Functional and biochemical characterization of a recombinant *Arabidopsis thaliana* 3-deoxy-D-manno-octulosonate 8-phosphate synthase

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An open reading frame, encoding for KDOPS (3-deoxy-D-manno-octulosonate 8-phosphate synthase), from *Arabidopsis thaliana* was cloned into a T7-driven expression vector. The protein was overexpressed in *Escherichia coli* and purified to homogeneity. Recombinant *A. thaliana* KDOPS, in solution, displays an apparent molecular mass of 76 kDa and a subunit molecular mass of 31.519 kDa. Unlike previously studied bacterial KDOPSs, which are tetrameric, *A. thaliana* KDOPS appears to be a dimer in solution. The optimum temperature of the enzyme is 65 °C and the optimum pH is 7.5, with a broad peak between pH 6.5 and 9.5 showing 90 % of maximum activity. The enzyme cannot be inactivated by EDTA or dipicolinic acid treatment, nor it can be

activated by a series of bivalent metal ions, suggesting that it is a non-metallo-enzyme, as opposed to the initial prediction that it would be a metallo-enzyme. Kinetic studies showed that the enzyme follows a sequential mechanism with $K_m = 3.6 \mu\text{M}$ for phosphoenolpyruvate and $3.8 \mu\text{M}$ for D-arabinose 5-phosphate and $k_{\text{cat}} = 5.9 \text{ s}^{-1}$ at 37 °C. On the basis of the characterization of *A. thaliana* KDOPS and phylogenetic analysis, plant KDOPSs may represent a new, distinct class of KDOPSs.

Key words: *Arabidopsis thaliana*, 3-deoxy-D-manno-octulosonate, 3-deoxy-D-manno-octulosonate 8-phosphate synthase, lipid A, lipopolysaccharide, phylogenetic tree.

INTRODUCTION

3-Deoxy-D-manno-octulosonate 8-phosphate synthase (KDOPS) (kdsA) catalyses the stereospecific condensation of A5P (D-arabinose 5-phosphate) and PEP (phosphoenolpyruvate) to form 3-deoxy-D-manno-octulosonate 8-phosphate (KDO-8-phosphate) and P_i . This enzyme is one of the five enzymes involved in the biosynthesis and utilization of 3-deoxy-D-manno-octulosonate (KDO), a key component of bacterial lipopolysaccharide and/or capsular polysaccharides [1,2]. KDO was originally considered to occur only in the cell wall and/or capsules of Gram-negative bacteria [3]. Previous studies have demonstrated that KDO exists in plants as a component of rhamnogalacturonan II, a structurally complex pectic polysaccharide released from enzymic liquefaction of monocots, dicots and gymnosperms [4–8]. KDO and 5-O-methyl KDO were also found to be the dominant residues in the scales and theca of the green alga *Tetraselmis striata* Butcher [9]. The existence of KDOPS in plants was first suggested by Doong et al. [10], who detected KDOPS-like activity in eight different plant species and partially purified and characterized KDOPS from spinach. Brabetz et al. [11] first identified a plant cDNA encoding KDOPS in *Pisum sativum* L. (pea) by complementing a temperature-sensitive *kdsA*^{ts} mutant of *Salmonella enteria*. They partially characterized a crude extract of *Escherichia coli* expressing the *kdsA* encoded by *P. sativum* L. cDNA.

The microbial non-metallo-KDOPSs from *E. coli* [12], *S. typhimurium* [13] and *Neisseria gonorrhoeae* [14], as well as microbial metallo-KDOPSs from *Aquifex aeolicus* [15], *Helicobacter pylori* [16] and *Chlamydia psittaci* [17] have been characterized. The structure and mechanism of the KDOPSs from *E. coli* (non-metallo) [18–22] and *A. aeolicus* (metallo) [23,24] have been investigated in great detail. Although the KDOPSs from microbial

sources have been extensively investigated, little is known about plant KDOPS. The isolation and detailed characterization of a homogeneous KDOPS from a plant species have not been reported. KDOPS from *Arabidopsis thaliana* has been predicted to require a metal for catalytic activity and occupies a unique and displaced branch in the KDOPS evolutionary tree [25], whereas its nearest neighbour, *P. sativum* KDOPS, has been reported not to require a metal for catalytic activity [11]. Therefore an investigation of a plant KDOPS is of interest to obtain further insight into the mechanism of this enzyme family, the potential difference between prokaryotic and eukaryotic enzymes, and the possibility of a unique evolutionary branch of KDOPSs. In the present study, the open reading frame (Atlg79500) from *A. thaliana*, homologous with *E. coli* KDOPS, was cloned and overexpressed in *E. coli*. The protein product was purified, characterized and confirmed to be a specific non-metallo-KDOPS.

EXPERIMENTAL

Materials

The plasmid pZL-1 containing the *A. thaliana* cDNA of putative *kdsA* (ABRC accession number Atlg79500; NCBI accession number NP_173084) was obtained from the ABRC (*Arabidopsis* Biological Resource Center, Ohio State University) and a separate plasmid was obtained from Dr T. Newman (Michigan State University). The Promega Wizard DNA purification kit was used for plasmid isolation and purification. The *E. coli* cells, Epicurian ColiTM XL1-Blue and BL21(DE3), were obtained from Stratagene Cloning System and Novagen respectively. Restriction enzymes and T₄ DNA ligase were purchased from New England Biolabs (Beverly, MA, U.S.A.). Thermal cycling was performed using an

Abbreviations used: A5P, D-arabinose 5-phosphate; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DPA, dipicolinic acid; 2dR5P, 2-deoxyribose 5-phosphate; E4P, D-erythrose 4-phosphate; KDO, 3-deoxy-D-manno-octulosonate; KDOPS, 3-deoxy-D-manno-octulosonate 8-phosphate synthase; MALDI-MS, matrix-assisted laser-desorption ionization-mass spectrometry; PEP, phosphoenolpyruvate; R5P, D-ribose 5-phosphate.

