A Reciprocal Single Mutation Affects the Metal Requirement of 3-Deoxy-α-manno-2-octulosonate-8-phosphate (KDO8P) Synthases from *Aquifex pyrophilus* and *Escherichia coli* *

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Smadar Shulami‡‡, Cristina Furdui‡, Noam Adir‡‡, Yuval Shoham‡‡¶, Karen S. Anderson‡, and Timor Baasov‡‡‡‡

From the ‡Department of Biotechnology and Food Engineering, the ‡Department of Chemistry, and the ‡Institute of Catalysis Science and Technology, Technion-Israel Institute of Technology, Haifa 32000, Israel, and the †Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520-8066

The enzyme 3-deoxy-α-manno-2-octulosonate-8-phosphate (KDO8P) synthase is metal-dependent in one class of organisms and metal-independent in another. We have used a rapid transient kinetic approach combined with site-directed mutagenesis to characterize the role of the metal ion as well as to explore the catalytic mechanisms of the two classes of enzymes. In the metal-dependent *Aquifex pyrophilus* KDO8P synthase, Cys11 was replaced by Asn (ApC11N), and in the metal-independent *Escherichia coli* KDO8P synthase a reciprocal mutation, Asn26 to Cys, was prepared (EcN26C). The ApC11N mutant retained about 10% of the wild-type maximal activity in the absence of metal ions. Addition of divalent metal ions did not affect the catalytic activity of the mutant enzyme and its catalytic efficiency (kcat/Km) was reduced by only ~12-fold, implying that the ApC11N KDO8P synthase mutant has become a bone fide metal-independent enzyme. The isolated EcN26C mutant had similar metal content and spectral properties as the metal-dependent wild-type *A. pyrophilus* KDO8P synthase. EDTA-treated EcN26C retained about 6% of the wild-type activity, and the addition of Mn2+ or Cd2+ stimulated its activity to ~30% of the wild-type maximal activity. This suggests that EcN26C KDO8P synthase mutant has properties similar to that of metal-dependent KDO8P synthases. The combined data indicate that the metal ion is not directly involved in the chemistry of the KDO8P synthase catalyzed reaction, but has an important structural role in metal-dependent enzymes in maintaining the correct orientation of the substrates and/or reaction intermediate(s) in the enzyme active site.

The enzyme 3-deoxy-α-manno-2-octulosonate-8-phosphate (KDO8P)* synthase (EC 4.1.2.16) catalyzes the condensation reaction between D-arabinose-5-phosphate (A5P) and phosphoenolpyruvate (PEP) to form KDO8P and inorganic phosphate (P) (see Scheme 1) (1). This enzymatic reaction plays an essential role in the assembly process of lipopolysaccharides of most Gram-negative bacteria, and therefore represents an attractive target for the design of novel antibacterial drugs (2, 3).

While earlier studies on *Escherichia coli* KDO8P synthase have established that this enzyme does not require metals (1), it has recently been demonstrated that enzymes from the hyperthermophilic bacteria *Aquifex aeolicus* (4), *Aquifex pyrophilus* (5), and from the pathogenic bacterium *Helicobacter pylori* (6), require a divalent metal cofactor for catalysis. Furthermore, phylogenetic analysis (7, 8) suggests that KDO8P synthases from other pathogenic bacteria, i.e. *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Campylobacter jejuni*, may also be metal-dependent enzymes. This is reminiscent of another class of enzymes, aldolases, which catalyze a similar aldol-type C-C bond formation (9), Class I aldolases, which are primarily found in animals and higher plants, do not require a metal cofactor for catalysis. In contrast, Class II aldolases, found predominantly in prokaryotes, use a divalent metal cofactor that functions as a Lewis acid. Thus, in addition to aldolases, KDO8P synthase represents another example of an enzyme that is metal-independent in one class of organisms (Class I) but metal-dependent in another (Class II). Therefore, KDO8P synthase represents a distinctive target for the development of selective, narrow-spectrum antibiotics.

The catalytic mechanism of *E. coli* KDO8P synthase has been studied extensively and earlier studies have established that the reaction occurs through the unusual cleavage of the C-O bond of PEP (10, 11). KDO8P synthesis was shown to be a sequential process in which the kinetically preferred order of binding involves the PEP preceding the binding of acyclic A5P, and the release of inorganic phosphate preceding the dissociation of the product KDO8P (12). The condensation step was shown to be stereospecific, with the si face of PEP attaching to the re face of the carboxyl group of A5P (see Scheme 1) (13, 14). More recent studies using rapid chemical quench techniques (15), together with the synthesis and evaluation of an acyclic bisubstrate inhibitor (16), supported the reaction pathway that involves the formation of an inherently unstable, acyclic bisphosphate intermediate I (Scheme 1). Moreover, the identification of I as a true reaction intermediate was provided...
and conducted an initial biochemical characterization of the recombinant enzyme (5). Sequence alignment revealed that the four amino acid residues that were identified in *A. aeolicus* KDOSP synthase as ligands of the metal ion are located at identical positions in the *A. pyrophilus* enzyme. Therefore, it is likely that these residues are also involved in metal binding in the *A. pyrophilus* KDOSP synthase. It is noteworthy that three out of the four metal binding residues, His5, Glu175, and Asp148 in metal-dependent KDOSP synthases, are completely conserved in all KDOSP synthases currently sequenced (7), implying that these residues are also involved in metal binding in the *A. pyrophilus* KDOSP synthase. In all KDOSP synthases, it is shown that a single amino acid replacement, Cys to Asn, could eliminate the requirement for metals in metal-dependent enzymes, and similarly, the reciprocal replacement in metal-independent *E. coli* KDOSP synthase (Asn286 to Cys) could result in metal-dependent activity.

