) pre-equilibrated with water. The column was first washed with 100 ml of water at a flow rate of 1 ml/min and then eluted with a linear gradient of 0 to 0.4 M potassium chloride (60 min at 1 ml/min). The fractions containing KDO 8-P were pooled and lyophilized. The lyophosphate was dissolved in 2 ml water and then desalted on a P2 column (2.0 × 60 cm) using water as the eluent.

**Isolation and Purification of Wild-type KDO 8-P Phosphatase from E. coli B**—KDO 8-P phosphatase was isolated and purified from *E. coli* B using a modification of the protocol originally described by Ray and Benedict (18). The frozen *E. coli* B cells (46 g) were thawed at 23 °C in 100 mM Tris-HCl (pH 7.4) in a final volume of 60 ml. The cell suspension was subjected to sonication at 4 °C (ice-water bath, 45-s pulses with a 2-min rest between pulses, four times) and the unbroken cells and cell debris were removed by centrifugation (40,000 × g, 30 min, 4 °C). The supernatant was saved. The pellet was suspended in 50 ml of 100 mM Tris-HCl (pH 7.4), sonicated and centrifuged as above. The two supernatants were combined. In order to
remove nucleic acids, a 2.2% (w/v) protamine sulfate solution (pH 7.0) was slowly added at 4 °C with gentle stirring to the supernatant to yield a final concentration of 0.267% (w/v) protamine sulfate. After continuous stirring for 15 min at 4 °C, the precipitated material was removed by centrifugation (40,000 × g, 30 min, 4 °C).

The pH of the above supernatant was adjusted to 5.2 (pH measured at 4 °C) by drop-wise addition of cold 1 N acetic acid. After continuously stirring the solution for 10 min at 4 °C, the precipitated protein was removed by centrifugation (29,000 × g, 20 min, 4 °C) and solid (NH₄)₂SO₄ was then slowly added to the supernatant. The protein fraction precipitating between 10% to 34% (w/v) (NH₄)₂SO₄ was collected by centrifugation (29,000 × g, 30 min, 4 °C) and dissolved in buffer A (20 mM Tris-HCl, pH 7.4). The sample was dialyzed against two liters of the same buffer overnight and the resulting protein solution was applied to a Q-Sepharose column (1.2 × 21 cm) pre-equilibrated with buffer A. The column was eluted at a flow rate of 2.0 ml/min using a linear gradient of 0 to 0.5 M potassium chloride in buffer A over a 60 min period. The fractions containing KDO 8-P phosphatase activity were pooled.

Solid (NH₄)₂SO₄ was slowly added to the pooled fractions to a final concentration of 20% (w/v). The sample was filtered (0.22 μm) and loaded onto a Phenyl Superose column (HR 10/10) pre-equilibrated with 20% (NH₄)₂SO₄ in buffer A. A reverse gradient from 20% to 0% (NH₄)₂SO₄ in buffer A was applied at a flow rate of 1.0 ml/min over a 60 min period. The fractions containing KDO 8-P phosphatase activity were pooled, dialyzed against one liter of buffer A overnight, and applied to a Mono Q (HR 5/5) column pre-equilibrated with buffer A. The column was eluted at a flow rate of 0.5 ml/min using a linear gradient of 0 to 0.3 M potassium chloride in buffer A over 30 min. The fractions containing KDO 8-P phosphatase activity were pooled and dialyzed against 500 ml buffer A overnight. The final preparation was
concentrated by ultrafiltration to 0.6 mg/ml (Centriprep YM-10 concentrator) and the resulting solution was stored at -80 °C. The purification process of wild-type KDO 8-P phosphatase is summarized in Table I.

Two Dimensional Gel Electrophoresis and N-terminal Amino Acid Sequencing—The 2D gel electrophoresis and N-terminal amino acid sequencing was performed by the University of Michigan Biomedical Resources Core Facility. The wild-type phosphatase preparation was subjected to electrophoresis in the first dimension on a 7 cm IPG strip (pH 3-10) using the Amersham-Pharmacia Multiphor II system. In the second dimension, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a NuPAGE Novex 4-12% Bis-Tris ZOOM Gel (Invitrogen) using the Invitrogen Xcell Surelock Mini-Cell system. The gel was electroblotted onto a polyvinylidene difluoride membrane (Applied Biosystems Mini ProBlott membrane) using the Bio-Rad Transblot Semi-Dry blotter system. The blotted protein was visualized by Coomassie blue R250. The N-terminal sequencing of the blotted proteins were performed on a Procise Protein Sequencing System.