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MJR Research Thermal Cycler. DNA sequencing and primer syntheses were performed by the University of Michigan Biomedical Resources Core Facility. Tris(hydroxymethyl)aminomethane, PEP mono(cyclohexylammonium) salt, A5P disodium salt, 2dR5P (2-deoxyribose 5-phosphate) sodium salt, R5P (D-ribose 5-phosphate) disodium salt, E4P (D-erythrose 4-phosphate) sodium salt and DPA (dipicolinic acid) were obtained from Sigma. Pura-tronic grade MgCl_2 , MnCl_2 , CuCl_2 and CdCl_2 were purchased from Alfa Aesar (Ward Hill, MA, U.S.A.). The EDTA disodium salt was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). High grade Spectra/Por® 7 dialysis tubing (10 000 molecular mass cut-off and metal free) was obtained from VWR Scientific (Chicago, IL, U.S.A.). The ceramic hydroxyapatite (CHT5-I) column was obtained from Bio-Rad Laboratories. The Mono Q (HR10/10), phenyl-Superose (HR10/10) and Superose 12 (HR10/30) columns were from Amersham Biosciences.

Cloning of the *A. thaliana* cDNA encoding KDOPS

The plasmid pZL-1 containing the *A. thaliana* cDNA of putative *kdsA* obtained from ABRC was transformed into chemically competent Epicurian Coli™ XL1-Blue cells. The isolated plasmids were sequenced using the universal sequencing primers T7 and Sp6, and the sequence was compared with the published sequence to confirm the presence of full-length putative *A. thaliana kdsA*. Multiple PCRs were performed to eliminate an internal *NdeI* restriction site located near the 5'-end of the open reading frame. The first PCR utilized the forward primer (P1) 5'-GATTCTAGAATTCATATGCGGCAACATCAC-3' and the reverse mutagenic primer (P2) 3'-CTCTTCCACTTCCAACGATACTAGACGGTATTCATTGACTAC-5' (the lower-case letter in the primers indicates the base that was mutated) to create a DNA fragment from the start codon to the internal *NdeI* site (nt 1–274 of the gene). The second PCR utilized the forward mutagenic primer (P3), 5'-GAGAAGGTGAAGGTTGCTTATGATCTGCCAATAGTAAGTGATG-3' and the reverse primer (P4), 5'-GATTCTGAATTCGGATCCAAGCTTAATCACGGTAGGGTGTAAGG-3' to create a DNA segment from the 'internal *NdeI* site' to the end of the gene (nt 232–873 of the gene). The full-length gene was produced by a third PCR utilizing the first two purified PCR products as templates with the above forward primer P1 and reverse primer P4, thus eliminating the internal *NdeI* restriction site but retaining the *NdeI* site at the beginning of the gene.

The final PCR product was restricted with *NdeI* and *Bam*HI (underlined) and ligated into the expression vector pT7-7, previously digested with the same restriction enzymes and treated with calf intestinal alkaline phosphatase. The ligation mixture was used to transform competent Epicurian Coli™ XL1-Blue cells. Plasmids from these clones containing the correct insert, confirmed by sequencing, were transformed into the expression cells, *E. coli* BL21(DE3).

Overexpression and purification of the recombinant protein

The *E. coli* BL21(DE3) cells harbouring *A. thaliana kdsA* was grown in 2YT medium [1.6 % (w/v) tryptone/1 % (w/v) yeast extract/0.5 % (w/v) NaCl] containing ampicillin (100 mg/l) at 37 °C with shaking (220 rev./min). When the culture reached the mid-exponential growth phase ($A_{600} \sim 1.5$), the culture was allowed to cool at 18 °C and induced with isopropyl β -D-thiogalactoside at a final concentration of 0.4 mM. After 16 h of growth at 18 °C, the cells were harvested by centrifugation (29 000 g for 20 min at 4 °C). The cell pellet was suspended in buffer A (20 mM Tris/HCl, pH 7.5) and subjected to sonication on ice (30 s pulses with a 2 min rest between pulses, five times). The crude extract was

centrifuged to remove cell debris (40 000 g for 30 min at 4 °C). The supernatant was loaded on to a Q-Sepharose anion-exchange column utilizing a 0–0.4 M KCl gradient in buffer A. The fractions containing KDOPS activity as identified by the Aminoff periodate–thiobarbituric acid assay [26] were pooled. Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the pooled fractions to a final concentration of 20 % (w/v). The sample was filtered (0.22 μm) and loaded on to a phenyl-Superose column (HR10/10), pre-equilibrated with 20 % $(\text{NH}_4)_2\text{SO}_4$ in buffer A. A reverse gradient from 20 to 0 % $(\text{NH}_4)_2\text{SO}_4$ in buffer A was applied at a flow rate of 1.0 ml/min for 100 min. The fractions containing KDOPS activity were pooled, dialysed against 1 litre of 10 mM phosphate buffer (pH 6.8) overnight and then applied to a ceramic hydroxyapatite (CHT5-I) column, pre-equilibrated with 10 mM phosphate buffer (pH 6.8). The column was eluted at a flow rate of 1 ml/min using a linear gradient of 10–50 mM phosphate buffer for 80 min. The fractions containing KDOPS activity were pooled and dialysed against 2 litres of 10 mM Tris/HCl (pH 7.1) for 48 h with two buffer changes. The purified enzyme (2.4 mg/ml) was divided into aliquots and stored at -80 °C.

Assay of KDOPS activity

Enzyme activity was determined either by a discontinuous colorimetric assay or a continuous spectrophotometric assay. For the standard discontinuous colorimetric assay, a 50 μl reaction mixture, in thin-walled PCR tube, containing 3 mM PEP, 3 mM A5P and 100 mM Tris/acetate buffer (pH 7.5) was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of enzyme. The reaction was quenched by adding 50 μl of 10 % (w/v) trichloroacetic acid. The amount of KDO produced was determined by the Aminoff periodate–thiobarbituric acid assay [26]. For the standard continuous spectrophotometric assay, which measures the disappearance of the α,β -unsaturated carbonyl absorbance ($\lambda = 232$ nm, molar absorption coefficient $\epsilon = 2840 \text{ M}^{-1} \cdot \text{cm}^{-1}$) of PEP, a 1 ml reaction mixture, containing 100 mM Tris/acetate buffer (pH 7.5), 300 μM PEP, 100 μM A5P and 20–50 nM KDOPS, was incubated at 37 °C. Progress of reaction was monitored by an HP 8453 UV–visible spectrophotometer. One unit of enzyme activity is defined as the production of 1 μmol of KDO-8-phosphate or the disappearance of 1 μmol of PEP/min at 37 °C.