In the present work, we describe the characterization of a C11N mutant of *A. pyrophilus* KDOSP synthase (ApC11N) and an N26C mutant of *E. coli* KDOSP synthase (EcN26C) with respect to metal binding and catalysis. The specific questions these studies were designed to address are as follows: (i) What is the role of the divalent metal ion in metal-dependent enzyme? Does the metal ion play a direct catalytic or structural role in catalysis? (ii) Can a single amino acid mutation be sufficient to convert the enzyme from a metal-dependent to a metal-independent and vice versa? Some important mechanistic implications of these observations are discussed.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—** ASP was prepared enzymatically according to the procedure of Whitesides and co-workers (25). The potassium salt of PEP was prepared in large quantities as previously described (26). Metal salts used in this study were obtained from Aldrich and all other chemicals were purchased from Aldrich or from Sigma and were used without further purification. Solutions and buffers were prepared through a column of Chelex 100 (Na+ from 100–200 mesh; Bio-Rad), to remove all traces of metal ion contamination. [1-14C]Pyruvate was obtained from American Radiolabeled Chemicals, and the HPLC Mono Q 5/5 anion exchange column was from Amersham Biosciences.

**[1-14C]PEP Synthesis—** Radiolabeled PEP was enzymatically synthesized from [1-14C]pyruvate (American Radiolabeled Chemicals) by coupling pyruvate phosphate dikinase (PPDK) obtained as a generous gift from Dr. Dunaway-Mariano, to inorganic pyrophosphatase reaction. After purification by Q-Sepharose anion-exchange chromatography using a linear gradient (20 mM to 1 M) of triethylammonium bicarbonate (TEAB) buffer, the fractions containing PEP were pooled and lyophilized. Final stock solutions (5.6 mM) contained 29,300 dpm/nmol.

**Bacterial Strains and Plasmids—** *E. coli* strain XL-1 Blue (Stratagene, La Jolla, CA) was used for general cloning. *E. coli* strain BL21(DE3) (Promega, Madison, WI) was used for protein production with the pET9d expression vector (Novagen, Madison, WI).

**DNA Manipulations—** Plasmid DNA was purified with the Wizard DNA Clean-Up system (Promega). DNA was transformed by electroporation using GeneZapper (IBI, New Haven, CT) for strains XL-1 Blue and BL21(DE3). DNA sequencing was performed at the University of Wisconsin, Madison, WI.

**Cloning and Mutagenesis—** The kdsA gene (GenBank accession number AT135560) from *A. pyrophilus* was cloned in the pET9d expression vector (Novagen, Madison, WI) as previously described (5). The kdsA gene from *E. coli* was amplified from the pJU1 vector (12), using

![Scheme 1. Proposed mechanism for KDOSP synthase-catalyzed reaction.](https://example.com/scheme1.png)
two PCR primers that allowed the in-frame cloning in the pET vector. The N-terminal primer (5′-GGGATCATAGGAACTACATTGGTCGGTAAGCGTGC-3′) was made to contain an ATG transnational start codon inside a BspHI restriction site (TCATG). The C-terminal primer (5′-GGGGATCCGGAATTCATGGTCGGTAAGCGTGC-3′) contained a stop codon (TAG) and a BglII restriction site (AGATCT) at the end of the gene. Following PCR amplification the gene was cloned into the T7 expression vector pET9d (linearized with NcoI and BamHI), resulting in plasmid pET9d-kdsA.

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers for C11N of KDO8P synthase from A. pyrophilus were as follows (the mutated nucleotides are shown in bold): 5′-GACTGGCTTCTCAGTGCCCTAGTGTGTGGCGG-3′ and 5′-GGGGACCCATATCGGATCGAGAGCGAGTC-3′. The primers for N26C of KDO8P synthase from E. coli were as follows (the mutated nucleotides are shown in bold): 5′-GATTCACACCGCATGCGGCCACAAGTGAC-3′ and 5′-GATCTGTTTGGCGGCATGTCCTUTTGGAATC-3′. The mutated genes were sequenced to confirm that only the desired mutation was inserted. The mutated genes were overexpressed and purified as described below.

**Purification of the kdsA Gene Products**—The recombinant kdsA genes from both E. coli (1) and A. pyrophilus bacteria (5) were expressed using similar procedures. Briefly, the expression was carried out by growing overnight cultures of E. coli BL21(DE3) carrying pET9d-kdsA in Luria-Bertani (LB) broth (27) with kanamycin (25 μg ml−1), without induction. Purification procedure of the kdsA gene from E. coli was similar to that of the thermostable enzyme but with the following modifications: all manipulations were carried out at 4 °C and without the heat treatment step. The overnight cultures (2 liters) were harvested, resuspended in 15 ml of 50 mm NaCl and 20 mm Tris-HCl buffer pH 7.5, and disrupted by two passages through a French Press (Spectronic Instruments, Inc., Rochester, NY) at room temperature. Cell extracts were centrifuged, and the resulting supernatant was applied to a column of Chelex 100–200 mesh, Bio-Rad). Additional purification steps for the formation of intermediate 1.

Steady-state Kinetic Analysis—The KDO8P synthase activity was determined either by a discontinuous colorimetric assay or a continuous spectrophotometric assay. All experiments were carried out in 100 mm HEPES pH 7.5 at 60 °C for A. pyrophilus wild-type and C11N KDO8P synthase and at 25 or 37 °C for E. coli wild-type and N26C KDO8P synthase in 50 mm Tris, pH 7.5. The dependence of A. pyrophilus wild-type and C11N-KDO8P synthase on metal chelator concentration was also analyzed for metal content, and the observed concentrations were subtracted from those obtained in the enzymes samples. The recombinant E. coli N26C—An aliquot of the EDTA-treated enzyme (20 μM) was diluted in 500 μl of metal-free buffer (50 mm Tris-HCl), pH 7.5, and various concentrations of CuSO4 were added. After 2 min of incubation at 35 °C, the absorbance spectra were recorded on a Biochrom 4060 spectrophotometer.