Sequence Analysis—Database searching was performed using the BLAST program (24) at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were performed using the program CLUSTAL W (http://www.ebi.ac.uk/clustalw) (25).

Cloning, Overexpression and Purification of KDO 8-P phosphatase—The yrbI gene (gi:16131088) was amplified from E. coli BL21 genomic DNA by standard polymerase chain reaction methodologies using Taq DNA polymerase as recommended by manufacturer. The forward primer was CATATGAGCAAAGCAGGTGCGTCGC and the reverse primer was GATTCTGAATTCGGATCC TCAATTCACCTTCACCC. The amplification product was isolated and ligated directly into the vector pCR®T7/CT-TOPO®. The ligation mixture was used
to transform chemically competent *E. coli* TOP10F’ cells. Plasmid DNA isolated from several transformants and identified by restriction analysis to contain the PCR product were subjected to DNA sequencing to confirm the sequence of the desired gene. One plasmid with the correct sequence, pT7CT-yrbI, was digested with the restriction endonucleases *Nde*I and *Bam*HI (underlined above). The restriction-digested product was ligated into the similarly restriction-digested expression vector, pT7-7, which had been treated with calf intestinal alkaline phosphatase. The ligation mixture was used to transform chemically competent *E. coli* XL1-Blue cells. The plasmid (pT7-yrbI) isolated from these transformants was first sequenced and then used to transform chemically competent *E. coli* BL21(DE3) cells.

The *E.coli* BL21(DE3) cells harboring the pT7-yrbI were grown in 2 × YT medium containing ampicillin (100 mg/L) at 37 °C with shaking (220 rpm). Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.4 mM when the culture reached an OD<sub>600</sub> of 1.5. The cells were harvested by centrifugation (29,000 × g, 20 min, 4 °C) 4 h post induction. The pellet was suspended in buffer A (20 mM Tris-HCl, pH 7.5), and subjected to sonication at 4 °C (ice-water bath, 30-s pulses with a 2-min rest between pulses, five times). The crude extract was centrifuged to remove cell debris (40,000 × g, 30 min, 4 °C).

The pH of the above supernatant (90 ml; 1,880 mg total protein from a 1.0-liter culture) was adjusted to 5.0 by slowly adding cold 1.0 N acetic acid, the solution was stirred for another 10 min at 4 °C and centrifuged to remove the precipitated protein (29,000 × g, 20 min, 4 °C). The supernatant (80 ml; 480 mg total protein) was dialyzed against two liters of buffer A overnight and applied to a Q-Sepharose column (1.2 × 21 cm) pre-equilibrated with buffer A. The column was eluted at a flow rate of 2.0 ml/min using a linear gradient from 0 to 0.5 M potassium chloride in the same buffer over 70 min. After the fractions containing KDO 8-P
phosphatase activity were pooled (37 ml; 230 mg of total protein), they were dialyzed against two liters of 10 mM HEPES buffer (pH 7.0) overnight. The final preparation was homogeneous as determined by SDS-PAGE. The total yield of homogeneous protein was 220 mg per liter of cell culture. The purified enzyme (6 mg/ml) was aliquoted, frozen in ethanol-dry ice, and stored at -80 °C.

**Molecular Weight (MW) Determinations**—The subunit MW of KDO 8-P phosphatase was determined by electrospray ionization mass spectrometry utilizing a Finnigan LCQ mass spectrometer. The native MW of KDO 8-P phosphatase was determined by gel filtration utilizing a Superose 12 column (HR10/30) according to the manufacturer's instructions (Sigma). The elution volume was determined in triplicate for all samples and standards.

**Kinetic Studies**—Reactions were carried out at 37 °C using the continuous spectrophotometric assay protocol as described above. Initial rates were determined in triplicate using the linear region (~ 30 s) of the reaction progress curve for six concentrations of KDO 8-P (between 0.1 and 10 × $K_m$). The values for $K_m$ and $k_{cat}$ were determined by fitting the reaction rate versus the substrate concentration to the Michaelis-Menten equation using the KaleidaGraph software.