Enzymic synthesis of KDO-8-phosphate

To a Chemtube (12 mm $\times$ 75 mm; Bio-Rad Laboratories,) containing a solution of BTP (1,3-bis[tris(hydroxymethyl)-methylamino]propane; 42.35 mg, 0.15 mmol) in water was added 13 mg of A5P (0.056 mmol), and 12 mg of PEP monocyclohexylamine salt (0.044 mmol) and the pH was adjusted to 6.8 using 1 M NaOH. *A. thaliana* KDOPS (3 mg, 95 nmol) was added to initiate the reaction and the final volume of the reaction mixture was adjusted to 2 ml. The reaction mixture was incubated at 37 °C for 2 h. The enzymic reaction was quenched by adding 0.5 ml of 10 % trichloroacetic acid, vortex-mixed for 30 s and centrifuged for 30 min (1500 g) to remove the precipitated protein. The supernatant was loaded on to a 5 ml Econo-Pac HighQ (Bio-Rad Laboratories) anion-exchange column (chloride form), pre-equilibrated with water. The column was washed with 30 ml of water at a flow rate of 1 ml/min. The phosphorylated monosaccharide was eluted from the column using a linear gradient of 0–0.5 M LiCl solution over a period of 1 h. Fractions containing the potential KDO-8-phosphate, as identified by periodate–thiobarbituric acid assay, were pooled and freeze-dried. The freeze-dried sample was dissolved in $^2\text{H}_2\text{O}$, and the ^1H , ^{13}C , ^{31}P -NMR

spectra were acquired on a Bruker Avance DRX 500 (operating at 500.132 MHz for ^1H , 125.7 MHz for proton-decoupled ^{13}C and 202.4 MHz for proton-decoupled ^{31}P).

Molecular-mass determinations

The subunit molecular mass of the enzyme was determined by MALDI-MS (matrix-assisted laser-desorption ionization-mass spectrometry) on a VESTEC-2000 instrument using a sinipinic acid matrix at the University of Michigan Protein Structure Facility. The native molecular mass was determined by gel filtration utilizing a Superose 12 column (HR10/30) according to the manufacturer's instructions. The elution volume was determined in triplicate for all samples and standards were obtained from Sigma.

Optimum temperature of KDOPS

The temperature dependence of enzyme activity was determined by measuring the activity between 20 and 80 °C with 3 mM PEP, 3 mM A5P, 100 nM enzyme in 100 mM Tris/acetate buffer using the discontinuous colorimetric assay. Since the pH of Tris buffer is temperature-dependent, the pH of the buffer was adjusted to 7.5 at the desired temperatures. At each temperature, the Tris/acetate buffer was preincubated for 2 min to allow it to reach the final pH of 7.5. PEP and A5P were then added and the entire reaction mixture was incubated for another 1 min. The reaction was initiated by the addition of the enzyme and allowed to proceed for an additional 2 min.

pH dependence of KDOPS

The pH dependence of the enzyme was measured between pH 4.0 and 10.0 at 37 °C by the discontinuous colorimetric assay described above using 1 mM PEP and 1 mM A5P in 100 mM succinic acid/sodium tetraborate (pH 4.0–5.5), 2-(*N*-morpholino)-ethanesulphonic acid (pH 5.5–6.5), BTP (pH 6.5–9.5) or glycine (pH 9.5–10.0) buffers. The pH of each buffer was measured at 25 °C.

Metal requirements of KDOPS

The enzyme (600 nM) was incubated with 3 mM PEP in 100 mM Tris/acetate (pH 7.5) in the presence of various bivalent metal ions (100 μM) or metal chelators at 25 °C for 5 min. The mixture was then incubated at 37 °C for 2 min before the initiation with A5P (3 mM) and monitored for 2 min. The activity of the enzyme was measured in triplicate using the discontinuous colorimetric assay.

Kinetic studies

Reactions were performed using the continuous spectrophotometric assay as described above. The assay mixture containing PEP (2–60 μM), A5P (5–50 μM) and 100 mM Tris/acetate (pH 7.5) buffer was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 32 nM KDOPS. Initial reaction velocities were calculated from the linear region (~30 s) of the reaction progress curve and measured in triplicate by varying the concentration of one substrate at various fixed concentrations of the other substrate. Apparent kinetic constants were calculated from the slopes and intercepts of the secondary plots deduced from the initial double-reciprocal plots ($1/v$ versus $1/[S]$). Linear regression analysis was performed using KaleidaGraph software. Results are the averages of triplicate assays.

Thermostability of *A. thaliana* KDOPS

The thermostability of *A. thaliana* KDOPS was determined by incubating the enzyme (6 μM) in 100 mM Tris/acetate (pH 7.5)

with 1 mM of either PEP or A5P, or without any substrate for 30 min at various temperatures. The incubated enzymes were allowed to cool to 25 °C in 5 min, centrifuged and subjected to the continuous spectrophotometric assay at 37 °C as described above.

In a separate experiment, KDOPS from *A. thaliana* or *E. coli* (both at 6 μM) were incubated with 1 mM PEP in 100 mM Tris/acetate (pH 7.5) for 7 min at 60 °C. Each enzyme solution was allowed to reach to 25 °C in 5 min, centrifuged and subjected to the continuous spectrophotometric assay at 37 °C. *E. coli* KDOPS was prepared from *E. coli* BL21(DE3) cells harbouring the pT7-7/kdsA plasmid as described previously [27].