Metal Content Analysis for the As-isolated and EDTA-treated KDO8P Synthase—A discontinuous assay was used to study the effects of metal chelators and divalent metal ions on enzymatic activities. The as-isolated assay, the amount of the KDO8P product was determined by the thionitrobenzoic acid assay as specified by Ray (1). One unit of activity is defined as the amount of enzyme required to produce 1 μmol of KDO8P per minute. The standard discontinuous assay was conducted in a final volume of 200 μl of 100 mm HEPES or 50 mm Tris pH 7.5, 100 μM PEP, 2 mm A5P, and appropriately diluted enzyme. After incubation, the reaction was quenched by adding trichloroacetic acid to a final concentration of 5%. To ensure initial velocity conditions, the enzyme concentration and the incubation time were chosen so that the substrate conversion was less than 5%, and the product formation was linear over the time course the measurements were made.

(i) Catalytic Activity of A. pyrophilus Wild-type and C11N KDO8P Synthase—The specific activities of wild-type and mutant KDO8P synthase from A. pyrophilus were determined at 60 °C in 100 mm HEPES buffer, pH 7.5. Aliquots (10 μl) of the wild-type enzyme (0.3 μM) or its C11N mutant (50 μM) were first preincubated with EDTA (10 μl, 4 mm) for 15 min at room temperature. In the case of wild-type enzyme, a buffer solution (160 μl) containing 0.48 mm Cd2+ was added to the enzyme followed by 20 μl of 20 mM PEP. In the case of the C11N mutant, Cd2+ was omitted from the reaction buffer. The resulting mixture was incubated for 10 min at 60 °C, and the reaction was initiated by adding 20 μl of A5P (20 mM). After incubation at 60 °C, the reaction was quenched by adding trichloroacetic acid to a final concentration of 5%. The effect of chelators on the activity of A. pyrophilus wild-type KDO8P synthase and the C11N mutant, was determined as follows: 10 μl of enzyme (2 μM) was incubated with 10 μl of EDTA or 1,10-phenanthroline at a final concentration of 10–500 μM for 15 min at room temperature. The assays were performed at 60 °C with the same final concentration of the chelator. The effect of various divalent metals was measured by preincubating the enzyme with 4 mM EDTA for 15 min at room temperature, followed by addition of reaction buffer (100 mm HEPES, pH 7.5, 160 μM) containing various metal concentrations, and the resulting mixture was again incubated for an additional 10 min at
and PEP (20 mM) were prewarmed for 10 min at 37 °C. The reaction was then quenched with 67 µl of 1M KOH. 60 µM of various metals and 20 µl of the mixture of substrates, A5P (20 mM) and PEP (20 mM) were prewarmed for 10 min at 37 °C. The reaction was initiated by adding 20 µl of wild type (0.3 µM) or EDTA-treated N26C mutant (6 µM), and the reaction was allowed to proceed under the initial velocity conditions.

More detailed kinetic studies were carried out using a continuous spectrophotometric assay. The reactions were performed in 100 mM Tris, pH 7.5 at 37 and 60 °C for E. coli KDO8P synthase and A. pyrophilus enzymes, respectively. Assay solutions contained appropriately diluted enzymes with varying concentrations (0.1 to 5 Kₘ, max) of one substrate while the other substrate was held constant (10 × Kₘ, max). Data were fit to Michaelis-Menten equation for metal dependence experiments or to the Hill equation when cooperative behavior was observed for substrate dependence experiments. The Hill coefficients (n_H) parameters for metal, PEP, and A5P were determined. The values of Kₘ and kₗin were determined by non-linear regression analysis using the program GRAFIT 5.0 or Kaleidagraph 3.52.

Pre-steady-state Experiments—Rapid quench experiments were performed using a Kintek RFQ-3 Rapid Chemical Quench (Kintek Instruments, Austin, TX) as previously described (28). The reaction was formed using a KDO8P synthase activity was assayed as follows: 160 µl of each sample was removed and analyzed by HPLC with on-line radioactivity detection (Packard Instruments, Downers Grove, IL). The products formed with a flow rate of 1 ml/min. Before entering the radioactivity detector the eluent was mixed with liquid scintillation mixture (Mone-flo V, National Diagnostics) at a flow rate of 5 ml/min. During the HPLC analysis a small amount of cold PEP was always injected as an internal standard as it co-elutes with hot PEP and its elution time can be monitored at 232 nm by absorbance detector. The retention times for KDO8P and PEP were 12.7 min and 16.5 min, respectively.

Pre-steady-state Burst Experiment for ApC11N—Pre-steady-state burst experiments in the presence of different metal concentrations: 20 µM N26C, 60 µM [1-14C]PEP, and 500 µM EDTA were mixed with 1 mM A5P in 50 mM Tris, pH 7.5 in a rapid-quench apparatus. The reaction was quenched with 0.3 M KOH, and the formation of KDO8P was followed by HPLC as described above. (ii) Pre-steady-state burst experiments in the presence of different metal concentrations: 20 µM N26C, 60 µM [1-14C]PEP, 500 µM EDTA, and different Cd²⁺ concentrations were mixed with 1 mM A5P in 50 mM Tris pH 7.5 in a rapid chemical quench apparatus. The total metal concentrations added into the solution were: 0.52 mM CdCl₂ (300 µM free metal), 1.2 mM (800 µM free metal), and 1.9 mM (1.4 mM free metal). The reaction was quenched with 0.3 M KOH, and the formation of KDO8P was followed by HPLC as described above. In this expression, C represents the product concentration at time t, the amplitude C₀ corresponds to active site concentration, kₗis the rate constant for product formation (s⁻¹) and kₘ is the rate of product release (s⁻¹). By dividing C₀ with the enzyme concentration, we determined the percentage of active enzyme. Data were analyzed using Kaleidagraph version 3.52, released June 17, 2002 by Synergy Software.