**Metal Requirements**—Recombinant KDO 8-P phosphatase was treated with EDTA to remove bound metal ions. The enzyme as isolated (6 mg/ml) was treated with 10 mM EDTA in 20 mM Tris-HCl (pH 7.0) for 2 h at 25 °C and dialyzed against one liter of metal-free 20 mM Tris-HCl (pH 7.0) for 24 h at 4 °C with two buffer changes. The metal-free Tris-HCl buffer was prepared directly using metal-free water (PURELAB plus system), ultra pure Trizma base and metal-free HCl. The divalent metals (1 mM, prepared in metal-free water) were added to the assay mixture to assess their effects on EDTA-treated (apo) KDO 8-P phosphatase activity.
pH Dependence of KDO 8-P Phosphatase—The enzymatic activity was measured between pH 4.0 and 9.0 at 37 °C by the discontinuous assay described above using sodium acetate (pH 4.0 to 5.0), 2-(N-morpholino)ethanesulfonic acid (pH 5.5 to 6.5), HEPES (pH 7.0 to 8.0), or glycyglycine (pH 8.5 to 9.0) at a concentration of 100 mM each. The pH of each reaction mixture was measured at 23 °C.

Electrophoresis—SDS-PAGE was performed under reducing conditions on a 12% polyacrylamide gel with a Mini-PROTEAN II electrophoresis unit (Bio-Rad). The gel was visualized with 0.25% Coomassie brilliant blue R250 stain. Isoelectric focusing of protein, under native state, was performed on a 5.5% (w/v) polyacrylamide gel containing “Carrier Ampholytes pH 3–10” (Bio-Rad) with a Model 111 Mini IEF Cell according to the manufacturer's instructions (Bio-Rad).

Miscellaneous Methods—Protein concentrations were determined using the Bio-Rad Protein Assay Reagent with bovine serum albumin (Sigma) serving as the standard. Optical spectroscopy was performed using a HP 8453 UV-visible spectrophotometer.

RESULTS

Purification of Wild-type KDO 8-P Phosphatase from E. coli B—A specific KDO 8-P phosphatase was purified from E. coli B cells grown in glucose minimal medium containing phosphate by monitoring the enzymatic hydrolysis of the phosphate group of KDO 8-P. The purification included ammonium sulfate fractionation as well as a combination of chromatographic separations utilizing Q-Sepharose, Phenyl Superose, and finally Mono Q chromatography. The purified wild-type enzyme preparation, which was purified approximately 850-fold (Table I), exhibited a specific activity of 34 units/mg in the presence of 1 mM Mg$^{2+}$ (16
units/mg in the absence of added Mg\(^{2+}\)). Under the discontinuous colorimetric assay conditions, neither D-ribose 5-phosphate nor D-glucose 6-phosphate served as a substrate. Thus, the substrate specificity and magnesium requirement of the present wild-type enzyme are identical to those originally reported by Ray and Benedict (18) for their wild-type KDO 8-P phosphatase. The examination of the "purified" wild-type KDO 8-P phosphatase from the present study by 2D gel electrophoresis revealed three protein spots which were further analyzed by N-terminal amino acid sequencing (Figure 2). Utilizing these N-terminal sequences, the *E. coli* K12 genome database at the NCBI web site was searched using the "search for short nearly exact matches" search algorithm. Based on these analyses, spot 1 was identified as 2-ketogluconate reductase \([\text{MW}_{\text{obs}} = 39 \text{ kDa}, \text{MW}_{\text{cal}} = 35,396]\), spot 2 as fructose-bisphosphate aldolase \([\text{MW}_{\text{obs}} = 42 \text{ kDa}, \text{MW}_{\text{cal}} = 39,147, \text{excluding the initial methionine}]\), and spot 3 as YrbI \([\text{MW}_{\text{obs}} = 52 \text{ kDa}, \text{MW}_{\text{cal}} = 19,866, \text{excluding the initial methionine}]\), a hypothetical protein of 188 amino acids (GenBank accession no. NP_417665). A BLASTP search of the NCBI databases with the YrbI sequence found that most matches were annotated as hypothetical proteins from Gram-negative bacteria (Table II). Since the lipopolysaccharide pathway exists primarily in Gram-negative bacteria and YrbI was the only *E. coli* protein that matched the N-terminal sequence of spot 3, the *yrbI* orf was considered the candidate gene encoding KDO 8-P phosphatase.