Substrate specificity of *A. thaliana* KDOPS

A 50 μl portion of the reaction mixture containing PEP (3 mM), a phosphorylated monosaccharide (3 mM 2dR5P, E4P or R5P) and Tris/acetate buffer (100 mM, pH 7.5) was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of recombinant *A. thaliana* KDOPS (6 μM) for 10 min. The reaction was quenched by the addition of 50 μl of 10 % trichloroacetic acid. The amount of potential monosaccharide produced was determined by a modified Aminoff periodate–thiobarbituric acid assay [26] in which the oxidation step was performed at 60 °C instead of 25 °C to ensure complete oxidation of the potential monosaccharide product.

Miscellaneous methods

Protein concentrations were determined using the Bio-Rad Protein Assay Reagent using BSA (Sigma) as the standard. SDS/PAGE (12 % gel) was performed under reducing conditions with a Mini-PROTEAN II electrophoresis unit (Bio-Rad Laboratories) and visualized with 0.25 % Coomassie Brilliant Blue R250 stain. Protein sequences were aligned using Clustal W [28].

RESULTS

Cloning, overexpression and purification of the enzyme

A BLASTp search of the *A. thaliana* genome database utilizing the protein sequence of *E. coli* KDOPS resulted in two homologous sequences annotated as KDOPS (ABRC accession numbers Atlg79500 and Atlg16340 or NCBI accession numbers NP_173084 and NP_178068). The two sequences share 92 % identity with each other and approx. 45 % identity with the *E. coli* sequence. The 873 bp open reading frame *kdsA* (Atlg79500 and NP_178068) was cloned into the T7-driven expression vector pT7-7 and the resulting protein product, *kdsA*, overexpressed in *E. coli* cells. The recombinant enzyme was purified by a combination of chromatographic separations, including Q-Sepharose, phenyl-Superose and hydroxyapatite chromatography. The purified protein was determined to be homogeneous by SDS/PAGE. The typical yield of purified enzyme was relatively low (5 mg/litre of cell culture), probably due to heterologous expression of a plant protein in bacteria.

Enzymic synthesis of KDO-8-phosphate

The ^1H -, ^{13}C - and ^{31}P -NMR spectra (results not shown) of the monosaccharide product generated from *A. thaliana* KDOPS catalysis (PEP and A5P as substrates) are consistent with the previously reported spectra for KDO-8-phosphate generated from *E. coli* KDOPS condensation [27].

Physical properties

The subunit molecular mass of the purified enzyme was 31.519 kDa as determined by MALDI-MS, which is consistent

Figure 1 Optimum temperature of *A. thaliana* KDOPS

Enzymic activity was measured using 3 mM PEP and 3 mM A5P in 100 mM Tris/acetate buffer (pH adjusted to 7.5 at each desired temperature) by the discontinuous colorimetric assay. Error bars represent S.D. for three determinations.

with the calculated molecular mass of 31.525 kDa based on the protein sequence minus the initial starter methionine. The native molecular mass was 76 kDa as determined by analytical gel-filtration chromatography. Since the native molecular mass is approx. 2.4 times that of the denatured, KDOPS from *A. thaliana* is predicted to have a dimeric structure in solution.

Optimum temperature

The optimum temperature curve shows that the enzyme activity increases with increasing temperature from 20 to 65 °C and decreases sharply from 65 to 80 °C (Figure 1). At the optimum temperature of 65 °C, the activity is 6-fold higher than that at 30 °C. An optimum temperature of approx. 53 °C, for the partially purified KDOPS from spinach, has been reported by Doong et al. [10]. Given that the activity of most bacterial [12–14] and plant KDOPSs [10,11] was assayed at 37 °C, the *A. thaliana* enzyme was assayed at 37 °C in the following experiments for comparative purposes.

Optimum pH

The optimum pH of recombinant *A. thaliana* KDOPS was approx. 7.5. A broad peak with high catalytic activity (90 % of maximum), however, was observed between pH 6.5 and 9.5 (Figure 2). It should be noted that the enzyme is more active in succinic acid/sodium tetraborate buffer when compared with that in 2-(*N*-morpholino)ethanesulphonic acid buffer at the same pH.

Metal requirement

To determine whether *A. thaliana* KDOPS requires a metal cofactor for activity, the enzyme was incubated with metal chelators (EDTA or DPA) or bivalent metal ions and then assayed for KDOPS activity by the discontinuous colorimetric assay. The enzymic activity was neither inhibited by EDTA or DPA nor activated by metal ions (Table 1), suggesting that there is no metal cofactor requirement for *A. thaliana* KDOPS.

Substrate kinetics

The kinetic constants of *A. thaliana* KDOPS were determined by the continuous PEP disappearance assay. The initial velocity was determined by varying the concentration of one substrate at

Figure 2 Optimum pH of KDOPS

Enzymic activity was measured using 1 mM PEP and 1 mM A5P in 100 mM succinic acid/sodium tetraborate (○, pH 4.0–5.5), 2-(*N*-morpholino)ethanesulphonic acid (□, pH 5.5–6.5), BTP (◇, pH 6.5–9.5) or glycine (△, pH 9.5–10.0) at 37 °C by the discontinuous colorimetric assay. Error bars represent S.D. for three determinations.

Table 1 Effects of metal chelators and bivalent metal ions on *A. thaliana* KDOPS activity

KDOPS (600 nM) was incubated with 3 mM PEP in 100 mM Tris/acetate (pH 7.5) in the presence of either metal chelators or bivalent metal ions at 25 °C for 5 min and then subjected to the discontinuous colorimetric assay.