Metal Content Analysis—For both E. coli and A. pyrophilus enzymes, metal content was analyzed by ICP-MS (Table I). The wild-type E. coli KDO8P synthase, as isolated, contained only 0.05 ± 0.01 mol of zinc and 0.05 ± 0.01 mol of magnesium per mol of enzyme. All of the other metals tested were below the detection limit. The EcN26C mutant, as isolated, contained ~0.2 mol of iron and 0.4 mol of zinc per mol of enzyme. Manganese, copper, chromium, nickel, cadmium, and cobalt were below the detection limit. This mutant enzyme exhibited a specific activity of 0.14 units/mg. Treatment with EDTA reduced the iron and zinc content to 0.009 mol per mol of enzyme. 0.15 mM EDTA was used as described above. Performing the reaction at higher enzyme concentrations was hindered by the formation of aggregates, which resulted in lower active site concentration and lower burst rate.

Pre-steady-state Burst Experiment for EcN26C—Pre-steady-state analysis of this mutant enzyme was complicated by a minor contaminant protein(s) possessing an activity that degraded the radiolabeled PEP to pyruvate as evidenced by HPLC analysis with radioactivity detection. This degradation of PEP occurred when the mutant enzyme was incubated with PEP for extended periods of time required to collect a time course for rapid chemical quench. Removal of the contaminating activity was accomplished by further purification using anion exchange chromatography. The specific activity after extended purification was somewhat lower. However, the contaminating activity causing PEP degradation was removed thus allowing further analysis using a transient kinetic strategy. (i) Pre-steady-state burst experiment in the absence of metals: 20 µM N26C, 60 µM [1-14C]PEP, and 500 µM EDTA were mixed with 1 mM A5P in 50 mM Tris, pH 7.5 in a rapid-quench apparatus. The reaction was quenched with 0.3 M KOH, and the formation of KDO8P was followed by HPLC as described above. (ii) Pre-steady-state burst experiments in the presence of different metal concentrations: 20 µM N26C, 60 µM [1-14C]PEP, 500 µM EDTA, and different Cd²⁺ concentrations were mixed with 1 mM A5P in 50 mM Tris pH 7.5 in a rapid chemical quench apparatus. The total metal concentrations added into the solution were: 0.52 mM CdCl₂ (300 µM free metal), 1.2 mM (800 µM free metal), and 1.9 mM (1.4 mM free metal). The reaction was quenched with 0.3 M KOH, and the formation of KDO8P was followed by HPLC as described above.

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The wild-type metal-dependent KDO8P synthase from A. pyrophilus, as isolated, contained 0.19 and 0.26 mol of iron
and zinc, respectively, per mol of enzyme (5), whereas metal analysis of the C11N variant revealed 0.22 mol of iron and 0.02 mol of zinc per mol of enzyme (Table I). Thus, it appears that the loss of Cys11 decreases the apparent affinity of KDO8P synthase for zinc but not for iron. Similar analysis of the wild-type and C11N mutant of A. pyrophilus KDO8P synthase, Cys11 to Asn, could eliminate the requirement for metal assistance. The consequence of C11N replacement in KDO8P synthase was investigated in direct coordination of the metal type and C11N mutant of E. coli KDO8P synthase—As mentioned above, the activity of ApC11N was not affected by metal chelators. Our working hypothesis assumes that the reciprocal mutation of the conserved Asn218 to Cys in the metal-independent E. coli enzyme might result in a metal-dependent KDO8P synthase (5). To examine this hypothesis the wild-type and the EDTA-treated EcN26C were separately incubated with various divalent metal ions and the respective catalytic activities were determined. Of the metals examined, Mn2+ and Cd2+ enhanced the original activity of EDTA-treated N26C by about 5-fold (Fig. 4), resulting in a 50% increase in the steady-state rate (kcat = 9.0 s−1) (Fig. 3A) with apparent KmMn2+ of 10 μM. However, when the same experiment was performed with Cd2+, it was found that although the kcat was only slightly lower than for the Mn2+-reconstituted enzyme (8.5 s−1 versus 9.0 s−1), there is a 16-fold difference in the Km values (0.6 μM versus 10 μM) (Fig. 3B).

According to the x-ray crystal structure of A. aeolicus KDO8P synthase, Cys11 is involved in direct coordination of the metal (22); therefore replacing Cys11 with Asn in KDO8P synthase from A. pyrophilus should affect metal binding. The consequence of C11N replacement on metal binding was evaluated by measuring enzyme activity in the presence of metal ions. Addition of Mn2+ up to 2 mM did not stimulate the reaction rate of C11N mutant. However, addition of Cd2+ increased the steady-state rate by about 3-fold (kcat = 1.5 s−1, Fig. 3C). The apparent KmCd2+ obtained from fitting the data to Michaelis-Menten equation was 640 μM.