**Cloning, Overexpression, and Purification of KDO 8-P phosphatase**—The *yrbI* orf, the putative KDO 8-P phosphatase coding region, is located in the *yrb* operon. This operon is comprised of ten genes to which no biological function has been assigned. In order to achieve true homologous expression in this study, the *yrbI* gene from *E. coli* BL21 was cloned into the expression plasmid pT7-7 and the protein was overexpressed in *E. coli* BL21(DE3). Crude extracts from cells harboring the recombinant plasmid showed KDO 8-P phosphatase activity of
38 units/mg protein in the presence of 1 mM Mg^{2+}, which was 380-fold higher than that of the crude extracts from plasmid-free *E. coli* BL21(DE3) cells. The recombinant enzyme was purified by acid precipitation followed by anion exchange chromatography on a Q-Sepharose column. The purified recombinant enzyme exhibited a specific activity of 460 units/mg in the presence of 1 mM Mg^{2+}. A single protein band observed on the SDS-PAGE gel demonstrated homogeneity (Figure 3). The typical yield of purified protein was 220 mg per liter of cell culture.

**Physical Properties of the Recombinant KDO 8-P Phosphatase**—The MW of the recombinant enzyme was 19,881 as determined by mass spectrometry. This value is in agreement with the calculated MW of 19,866. The results from SDS-PAGE suggested a subunit MW of 23 kDa (Figure 3). The native MW of the recombinant enzyme was 89 kDa (analytical gel filtration chromatography). Since the native MW is about 4 times that of the denatured MW determined, the recombinant KDO 8-P phosphatase is predicted to have a tetrameric structure. The pI of the recombinant enzyme was 4.7 as determined by isoelectric focusing.

**Catalytic Properties of the Recombinant KDO 8-P Phosphatase**—In the presence of 1 mM Mg^{2+}, the recombinant enzyme exhibited a pH optimum around 5.5, however, a broad peak with high catalytic activity (90% of maximum) was observed between pH 5.5 and 7.0 (Figure 4). Based on these results, the reported increased stability of enzyme activity at pH 7.0 in 100 mM HEPES (18), and to allow comparison of the present results with those previously reported (18), all subsequent assays were performed in HEPES buffer (100 mM, pH 7.0).

In order to compare with previous reports, the substrate specificity of the recombinant KDO 8-P phosphatase was determined by the discontinuous colorimetric assay in the presence of 1 mM Co^{2+} (18). As shown in Table III, of the seven potential substrates tested, only KDO 8-P
was hydrolyzed at a detectable rate. The kinetic constants of recombinant KDO 8-P phosphatase were determined for KDO 8-P using the continuous coupled assay in the presence of 1 mM Mg\(^{2+}\) at 37 °C. The enzyme exhibited Michaelis-Menten kinetics with an apparent \(K_m\) of 75 ± 5 \(\mu\)M and a \(k_{cat}\) of 175 ± 7 s\(^{-1}\).

Requirement for Divalent Metals—During the purification of wild-type KDO 8-P phosphatase, it was observed that the presence of a divalent metal increased phosphatase activity. To investigate the divalent metal requirements for the recombinant enzyme, the enzyme as isolated was first treated with 10 mM EDTA and the mixture was extensively dialyzed against metal-free buffer to prepare the apoenzyme. The effect of divalent metal on apoenzyme was assessed by adding the divalent metals to the assay mixture to a final concentration of 1 mM (Figure 5). The phosphatase activity was stimulated by Co\(^{2+}\) and Mg\(^{2+}\) about nine fold versus apoenzyme, while Ba\(^{2+}\), Zn\(^{2+}\), and Mn\(^{2+}\) were less effective stimulators and Ca\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\), and Cu\(^{2+}\) had an inhibitory effect on activity. The phosphate hydrolyzing activity of apo-KDO 8-P phosphatase increased with increasing Mg\(^{2+}\) concentrations up to 1 mM (Figure 5 inset). The apoenzyme exhibited a high affinity for Mg\(^{2+}\).