	Specific activity (units/mg)
Enzyme as isolated	10.7 ± 0.5
1 mM EDTA	11.5 ± 0.3
10 mM EDTA	11.8 ± 0.9
10 mM EDTA + 1 mM DPA	11.7 ± 0.7
100 µM Mn ²⁺	10.5 ± 0.5
100 µM Mg ²⁺	10.3 ± 0.7
100 µM Zn ²⁺	9.8 ± 0.2
100 µM Cu ²⁺	4.2 ± 0.3
100 µM Cd ²⁺	2.5 ± 0.4

various fixed concentrations of the other substrate. The double-reciprocal plots of the velocity against substrate concentration with either A5P or PEP as the fixed substrate showed straight lines intersecting at a common point to the left of the vertical axis (Figures 3A and 4A), indicating a sequential mechanism in which the substrates must bind to the enzyme before any product is released. Further experiments will be conducted to determine whether the reaction follows an ordered or random sequential mechanism. The K_m values obtained from the secondary plots were $3.6 \pm 0.4 \mu\text{M}$ for PEP and $3.8 \pm 0.5 \mu\text{M}$ for A5P (Figures 3B and 4B). The k_{cat} of the enzyme was $5.9 \pm 0.1 \text{ s}^{-1}$.

Thermostability of *A. thaliana* KDOPS

Thermostability of *A. thaliana* KDOPS was determined by measuring enzyme activity after 30 min of heat treatment at various temperatures. In the absence of any substrates or in the presence of A5P, the enzyme lost 30 % of activity after incubation at 40 °C and was completely inactive after incubation at 50 °C (Figure 5A). In the presence of PEP, however, the enzyme retained 100 % of activity after incubation at 50 °C (Figure 5A). To compare the stabilization effect of PEP on KDOPSs from *A. thaliana* and *E. coli*, both enzymes were individually incubated

Figure 3 PEP kinetics of *A. thaliana* KDOPS

(A) Double-reciprocal plots of initial velocities of KDOPS as a function of PEP concentration. A5P concentrations were 2 μM ($\circ$), 5 μM ($\diamond$), 15 μM ($\square$) and 60 μM ($\times$). (B) Secondary plot of $[PEP]/V^{\text{app}}$ against $[PEP]$, deduced from the double-reciprocal plots. Error bars represent S.D. for three determinations.

at 60 °C for 7 min in the presence of PEP. Whereas *E. coli* KDOPS retained only 10 % of activity, *A. thaliana* KDOPS showed a surprising 20 % increase in activity (Figure 5B).

Substrate specificity of *A. thaliana* KDOPS

A. thaliana KDOPS can utilize 2dR5P as a substrate with low catalytic efficiency. Neither E4P nor R5P served as substrates, as determined by the sensitive modified Aminoff periodate–thiobarbituric acid assay [26], in which the oxidation of a potential monosaccharide product was enhanced to ensure the detection of lower-level products.

DISCUSSION

Bacterial KDOPSs have been extensively investigated [12–24]; however, little information is available for plant KDOPSs. In the present study, the *kdsA* from *A. thaliana* is overexpressed,

Figure 4 A5P kinetics of *A. thaliana* KDOPS

(A) Double-reciprocal plots of the initial velocities of KDOPS as a function of A5P concentration. PEP concentrations were 5 μM ($\circ$), 10 μM ($\diamond$), 25 μM ($\square$) and 50 μM ($\times$). (B) Secondary plot of $[A5P]/V^{\text{app}}$ against $[A5P]$, deduced from the double-reciprocal plots. Error bars represent S.D. for three determinations.

purified and characterized. The NMR spectra of the condensation product between PEP and A5P catalysed by the recombinant *A. thaliana* enzyme is identical with that of the *E. coli* KDOPS product, confirming that the recombinant *A. thaliana* enzyme is a KDOPS. The catalytic properties of *A. thaliana* KDOPS are similar to its microbial counterparts. The enzyme exhibits a sequential mechanism similar to that observed for the *E. coli* enzyme [22]. The catalytic efficiency (k_{cat}) of the plant enzyme is similar to that of the *E. coli* enzyme, but is higher than that of the *A. aeolicus* enzyme (Table 2), and it has significantly lower K_m values (for both substrates) when compared with either its *E. coli* or *A. aeolicus* counterparts. The *A. thaliana* enzyme is capable of utilizing 2dR5P as a substrate, although a poor one, but neither R5P nor E4P (results not shown), again identical with *E. coli* KDOPS [14].

The stability of the *A. thaliana* KDOPS against thermal denaturation is enhanced by the presence of PEP, whereas A5P, the other substrate, has no effect on thermal stability (Figure 5A). The PEP-induced stability may be explained by the ability of PEP

Figure 5 Stability of *A. thaliana* KDOPS against thermal denaturation

(A) Effects of substrates on the stability of KDOPS against heat denaturation. The enzyme (6 μ M) was incubated in 100 mM Tris/acetate (pH 7.0) either with 1 mM PEP (○) or 1 mM A5P (◇) or without substrate (□) for 30 min at the indicated temperatures and assayed for residual activity. (B) Comparison of the thermostabilities of *A. thaliana* and *E. coli* KDOPSs. The enzymes (both at 6 μ M) were incubated with 1 mM PEP in 100 mM Tris/acetate (pH 7.5) for 7 min at 60 °C and assayed for residual activity. Grey column, before incubation; black column, after incubation. Error bars represent S.D. for three determinations.

Table 2 Comparison of the biochemical properties of KDOPSs from *A. thaliana*, *E. coli* and *A. aeolicus*

Enzyme property	<i>A. thaliana</i> KDOPS	<i>E. coli</i> KDOPS*	<i>A. aeolicus</i> KDOPS*
Molecular mass			
Subunit (calculated, kDa)	31.525	30.833	29.734
Gel filtration	Dimer	Tetramer	Tetramer
Crystallography	NA†	Tetramer	Tetramer
Metal requirement	No	No	Yes
Kinetic constants			
K_m^{PEP} (μ M)	3.6	19	28–43
K_m^{A5P} (μ M)	3.8	29	8–74
k_{cat} (s^{-1})	5.9	6.8	0.38–2

* Results of previous studies [15,19,23,32,33,43].