Metal Requirement for the Wild-type and N26C Mutant E. coli KDO8P Synthase—As mentioned above, the activity of ApC11N was not affected by metal chelators. Our working hypothesis assumes that the reciprocal mutation of the conserved Asn218 to Cys in the metal-independent E. coli enzyme might result in a metal-dependent KDO8P synthase (5). To examine this hypothesis the wild-type and the EDTA-treated EcN26C were separately incubated with various divalent metal ions and the respective catalytic activities were determined. Of the metals examined, Mn2+ and Cd2+ enhanced the original activity of EDTA-treated N26C by about 5-fold (Fig. 4), resulting in a 50% increase in the steady-state rate (kcat = 9.0 s−1) (Fig. 3A) with apparent KmMn2+ of 10 μM. However, when the same experiment was performed with Cd2+, it was found that although the kcat was only slightly lower than for the Mn2+-reconstituted enzyme (8.5 s−1 versus 9.0 s−1), there is a 16-fold difference in the Km values (0.6 μM versus 10 μM) (Fig. 3B).

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Metal Chelators Have No Effect on the Activity of ApC11N—To examine whether a single amino acid replacement, Cys11 to Asn, could eliminate the requirement for metal ions in A. pyrophilus KDO8P synthase, the activity of ApC11N was determined in the presence of metal chelators. In the presence of EDTA, the specific activity of ApC11N (0.8 units/mg) was 10% of the maximal activity of Cd2+-reconstituted wild type (8.0 units/mg, Table I). Unlike the wild-type A. pyrophilus KDO8P synthase, neither EDTA nor 1,10-phenanthroline influenced the activity of ApC11N (Fig. 2). When subjected to ICP-MS analysis, the EDTA-treated mutant and wild-type enzymes, showed almost identical metal content, less than 0.15 mol of metals per mol of enzyme (Table I). These data indicate that the ApC11N can catalyze the reaction without metal assistance.

Metal Requirement for the Wild-type and C11N Mutant A. pyrophilus KDO8P Synthase—The EDTA treatment of wild-type A. pyrophilus KDO8P synthase, almost completely abolishes its activity, resulting in kcat of 0.045 ± 0.011 s−1. By following the dependence of the steady-state rate on metal ion concentration and fitting the data to a Michaelis-Menten equation, it was found that the addition of Mn2+ to the wild-type KDO8P synthase resulted in a 200-fold increase in the steady-state rate (kcat = 9.0 s−1) (Fig. 3A) with apparent KMMn2+ of 10 μM. However, when the same experiment was performed with Cd2+, it was found that although the kcat was only slightly lower than for the Mn2+-reconstituted enzyme (8.5 s−1 versus 9.0 s−1), there is a 16-fold difference in the Km values (0.6 μM versus 10 μM) (Fig. 3B).

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Metal Requirement for the Wild-type and N26C Mutant E. coli KDO8P Synthase—As mentioned above, the activity of ApC11N was not affected by metal chelators. Our working hypothesis assumes that the reciprocal mutation of the conserved Asn218 to Cys in the metal-independent E. coli enzyme might result in a metal-dependent KDO8P synthase (5). To examine this hypothesis the wild-type and the EDTA-treated EcN26C were separately incubated with various divalent metal ions and the respective catalytic activities were determined. Of the metals examined, Mn2+ and Cd2+ enhanced the original activity of EDTA-treated N26C by about 5-fold (Fig. 4), resulting in a 50% increase in the steady-state rate (kcat = 9.0 s−1) (Fig. 3A) with apparent KmMn2+ of 10 μM. However, when the same experiment was performed with Cd2+, it was found that although the kcat was only slightly lower than for the Mn2+-reconstituted enzyme (8.5 s−1 versus 9.0 s−1), there is a 16-fold difference in the Km values (0.6 μM versus 10 μM) (Fig. 3B).

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parameters of the wild-type and the C11N mutant KDO8P synthase, we followed the dependence of the steady-state rate on PEP and A5P concentrations. The results of these kinetic studies are summarized in Table II. For the wild-type Mn2+/H11001-KDO8P synthase the $k_{\text{cat}}$ value was 9.0 s$^{-1}$, and the $K_m$ for PEP and A5P were 26 M and 67 M, respectively (Fig. 5, A and B).

We performed the same experiment with the C11N KDO8P synthase (Fig. 5, C and D). The $k_{\text{cat}}$ for the mutant enzyme was 0.42 s$^{-1}$ and the $K_m$ for PEP and A5P were 17 and 140 M, respectively. Cooperative behavior was noted for the Mn2+/H11001-reconstituted wild-type A. pyrophilus KDO8P synthase as well as for the C11N mutant. The respective Hill coefficients are presented in Table II. The catalytic rates were not affected by the order of substrates addition.

Steady-state Kinetic Parameters for Wild-type and N26C Mutant E. coli KDO8P Synthase—Kinetic analysis of the native and N26C mutant of E. coli KDO8P synthase were carried out with or without reconstitution with metals and the kinetic parameters are presented in Table II. Comparison between the kinetic parameters of the wild-type E. coli KDO8P synthase and its N26C mutant reveals the following: (i) EDTA-treated N26C mutant exhibits a 17-fold lower $k_{\text{cat}}$, yet the presence of metals increases the $k_{\text{cat}}$ values 5-fold. (ii) In the absence of metals, the N26C mutant has about 4–5-fold higher $K_m$ for both A5P and PEP as compared with the wild-type enzyme; however, in the presence of Mn2+ the $K_m$ for PEP decreases, while in the presence of Cd2+ the $K_m$ for PEP is equal to the $K_m$ for the wild-type enzyme. iii) The $k_{\text{cat}}/K_m$ for PEP in the presence of metals was elevated ~20-fold, suggesting the important role played by the metals in the PEP interaction with the enzyme. The value for $k_{\text{cat}}/K_m$ for A5P is also changed although only a factor of 4–6.