DISCUSSION

KDO is an essential component of the lipopolysaccharide which is present in Gram-negative bacteria while absent in Gram-positive microorganisms. KDO 8-P phosphatase is the only enzyme in the KDO biosynthetic pathway for which the gene responsible for expression has not been identified. The YrbI protein from \textit{E. coli} has been defined as KDO 8-P phosphatase based on the following findings: (i) the N-terminal sequence of the wild-type KDO 8-P phosphatase isolated in the present work corresponds to the \textit{yrbI} orf to which no biological
function had been previously assigned, (ii) a BLASTP search of the NCBI genomic databases using the YrbI sequence identified matches to only hypothetical proteins from Gram-negative bacteria (there were no matches to any proteins from Gram-positive microorganisms), (iii) the recombinant gene product of \textit{yrbI} has a low apparent \( K_m \) of 75 \( \mu \)M for KDO 8-P and a high \( k_{cat} \) of 175 s\(^{-1}\) for KDO 8-P hydrolysis, (iv) no other phosphorylated monosaccharide or compound tested in the present study served as an alternate substrate.

The substrate specificity, kinetic constants, divalent metal requirement, as well as the relatively low pH activity optimum of the recombinant KDO 8-P phosphatase (Table IV) are virtually identical to those reported for the wild-type KDO 8-P phosphatase (18). The pIs and the native MWs of recombinant KDO 8-P phosphatase and the previously reported wild-type KDO 8-P phosphatase (18) are identical. A discrepancy in the denatured MWs was observed, however, between the wild-type and the recombinant protein. The calculated MW of YrbI (188 amino acids) is 19,866. The MW of the recombinant enzyme is 23 kDa as determined by SDS-PAGE and 19,881 by mass spectrometry. The apparent MW of the wild-type enzyme isolated in this report was 52 kDa as determined from 2D gel, as opposed to 40-43 kDa as determined by Ray and Benedict from SDS-PAGE (18). There are several possible explanations for this discrepancy. One possibility could be that wild type and recombinant KDO 8-P phosphatase differ in their inter-subunit interactions due to the fact that one is highly overexpressed while the other is expressed at physiological levels. These differences in quaternary structure between the wild-type enzyme and the recombinant enzyme may account for the variation seen in the denaturation states under the conditions used for SDS-PAGE analysis. Another possibility could be that the wild-type and recombinant KDO 8-P phosphatases actually differ in their amino acid
sequence length/composition. The recombinant enzyme encoded by \textit{yrbI} may simply comprise only the N-terminal segment of the wild-type KDO 8-P phosphatase.

A close examination of the \textit{yrb} operon reveals that the termination codon of the \textit{yrbI} orf overlaps the initiation codon of the downstream \textit{yrbK} orf (ATGA). The \textit{yrbK} orf encodes a 21 kDa protein (191 amino acids). The \textit{yrbI} stop codon, TGA, has long been recognized as a dual signal for either termination or frame shifting (26,27). Translational frame shifting has been observed in retroviruses, retrotansposons, bacterial insertion sequences, bacterial cellular genes, and eukaryotic genes (26,28). Frame shifting events at some recoding sites yield two protein products from one orf or one fusion protein in which the N- and C-terminal regions are encoded by two overlapping orfs respectively. In some instances, this process serves as a control mechanism for the expression of specific genes (27-29). It is therefore attractive to postulate that the wild-type KDO 8-P phosphatase is the product of a -1 translational frame shifting event that occurred at the overlapping sites of \textit{yrbI} and \textit{yrbK}. If this were the case, the cell might express three protein products in varying concentrations, the \textit{yrbI} product (20 kDa), the \textit{yrbK} product (21 kDa), and the \textit{yrbI}-\textit{yrbK} transframe fusion protein (41 kDa). This frame shifting event, which would occur under certain physiological circumstances, could be a regulatory mechanism for the production and/or transport of KDO and, therefore, would serve as a control point in the biosynthesis of LPS region of Gram-negative bacteria. It should be noted that the \textit{E. coli} cells used for isolating the wild-type KDO 8-P phosphatases in both the present study and Ray and Benedict’s study were grown in a glucose minimal medium supplemented with high levels of inorganic phosphate in order to repress the synthesis of alkaline phosphatase. This phosphate-rich growth condition may have induced the production of the putative high MW \textit{yrbI}-\textit{yrbK} transframe protein. Further studies are under way to distinguish between these two possible
explanations as well as other possible scenarios for the observed discrepancies in the denatured MWs.