† NA, not available.

to bind tightly to the active site of the plant enzyme, thereby shifting the ligand-binding equilibrium towards the native enzyme–PEP complex. Le Chatelier's principle has been invoked by other authors for proteins to explain similar observations [29–31]. Indeed, it has been reported that *E. coli* KDOPS, as isolated, contains a tightly associated PEP [20]. In the proposed catalytic mechanism of KDOPS [22], PEP binds to the enzyme before the

binding of A5P. It is reasonable to predict that while the enzyme is not catalysing the condensation reaction, A5P is not bound to the enzyme and thus A5P does not have any stabilization effect. PEP has a more stabilizing effect on *A. thaliana* KDOPS than on *E. coli* KDOPS (Figure 5B), suggesting a possible tighter PEP–enzyme complex in the *A. thaliana* enzyme.

On the basis of the phylogenetic analysis, KDOPSs have been separated into two classes [25]. The difference between Class I and Class II KDOPSs has been suggested to be metal requirement, based mainly on the fact that the *E. coli* KDOPS (Class I) is a non-metallo-enzyme [12] and that the *A. aeolicus* KDOPS (Class II) is a metallo-enzyme [32]. In *A. aeolicus* as well as in all Class II KDOPSs, four amino acid residues, Cys-11, His-185, Glu-222 and Asp-233, form the metal-binding sites [23]. Sequence alignment shows that three of these four residues (His-185, Glu-222 and Asp-233 in *A. aeolicus* KDOPS) are absolutely conserved in both Class I and Class II KDOPSs; however, the fourth metal-chelating residue, Cys-11, is absolutely conserved only in the microbial Class II KDOPSs. In all the Class I KDOPSs, this cysteine residue is replaced by asparagine (Figure 6A). A BLASTp search of the NCBI database with the *E. coli* kdsA finds significant homologues (44–47% identity) in *A. thaliana*, *P. sativum*, *Oryza sativa* and *Lycopersicon esculentum* (Table 3). The kdsA homologues are highly conserved within plants, with >82% identity and 90% similarity to each other, but they share only 44–51% sequence identity with their *E. coli* and *A. aeolicus* counterparts. All the plant KDOPSs listed above contain an asparagine residue instead of a cysteine as the fourth ligand (Figure 6A), thus suggesting that plant KDOPSs are non-metallo. In the present study, the activity of *A. thaliana* KDOPS was neither inhibited by EDTA or DPA nor activated by various bivalent metal ions (Table 1), suggesting that the enzyme is non-metallo. Indeed, KDOPS from *P. sativum* was also reported to be a non-metallo-enzyme [11]. Therefore it is probable that plant KDOPSs are non-metallo.

The quaternary structure of *A. thaliana* KDOPS seems to be different from that of microbial KDOPSs (Table 2). *A. thaliana* KDOPS is dimeric in solution, whereas both the *E. coli* and *A. aeolicus* KDOPSs have been reported to be tetrameric in solution [15,33] and in the crystals [19,23]. In *E. coli* KDOPS [19], two loops were reported to be involved in the assembly of the tetramer: loop L2 (residues 58–72) and loop L6 (residues 170–182). Sequence alignment (Figure 6B) shows that the residues within both the loops are reasonably conserved in bacterial and plant KDOPSs, except Ile-66 in loop L2 and Asn-176 in loop L6, which are conserved in bacterial KDOPSs, but are replaced by serine and aspartic residues respectively in all the plant source enzymes available to date. It has been reported that single amino acid replacements may result in changes in quaternary structure [34]. Therefore it is probable that two amino acid changes noted above may cause the different quaternary structures predicted between plant and bacterial KDOPSs. More experiments are required to test this hypothesis. Furthermore, it has been suggested that, in the microbial tetrameric KDOPSs from *E. coli* and *A. aeolicus* [23,35], PEP binds in all four active sites, whereas A5P binds only in the two alternative active sites initiating the reaction and possibly triggers a conformational change in the two alternative subunits to facilitate A5P binding for subsequent reaction in those active sites. Since *A. thaliana* KDOPS is a dimer in solution, it will be of interest to understand how the two subunits interact during catalysis. Is the reaction catalysed alternatively by subunit interaction or do both subunits work in unison? Studies on the subunit interaction as well as solving the crystal structure of this enzyme, both of which are currently in progress, should clarify the potential difference(s) in catalytic mechanisms between plant and microbial KDOPSs.

Figure 6 Alignment of KDOPS sequences from plant and microbial sources

Sequences were aligned by Clustal W [28]. (A) Putative metal-binding residues. Invariant residues corresponding to the metal-binding sites are shaded grey (Cys-11, His-185, Glu-222 and Asp-333; numbering based on the *A. aeolicus* KDOPS sequence) [23]. The conserved asparagine residues (corresponding to *A. aeolicus* KDOPS Cys-11) in the plant and Class I sequences are shaded black. (B) Putative tetramer assembly regions (loops L2, L6 in the crystal structure of *E. coli* KDOPS) [19]. The conserved amino acids that differed significantly between bacterial and plant KDOPSs are boxed.

Table 3 kdsA-like sequences from plant sources

Source	Accession no.	Definition	Identity with <i>E. coli</i> KdsA (%)	Sequence length
<i>A. thaliana</i>	NP_178068	2-Dehydro-3-deoxyphosphooctonate aldolase-related	46	290
	NP_173084	KDOPS-related	44	291
<i>P. sativum</i>	CAA74644	KDOPS	47	290
<i>O. sativa</i>	AA072599	Putative 2-dehydro-3-deoxyphosphooctonate aldolase	47	340
<i>L. esculentum</i>	CAC35366	KDOPS	46	290

In the phylogenetic trees published to date [25,36], the two plant KDOPSs from *A. thaliana* and *P. sativum*, whose activities were only predicted from their DNA sequences at that time, although located in a distinct branch between Class I and II, were included in Class II, which is considered to be metallo. On the basis of the metal independence of *A. thaliana* KDOPS and its dimeric solution structure, as well as the present phylogenetic analysis, it is proposed in the present study that plant KDOPSs may represent a new, distinct class of KDOPSs, tentatively named Class III. Indeed, in other phylogenetic studies, quaternary structures have been the prime property used to predict the evolution of proteins [37]. When additional plant KDOPS sequences become available, a new phylogenetic tree will be generated to provide further insight into the possible presence of this third class of KDOPS and their evolution. In a related family of enzymes, DAHPSs (3-deoxy-D-

arabino-heptulosonate 7-phosphate synthases), which catalyse a similar aldol condensation reaction except between PEP and E4P, a distinction between plant and bacterial DAHPSs has also been suggested [36]. Actually, bacterial KDOPSs are included in the generation of the tree for the DAH7Ps, whereas the plant DAH7Ps are not included in the data. KDOPS and DAHPS have a similar three-dimensional structure [19,23,38] and are believed to have evolved from a common ancestor, probably the ancient DAHPS [25,36].