Pre-steady-state Burst Experiments for Mn2+/H11001- and Cd2+/H11001-reconstituted Wild-type A. pyrophilus KDO8P Synthase and the C11N Mutant—The purpose of the pre-steady-state burst experiments was to determine the rate-limiting step of the reaction, the active site concentration, and the rate constant of product formation for the Mn2+/H11001- and Cd2+/H11001-reconstituted KDO8P synthase. Mn2+ - and Cd2+ -reconstituted enzymes have almost the same $k_{\text{cat}}$ and a burst in product formation was observed for both the Mn2+/H11001- and Cd2+/H11001-KDO8P synthase. The metal ion addition resulted in a fully active enzyme, the burst amplitude showing close to 100% active site concentration. The linear rate of product formation determined from the pre-steady-state burst experiments matched the steady-state rate ($k_{\text{cat}}$) determined from steady-state experiments with almost no difference observed between the Mn2+/H11001 (8.0 s$^{-1}$) and Cd2+/H11001 (9.9 s$^{-1}$) enzyme. However, the major differences were in the rate constants of the burst phase that strictly determine the rate constants of chemical catalysis. This value for the Mn2+/H11001-
KDO8P Synthase from A. pyrophilus and E. coli

KDO8P synthase represents one of the rare examples of an enzyme that is metal-dependent in one class of organisms and metal-independent in another (7, 8). In this study, a metal-dependent enzyme, the A. pyrophilus KDO8P synthase, and a metal-independent enzyme, the E. coli KDO8P synthase, were used to define the function of the divalent metal ion, as well as to identify similarities and/or differences between the catalytic mechanisms of the two classes of enzymes. For this purpose, two mutant enzymes ApC11N and EcN26C were prepared and characterized with respect to metal effects and catalysis.

The three-dimensional structure of KDO8P synthase of A. pyrophilus used in this study is assumed to be similar to that of the highly homologous A. aeolicus enzyme, for which high resolution structures have been determined by x-ray crystallography (22, 23). When superimposed, the A. aeolicus (23) and E. coli (20) structures are almost identical in their general fold (root mean square deviation of 1.2 Å between comparable α carbons). In Fig. 9, the residues serving as ligands to the bound metal ion (Cd^{2+}) in the A. aeolicus structure (Aa, shown in green stick representation) are shown superimposed on the homologous residues from the E. coli enzyme (Ec, shown in gray stick representation). Three residues are very similar in their orientation, although no metal ion is present in the E. coli enzyme. The EcD250 residue and the homologous AaD233 residue (Fig. 9), are located on a flexible loop and have a dissimilar orientation in the two crystals. Crystallographic determination of the EcN26C in the presence of metal ions will be necessary to determine whether under these conditions the Asp^{250} residue flips over to ligate the metal ion.

ApC11N: A Metal-independent KDO8P Synthase Enzyme—As shown in Table I, the C11N mutant enzyme contains the similar amount of iron (~0.2 mol) as the wild-type enzyme; however this metal does not affect the activity of the mutant enzyme (~0.8 units/mg), as it remained unchanged before and after treatment with EDTA or 1,10-phenanthroline (Table I and Fig. 2). Addition of exogenous divalent metal cations such as Zn^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}, which typically stimulate the activity of the wild-type enzyme at different levels (5), had no effect on the activity of C11N, and only Cd^{2+} (0.6 mM) stimulated its activity by 1.5-fold (1.2 units/mg; Table I). These results indicate that the replacement of Cys^{11} by Asn in A. pyrophilus KDO8P synthase eliminates the requirement of a divalent metal cation for catalysis, resulting in an active metal-independent variant.

Additional support to this conclusion was gained from detailed kinetic characterization of both the wild-type and C11N A. pyrophilus KDO8P synthases and their comparison to the kinetics of other Class II and Class I KDO8P synthases. Significantly, the apparent affinity to Cd^{2+} ions by the C11N mutant (K_{M} = 640 μM) is reduced by about three orders of magnitude versus that of the wild-type enzyme (K_{M} = 0.6 μM) (Fig. 3). In contrast, the catalytic efficiency (k_{cat}/K_{M}) of the EDTA-treated C11N mutant enzyme toward the PEP substrate is reduced by only about 12-fold, versus that of the Cd^{2+}-reconstituted wild-type enzyme (Table II). Interestingly, very similar (8-fold) reduction in catalytic efficiency toward the PEP substrate of the native H. pylori enzyme (Class II enzyme) versus E. coli enzyme (Class I enzyme) was recently reported (30). This difference was further reduced to 4.5-fold when comparing H. pylori Cd^{2+}-KDO8P synthase and E. coli KDO8P synthase. As seen from the data in Table II, the A. pyrophilus Cd^{2+}-KDO8P synthase is also about 3-fold less efficient than the wild-type E. coli enzyme. It is noteworthy that similar to the H. pylori KDO8P synthase (30), the Mn^{2+}-reconstituted wild-type A. pyrophilus and C11N mutant displayed cooper-
tive behavior. Moreover, it was observed that the metal may influence stability since the C11N mutant enzyme aggregates and is less active at higher protein concentrations (H11022/H9262 M).

Transient kinetic analysis of the wild-type A. pyrophilus enzyme revealed that the overall rate-limiting step for the reaction was product release and that the rate constant of chemical catalysis was influenced by the type of metal with Cd²⁺-KDO8P synthase being 3-fold faster than Mn²⁺-KDO8P synthase (Fig. 6). Product release was rate-limiting for the C11N mutant as well although the rate constant of chemical catalysis was slower (Fig. 7).