Based on the biochemical characteristics presented here, KDO 8-P phosphatase can be classified as a specific, low molecular weight acid phosphatase. Amino acid sequence homology analysis has also been utilized to classify phosphatase families (30,31). Such analysis places KDO 8-P phosphatase into the haloacid dehalogenase (HAD) superfamily of hydrolases. This family is comprised of haloacid dehalogenases, epoxide hydrolases, ATPases, phosphomutases and a variety of phosphatases, including phosphoserine phosphatase, phosphoglycolate phosphatase, sucrose-6\(^F\)-phosphate phosphohydrolase and trehalose-6-phosphatase (32-35). KDO 8-P phosphatase, the translated \textit{YrbI} orf, shares the three highly conserved motifs generally observed in this superfamily of enzymes (32,34,36): motif I: DXDX[T/V]; motif II: [S/T]XX; and motif III: K-[G/S][D/S]XXX[D/N] (Figure 6). Although the overall sequence similarity between members of the HAD superfamily is generally low, a comparison of the structures of several members of the family demonstrates a conserved fold and suggests that enzymes in this superfamily most likely evolved from a common ancestor (37). An additional signature sequence motif (GGXGAXRE), unique to the KDO 8-P phosphatase-like sequences identified in the gene data bank, is located in the C-terminal region (Figure 6). Whether this signature sequence plays a role in structure and function unique to KDO 8-P phosphatases remains to be determined.

During the completion of this study, Parsons et al. (38) published the crystal structure of the \textit{YrbI} protein from \textit{Haemophilus influenzae} (HI1679, \textit{MW}_{cal} = 19,432) solved to 1.67-Å resolution. The \textit{H. influenzae} \textit{YrbI} was tetrameric and the monomer subunits exhibited a \(\alpha/\beta\)-hydrolase fold. The active site of each monomer was located at the subunit interface. The active
site was formed mainly by the three conserved motifs characteristic of the HAD superfamily to which KDO 8-P phosphatase belongs. A cobalt ion, used for crystallization, was coordinated at each of the four active sites. Based on structural and sequence analysis as well as enzymatic assays, the authors tentatively assigned the function of the protein to be that of a small molecule phosphatase. Unfortunately, no experimental data on the MW of their recombinant protein was reported to allow comparison. Although the true physiological substrate of their phosphatase was not identified, the authors correctly predicted YrbI to be the sugar phosphatase. The \textit{H. influenzae} and \textit{E. coli} YrbI are 39\% identical; thus, the present study not only confirms their prediction that the \textit{H. influenzae} YrbI is a phosphatase but also suggests the substrate for their enzyme.

In summary, the \textit{E. coli} \textit{yrb}I orf encodes for the protein KDO 8-P phosphatase. This is the first characterized gene in the \textit{yrb} operon in \textit{E. coli} and may help provide a clue to the function of other gene products in this operon. Further studies on this enzyme may provide useful information in the study of the evolution and structure/function of the entire HAD superfamily of enzymes. Additional experiments are in progress to better understand the discrepancies between the MWs of the recombinant and wild-type KDO 8-P phosphatases. Based on the importance of KDO in the lipopolysaccharide biosynthetic pathway, inhibition of KDO 8-P phosphatase will present an attractive target for the design of new generation antibiotics. The determination of the three-dimensional structure of the \textit{E. coli} KDO 8-P phosphatase, now in progress, as well as the structure of various site-directed mutants, in the presence of substrate and/or substrate analogues, will further assist in the elucidation of the mechanism of KDO 8-P phosphatase catalysis which will prove invaluable in the design of inhibitors.
ACKNOWLEDGEMENTS

This investigation was supported by National Institutes of Health Grant GM 53069 (RWW). We thank Dr. George A. Garcia for kindly providing *E. coli* BL21 genomic DNA, Dr. Michael Bly for performing 2D gel electrophoresis, and Ms. Sherry Williams for performing N-terminal amino acid sequencing. We also thank other members of the Woodard group for helpful discussions.