Although the monosaccharide KDO has been isolated from plants, its exact physiological role(s) remains unclear [2,7,8]. A BLASTp search of the *A. thaliana* genome database utilizing the *E. coli* protein sequences of the enzymes (total 13) involved in KDO-lipid A synthesis reveals the presence of the homologues to kdsD (formally yrbH) [39], kdsA, kdsB, LpxA, LpxC, LpxD,

Table 4 KDO-lipid A biosynthesis pathway enzyme homologues in *A. thaliana*

Enzyme	Accession no. (<i>A. thaliana</i> / <i>E. coli</i>)	Identity with <i>E. coli</i> counterpart	Sequence length (<i>A. thaliana</i> / <i>E. coli</i>)
kdsD (A5P isomerase)	NP_191029/NP_417664	33	350/328
kdsA (KDOPS)	NP_178068/NP_415733	46	290/284
	NP_173084/NP_415733	44	291/284
kdsC (KDO-8-phosphate phosphatase)	NA*/NP_417665	NA	NA/188
kdsB (CMP-KDO synthetase)	NP_175708/NP_415438	41	290/248
LpxA (acyl-carrier protein-UDP- <i>N</i> -acetylglucosamine acyltransferase)	NP_194683/NP_414723	37	334/262
	NP_192430/NP_414723	27	299/262
LpxC (UDP-3- <i>O</i> -acyl- <i>N</i> -acetylglucosamine deacetylase)	NP_849706/NP_414638	32	955/305
	NP_173884/NP_414638	30	949/305
	NP_173874/NP_414638	30	925/305
	NP_173878/NP_414638	27	905/305
LpxD (UDP-3- <i>O</i> -acyl- <i>N</i> -acetylglucosamine acyltransferase)	NP_192430/NP_414721	40	299/341
	NP_194638/NP_414721	26	334/341
LpxH (UDP-2,3-diacylglucosamine hydrolase)	NA/P43341	NA	NA/240
LpxB (lipid A disaccharide synthase)	NP_178531/NP_414724	34	161/382
LpxK (tetra-acyl-disaccharide 4'-kinase)	NP_566663/P27300	26	395/328
WaaA (KdtA) (KDO transferase)	NP_195997/P23282	34	447/425
LpxL (htrB) (lipid A biosynthesis lauroyl acyltransferase)	NA/P24187	NA	NA/306
LpxM (msbB) (lipid A biosynthesis (KDO) ₂ -(lauroyl)-lipid IVA acyltransferase)	NA/P24205	NA	NA/323

* NA, not applicable.

LpxB, LpxK and WaaA, whereas the remaining four sequences, LpxH, LpxL, LpxM and the newly identified kdsC (formally yrbI) [40], have no significant homologues (Table 4). The presence of these homologues of KDO biosynthetic genes has prompted the hypothesis for the existence of a KDO-lipid A-like molecule that may function as a structural component of chloroplast outer membranes in plants [2]. Insight into the role of KDO in plants awaits further experiments.

At the time of completion of this study, Matsuura et al. [41] published a report on the cloning and tissue expression analysis of *A. thaliana* KDOPS. The *AtkdsA1* (At1g79500) gene was found to be mainly transcribed in the shoots, whereas the *AtkdsA2* (At1g16340) was mainly transcribed in the roots. Additionally, Chevalier and co-workers [42] have isolated a cDNA encoding tomato (*L. esculentum*) KDOPS and analysed the expression of this *kdsA* gene during cell cycle. Studies of these two research groups may aid in elucidating the potential physiological function of plant KDOPS and KDO-lipid A-like molecules.

In summary, KDOPS (kdsA) from *A. thaliana* has been cloned, overexpressed and characterized in detail. This is the first full characterization of a homogeneous plant KDOPS. The enzyme is functionally similar to its bacterial counterparts in terms of catalytic and kinetic properties, is more stable against thermal denaturation, is a dimer in solution and is a non-metallo-enzyme. The plant enzymes seem to be distinct from the bacterial Class I and Class II enzymes and are in this study proposed to represent a third class of KDOPSs. Investigations are currently in progress to elucidate further the differences between plant and microbial enzymes as well as to understand the evolution of this enzyme family.