Is the EcN26C a Metal-requiring Enzyme?—Several lines of evidence obtained in this study substantiate that N26C mutant of E. coli KDO8P synthase has properties similar to that of metal-dependent Class II KDO8P synthase. First, the observed spectral properties of the N26C mutant enzyme are very similar to those of Class II enzymes. Thus, unlike the wild-type E. coli enzyme which is colorless even at concentrations of 20 mg/ml, the N26C mutant as isolated displayed a characteristic pinkish color at concentrations above 4 mg/ml as reflected by a broad absorption band centered at 575 nm (curve 2 in Fig. 1), similar to that observed for the wild-type A. aeolicus (4) and A. pyrophilus (5) enzymes. This spectral property is suggestive of the presence of protein thiolate associated ferric ion (4, 5) in the isolated N26C enzyme, which was further confirmed by metal analysis (Table I). The elimination of the 575 nm absorp-

| Source | Enzyme              |  k<sub>cat</sub> | K<sub>A5P</sub> | K<sub>PEP</sub> | k<sub>cat</sub>/K<sub>A5P</sub> | k<sub>cat</sub>/K<sub>PEP</sub> |
|--------|---------------------|----------------|----------------|----------------|-----------------------------|-----------------------------|
| A. pyrophilus | Cd<sup>2+</sup>-wild type | 6.0 ± 0.8 | 18 ± 2 | 16 ± 2 | 0.33 | 0.37 |
|          | Mn<sup>2+</sup>-wild type | 9.0 ± 0.8 | 67 ± 6 (1.6)<sup>a</sup> | 26 ± 3 (2)<sup>a</sup> | 0.13 | 0.34 |
|          | EDTA-treated C11N | 0.42 ± 0.03 | 140 ± 12 (1.66)<sup>a</sup> | 17 ± 2 (2.5)<sup>a</sup> | 0.003 | 0.03 |
|          | Wild-type | 6.1 ± 0.6 | 20 ± 2 | 6.0 ± 0.8 | 0.3 | 1.0 |
|          | EDTA-treated N26C | 0.36 ± 0.04 | 75 ± 11 | 32 ± 4 | 0.005 | 0.01 |
|          | Mn<sup>2+</sup>-N26C | 1.9 ± 0.1 | 70 ± 9 | 19 ± 2 | 0.03 | 0.1 |
|          | Cd<sup>2+</sup>-N26C | 1.9 ± 0.1 | 110 ± 14 | 5.8 ± 1.3 | 0.02 | 0.33 |
| E. coli | Wild-type | 6.1 ± 0.6 | 20 ± 2 | 6.0 ± 0.8 | 0.3 | 1.0 |
|          | EDTA-treated N26C | 0.36 ± 0.04 | 75 ± 11 | 32 ± 4 | 0.005 | 0.01 |
|          | Mn<sup>2+</sup>-N26C | 1.9 ± 0.1 | 70 ± 9 | 19 ± 2 | 0.03 | 0.1 |
|          | Cd<sup>2+</sup>-N26C | 1.9 ± 0.1 | 110 ± 14 | 5.8 ± 1.3 | 0.02 | 0.33 |

<sup>a</sup>Hill coefficient numbers (n<sub>H</sub>) are given in parentheses.
tion band upon addition of excess CuSO₄ and concomitant appearance of the new peak at 385 nm suggests displacement of the enzyme bound ferric ion by the copper (4, 29). Titration of the EDTA-treated N26C with CuSO₄ generates the same 385-nm band with the stoichiometry of one Cu²⁺ ion per enzyme subunit (Fig. 1).

Second, the performance of N26C in terms of its apparent affinity to divalent metal ions and treatment with metal chelators is also similar to that of Class II enzymes. For example, the isolated N26C displays similar content of zinc and iron as the wild-type A. pyrophilus enzyme (Table I), and treatment with EDTA removes these metals from the enzyme, as confirmed by metal analysis (Table I) and by disappearance of the characteristic 575 nm absorption band (Fig. 1). Furthermore, while the EDTA-treated N26C enzyme retains ~6% of the wild-type activity, addition of Cd²⁺ or Mn²⁺ stimulate its activity up to ~30% of the wild-type activity (Fig. 4), indicating significant role of metal ions on its catalytic performance.

Third, kinetic characterization of N26C strengthens the above conclusion. As seen from Table II, while the EDTA-treated N26C is 100-fold less efficient (with respect to PEP) than the wild-type E. coli KDO8P synthase, this difference was reduced to 10-fold and 3-fold in the Mn²⁺- and Cd²⁺-reconstituted N26C enzymes, respectively. These differences are due to variations in both the $k_{cat}$ and the $K_m$ values. The $k_{cat}$ of the EDTA-treated N26C was determined to be 17-fold lower than the wild-type E. coli enzyme. However, the $k_{cat}$ values of both the Cd²⁺-N26C and Mn²⁺-N26C were the same, about 3-fold lower than that of the wild-type enzyme. In general, the apparent affinity of the mutant enzyme for both substrates, PEP and A5P, is reduced by a similar extent (either in EDTA-treated or metal-reconstituted form) and only Cd²⁺-N26C has the same $K_m^{PEP}$ value as the wild-type enzyme. Importantly, the catalytic efficiency of Cd²⁺-N26C toward PEP is the same (0.35 μM⁻¹ s⁻¹) as that of the A. pyrophilus Cd²⁺-KDO8P synthase (0.37 μM⁻¹ s⁻¹).

Transient kinetic analysis of the N26C mutant revealed that the overall rate-limiting step is influenced by the presence of metal since it was shown that the rate-limiting step was chemical catalysis in the EDTA-treated enzyme before metal reconstitution. The Cd²⁺-reconstituted N26C enzyme showed a characteristic burst of product indicating that product release was now the overall rate-limiting step. The enzyme/metal stoichiometry also affected the relative rates for chemical catalysis and product release.

In summary, these results indicate that the EcN26C has properties similar to that of metal-dependent Class II KDO8P synthase and that the engineered Cys³⁰⁶ residue in N26C fulfills the role of the native, metal-ligated cysteine in Class II enzymes.