REFERENCES


Figure legends:

Figure 1. The biosynthesis of lipid A-KDO. The enzymes that catalyze these reactions are: (1) D-arabinose 5-phosphate isomerase (KpsF), (2) KDO 8-P synthase (KdsA), (3) KDO 8-P phosphatase, (4) cytidine 5'-monophosphate-KDO synthetase (KdsB), (5) KDO transferase (WaaA).

Figure 2. The 2D gel electrophoresis of wild-type KDO 8-P phosphatase purified from *E. coli* B. Protein spots indicated by arrows were identified by N-terminal sequencing: 1, 2-ketogluconate reductase (TKRA) (N-terminal sequence MKPSVILYKA); 2, fructose-bisphosphate aldolase (fba) (N-terminal sequence SKIFDFVKPG); 3, hypothetical protein (YrbI) (N-terminal sequence SKAGASLAT).

Figure 3. The SDS-PAGE analysis of the purified recombinant KDO 8-P phosphatase. Lane 1, molecular weight standards; lane 2, recombinant phosphatase preparation (10 µg); lane 3, recombinant phosphatase preparation (20 µg). The gel was stained with Coomassie brilliant blue R250.

Figure 4. The pH optimum of the recombinant KDO 8-P phosphatase. Enzymatic activity was measured in the presence of 1 mM KDO 8-P and 1 mM Mg²⁺ by the discontinuous assay at different pH values as described in the “Experimental Procedures”. The results are the averages of triplicate assays.
Figure 5. The metal requirement of recombinant KDO 8-P phosphatase. Enzyme activity was measured in the presence of each indicated metal (final concentration, 1 mM) by the discontinuous assay. None: the activity of the apoenzyme was measured in the absence of added metals. *Inset*, The concentration dependence of apoenzyme activation by Mg$^{2+}$, enzymatic activity was measured by the continuous assay.

Figure 6. Alignment of KDO 8-P phosphatase-like sequences. Sequences were aligned using CLUSTAL W (25). The GenBank accession numbers of the sequences are listed in Table II. Absolute conserved residues are marked with an asterisk (*). Residues that are homologous to conserved residues in motifs I, II, and III of the HAD superfamily are highlighted in black. Conserved residues in the unique signature sequence of KDO 8-P phosphatase (C-terminal region) are highlighted in gray.
Table I

**Purification of wild-type KDO 8-P phosphatase from *E. coli* B**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity b (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>144</td>
<td>3610</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>113</td>
<td>214</td>
<td>0.53</td>
<td>13</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>65</td>
<td>38</td>
<td>1.7</td>
<td>43</td>
</tr>
<tr>
<td>Phenyl Superose</td>
<td>17</td>
<td>4.5</td>
<td>3.7</td>
<td>93</td>
</tr>
<tr>
<td>Mono Q</td>
<td>3.4</td>
<td>0.1</td>
<td>34</td>
<td>850</td>
</tr>
</tbody>
</table>

a The *E. coli* B cells were grown to mid-logarithmic phase in a glucose minimal medium supplemented with inorganic phosphate to repress the synthesis of alkaline phosphatase.
b Enzymatic activity and protein concentration were determined for each purification step post dialysis.