Mechanistic Implications—The results described above show that reciprocal single mutations have the potential to convert metal-dependent class KDO8P synthase to a metal-independent variant and vice versa, as demonstrated here for the A. pyrophilus and E. coli enzymes. However, perhaps the most important questions to be considered are the mechanistic implications of these results on KDO8P synthase catalysis.

The demonstration that ApC11N can act in the absence of metal ion and with significant efficiency implies that the metal ion is not directly involved in the chemistry of the metal-dependent KDO8P synthase-catalyzed reaction. Thus, the suggested role of the divalent metal ion in catalysis (22, 23), that of providing a highly activated hydroxide ion that initiates the condensation reaction between PEP and A5P (Scheme 2, path b), seems less likely. If this was indeed the case, then such a step would be an essential elementary step for catalysis to occur, and in the absence of metal ion there should be absolutely no catalysis. In addition, it is also of note that the
formation was observed for 0.02 and 0.3 mM Cd2+

cation 0.14

tration (60% active site concentration, rate constant for product forma-

quench apparatus. No burst in product formation was observed (r, Scheme 2, 1b,

stepwise formation of a transient C3 carbanionic species at C3

of the catalytic cycle. Thus, unlike the metal-dependent Class

the carbonyl oxygen, which is too far to directly affect this stage

undergo rapid inactivation, the addition of metal ions to form

sulfur are shown in

desired active site cavity is maintained, allowing for the correct

orientation of the substrates and/or reaction intermediate(s). A similar role in the metal-independent Class I enzymes might be

achieved by the conserved Asn residue (4, 5). Thus, the ob-

served ability of the EcN26C to act to some extent in a metal-

dependent fashion does not appear to be a consequence of a

change in the catalytic mechanism in the mutant enzyme, but

is more likely to be a direct consequence of the loss of function

associated directly with Asn26 in the wild-type enzyme and that

this function is partially fulfilled by the metal ion in the N26C

mutant. According to the recently solved crystal structure of the

E. coli KO6P synthase in its binary complex with PEP (32), Asn26 lies 3.8 Å away from N4 of His202 and 5.4 Å away from

the carboxylate of PEP. However, His202 is largely involved in PEP binding due to interactions with both PEP-phosphate and PEP-carboxylate, and plays a very important

role in selection of a particular PEP conformer in which the

phosphate group of PEP extends toward the si face. Indeed, the

mutation of His202 with glycine rendered the enzyme virtually inactive (32). Thus, despite the lack of direct contact to the PEP

substrate, Asn26 plays an important structural role by orienting

His202 in such a way that allows the binding and recogni-

tion of the correct conformation of the PEP substrate.

Taken together, the observed data suggest that the role of Asn26 in E. coli enzyme is similar to that of the metal ion in the A. pyrophilus enzyme, and that this role is primarily structural. The suggested structural role of metal ion in Class II KO6P synthases has also been indicated for the H. pylori KO6P synthase (30), and for the structurally and mechanis-
tically closely related enzyme, 3-deoxy-D-arabino-2-heptulosonate-7-phosphate synthase (DAHP synthase) (33). These two studies showed that while aponzymes were unstable and undergo rapid inactivation, the addition of metal ions to form

the holoenzymes promoted their stability. Analogously, in the
case of the E. coli KO6P synthase, it was previously demon-

strated that tightly bound PEP substrate stabilizes the enzyme
during purification and storage and protects against heat in-

activation (15).

Nevertheless, although at this stage of investigation it is not clear whether the formation of new C-C and C-O bonds during the condensation step of KO6P synthase reaction is a syn-

chronous or stepwise process, the observed data in this work

...
suggest that both Class I and Class II enzymes operate by the same stepwise fashion through the intermediary of a transient oxocarbenium ion at C2 of PEP (1a, Scheme 2, path a). The intermediate 1a can be captured by bulk water to lead to the formation of acyclic hemiketal phosphate 1, which then rapidly decomposes to yield the products KDO8P and Pi. This mechanism is further indicated by results obtained through structural data on the E. coli KDO8P synthase in complex with the substrate PEP and with a mechanism-based inhibitor (20), with analogues of PEP (34), with various experiments using a mechanism-based inhibitor, and with intramolecular models of the KDO8P synthase-catalyzed reaction (21). Additionally, the same oxocarbenium ion transition state of PEP has been suggested earlier to account for the enzymatic reactions of UDP-GlcNAc enolpyruvoyl transferase (35) and 5-enolpyruvoylshikimate-3-phosphate synthase (36). These enzymes catalyze the enol ether transfer from PEP to their respective cosubstrate alcohols, and represent a different distinct class of enzymatic reaction involving the same C-O bond cleavage of PEP and the same stereospecific 2-st face addition of an electrophile at C3 of PEP, which is observed for KDO8P synthase (37).

In conclusion, the results presented in this study imply that the metal ion may not be directly involved in the chemistry of the KDO8P synthase-catalyzed reaction, but has important structural roles in Class II enzymes through maintaining correct orientation of the substrates and/or reaction intermediate(s) in the enzyme active site to allow the catalysis. The observed data also supports the notion that the elementary steps of catalysis in both Class I and Class II enzymes may follow the same oxocarbenium ion mechanism as illustrated in Scheme 2, path a.

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A Reciprocal Single Mutation Affects the Metal Requirement of 3-Deoxy-d-manno-2-octulosonate-8-phosphate (KDO8P) Synthases from *Aquifex pyrophilus* and *Escherichia coli*

Smadar Shulami, Cristina Furdui, Noam Adir, Yuval Shoham, Karen S. Anderson and Timor Baasov

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