Table II

**YrbI-like sequences from other sources**

<table>
<thead>
<tr>
<th>Sources</th>
<th>Accession number</th>
<th>Definition</th>
<th>Identities to <em>E. coli</em> YrbI (%)</th>
<th>Sequence length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella enterica</em></td>
<td>NP_457695</td>
<td>conserved hypothetical protein</td>
<td>96</td>
<td>188</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>NP_407035</td>
<td>conserved hypothetical protein</td>
<td>75</td>
<td>187</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>NP_232153</td>
<td>conserved hypothetical protein</td>
<td>58</td>
<td>185</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>NP_253148</td>
<td>conserved hypothetical protein</td>
<td>44</td>
<td>179</td>
</tr>
<tr>
<td><em>Ralstonia solanacearum</em></td>
<td>NP_518533</td>
<td>conserved hypothetical protein</td>
<td>43</td>
<td>195</td>
</tr>
<tr>
<td><em>Neisseria meningitidis MC58</em></td>
<td>NP_284827</td>
<td>conserved hypothetical protein</td>
<td>43</td>
<td>178</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>NP_638146</td>
<td>conserved hypothetical protein</td>
<td>40</td>
<td>182</td>
</tr>
<tr>
<td><em>Xylella fastidiosa 9a5c</em></td>
<td>NP_298701</td>
<td>conserved hypothetical protein</td>
<td>39</td>
<td>182</td>
</tr>
<tr>
<td><em>Haemophilus influenzae Rd</em></td>
<td>NP_439821</td>
<td>conserved hypothetical protein</td>
<td>39</td>
<td>180</td>
</tr>
<tr>
<td><em>Thauera aromatica</em></td>
<td>CAC12689</td>
<td>hypothetical protein</td>
<td>38</td>
<td>169</td>
</tr>
<tr>
<td><em>Helicobacter pylori 26695</em></td>
<td>NP_208361</td>
<td>conserved hypothetical protein</td>
<td>36</td>
<td>164</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>NP_245461</td>
<td>unknown</td>
<td>34</td>
<td>181</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>NP_281830</td>
<td>hypothetical protein Cj0647</td>
<td>32</td>
<td>162</td>
</tr>
<tr>
<td><em>Aquifex aeolicus</em></td>
<td>NP_214489</td>
<td>hypothetical protein</td>
<td>32</td>
<td>163</td>
</tr>
<tr>
<td><em>Chlorobium tepidum TLS</em></td>
<td>NP_661718</td>
<td>conserved hypothetical protein</td>
<td>29</td>
<td>172</td>
</tr>
</tbody>
</table>
Table III

Substrate specificity of recombinant E. coli KDO 8-P phosphatase

Recombinant KDO 8-P phosphatase (3 ng for KDO 8-P, and 6 µg for other compounds) was incubated with each indicated substrate and assayed for phosphatase activity by the discontinuous assay. The 50 µl reaction mixture contained 100 mM HEPES (pH 7.0), 5 mM substrate and 1 mM CoCl₂. The results are the averages of triplicate assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDO 8-P</td>
<td>460</td>
</tr>
<tr>
<td>p-Nitrophenylphosphate</td>
<td>0.05</td>
</tr>
<tr>
<td>D-Ribose 5-phosphate</td>
<td>0.02</td>
</tr>
<tr>
<td>D-Arabinose 5-phosphate</td>
<td>0.02</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D-Glucose 6-phosphate</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D-Mannose 6-phosphate</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table IV

Comparison of recombinant KDO 8-P phosphatase and wild-type KDO 8-P phosphatase as reported by Ray and Benedict (18)

<table>
<thead>
<tr>
<th>Property</th>
<th>Recombinant KDO 8-P phosphatase</th>
<th>Wild-type KDO 8-P phosphatase reported by Ray and Benedict</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetic constants</td>
<td>$K_m = 75$ µM</td>
<td>$K_m = 91$ µM</td>
</tr>
<tr>
<td></td>
<td>$V_{max} = 500$ units/mg</td>
<td>$V_{max} = 480$ units/mg</td>
</tr>
<tr>
<td>Alternate substrates</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Metal requirement</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>pH optimum</td>
<td>5.5-7.0</td>
<td>5.5-6.5</td>
</tr>
<tr>
<td>pI</td>
<td>4.6</td>
<td>4.7</td>
</tr>
<tr>
<td>MW (kDa)</td>
<td>89 (gel filtration)</td>
<td>80 (gel filtration)</td>
</tr>
<tr>
<td></td>
<td>23 (SDS-PAGE)</td>
<td>40–43 (SDS-PAGE)</td>
</tr>
<tr>
<td></td>
<td>19.881 (mass spectrometry)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Tetramer</td>
<td>Dimmer</td>
</tr>
</tbody>
</table>
Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

[Graph showing specific activity as a function of divalent cations and log Mg^{2+} (mM) concentration.]

- Co, Mg, Mn, Zn, Ba, Cu, Hg, Cd, Ca, None

Specific Activity (units/mg)

Divalent Cation

log_{10} Mg^{2+} (mM)