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REFERENCES

- Raetz, C. R. (1990) Biochemistry of endotoxins. *Annu. Rev. Biochem.* **59**, 129–170
- Raetz, C. R. and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**, 635–700
- Whitfield, C. and Valvano, M. A. (1993) Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. *Adv. Microb. Physiol.* **35**, 135–246
- York, W. S., Darvill, A. G., McNeil, M. and Albersheim, P. (1985) Structure of plant-cell walls. 16. 3-Deoxy-D-manno-2-octulosonic acid (Kdo) is a component of rhamnogalacturonan-II, a pectic polysaccharide in the primary-cell walls of plants. *Carbohydr. Res.* **138**, 109–126
- Doco, T., Williams, P., Vidal, S. and Pellerin, P. (1997) Rhamnogalacturonan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables. *Carbohydr. Res.* **297**, 181–186
- O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G. and Albersheim, P. (1996) Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester – *in vitro* conditions for the formation and hydrolysis of the dimer. *J. Biol. Chem.* **271**, 22923–22930
- Stevenson, T. T., Darvill, A. G. and Albersheim, P. (1988) Structure of plant-cell walls. 23. Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydr. Res.* **182**, 207–226
- Stevenson, T. T., Darvill, A. G. and Albersheim, P. (1988) Structure of plant-cell walls. 22. 3-Deoxy-D-lyxo-2-heptulosaric acid, a component of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydr. Res.* **179**, 269–288
- Becker, B., Lommerse, J. P. M., Melkonian, M., Kamerling, J. P. and Vliegenthart, J. F. G. (1995) The structure of an acidic trisaccharide component from a cell-wall polysaccharide preparation of the green-alga *Tetraselmis striata* Butcher. *Carbohydr. Res.* **267**, 313–321
- Doong, R. L., Ahmad, S. and Jensen, R. A. (1991) Higher-plants express 3-deoxy-D-manno-octulosonate 8-phosphate synthase. *Plant Cell Environ.* **14**, 113–120
- Brabetz, W., Wolter, F. P. and Brade, H. (2000) A cDNA encoding 3-deoxy-D-manno-oct-2-ulosonate-8-phosphate synthase of *Pisum sativum* L. (pea) functionally complements a *kdsA* mutant of the Gram-negative bacterium *Salmonella enterica*. *Planta* **212**, 136–143
- Ray, P. H. (1980) Purification and characterization of 3-deoxy-D-manno-octulosonate 8-phosphate synthetase from *Escherichia coli*. *J. Bacteriol.* **141**, 635–644
- Taylor, W. P., Sheflyan, G. Y. and Woodard, R. W. (2000) A single point mutation in 3-deoxy-D-manno-octulosonate-8-phosphate synthase is responsible for temperature sensitivity in a mutant strain of *Salmonella typhimurium*. *J. Biol. Chem.* **275**, 32141–32146
- Sheflyan, G. Y., Sundaram, A. K., Taylor, W. P. and Woodard, R. W. (2000) Substrate ambiguity of 3-deoxy-D-manno-octulosonate 8-phosphate synthase from *Neisseria gonorrhoeae* revisited. *J. Bacteriol.* **182**, 5005–5008
- Duewel, H. S., Sheflyan, G. Y. and Woodard, R. W. (1999) Functional and biochemical characterization of a recombinant 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase from the hyperthermophilic bacterium *Aquifex aeolicus*. *Biochem. Biophys. Res. Commun.* **263**, 346–351
- Krosky, D. J., Alm, R., Berg, M., Carmel, G., Tummino, P. J., Xu, B. and Yang, W. (2002) *Helicobacter pylori* 3-deoxy-D-manno-octulosonate-8-phosphate (KDO-8-P) synthase is a zinc-metalloenzyme. *Biochim. Biophys. Acta* **1594**, 297–306

- 17 Brabetz, W. and Brade, H. (1997) Molecular cloning, sequence analysis and functional characterization of the gene *kdsA*, encoding 3-deoxy-D-manno-2-octulosonate-8-phosphate synthase of *Chlamydia psittaci* 6BC. *Eur. J. Biochem.* **244**, 66–73
- 18 Radaev, S., Dastidar, P., Patel, M., Woodard, R. W. and Gatti, D. L. (2000) Preliminary X-ray analysis of a new crystal form of the *Escherichia coli* KDO8P synthase. *Acta Crystallogr. D* **56**, 516–519
- 19 Radaev, S., Dastidar, P., Patel, M., Woodard, R. W. and Gatti, D. L. (2000) Structure and mechanism of 3-deoxy-D-manno-octulosonate 8-phosphate synthase. *J. Biol. Chem.* **275**, 9476–9484
- 20 Liang, P. H., Lewis, J., Anderson, K. S., Kohen, A., D'Souza, F. W., Benenson, Y. and Baasov, T. (1998) Catalytic mechanism of Kdo8P synthase: transient kinetic studies and evaluation of a putative reaction intermediate. *Biochemistry* **37**, 16390–16399
- 21 Baasov, T., Sheffer-Dee-Noor, S., Kohen, A., Jakob, A. and Belakhov, V. (1993) Catalytic mechanism of 3-deoxy-D-manno-2-octulosonate-8-phosphate synthase. The use of synthetic analogues to probe the structure of the putative reaction intermediate. *Eur. J. Biochem.* **217**, 991–999
- 22 Kohen, A., Jakob, A. and Baasov, T. (1992) Mechanistic studies of 3-deoxy-D-manno-2-octulosonate-8-phosphate synthase from *Escherichia coli*. *Eur. J. Biochem.* **208**, 443–449
- 23 Duewel, H. S., Radaev, S., Wang, J., Woodard, R. W. and Gatti, D. L. (2001) Substrate and metal complexes of 3-deoxy-D-manno-octulosonate-8-phosphate synthase from *Aquifex aeolicus* at 1.9-angstrom resolution – implications for the condensation mechanism. *J. Biol. Chem.* **276**, 8393–8402
- 24 Wang, J., Duewel, H. S., Woodard, R. W. and Gatti, D. L. (2001) Structures of *Aquifex aeolicus* KDO8P synthase in complex with R5P and PEP, and with a bisubstrate inhibitor: role of active site water in catalysis. *Biochemistry* **40**, 15676–15683
- 25 Birck, M. R. and Woodard, R. W. (2001) *Aquifex aeolicus* 3-deoxy-D-manno-2-octulosonic acid 8-phosphate synthase: a new class of KDO 8-P synthase? *J. Mol. Evol.* **52**, 205–214
- 26 Sheflyan, G. Y., Howe, D. L., Wilson, T. L. and Woodard, R. W. (1998) Enzymatic synthesis of 3-deoxy-D-manno-octulosonate 8-phosphate, 3-deoxy-D-altro-octulosonate 8-phosphate, 3,5-dideoxy-D-gluc(manno)-octulosonate 8-phosphate by 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J. Am. Chem. Soc.* **120**, 11027–11032
- 27 Dotson, G. D., Dua, R. K., Clemens, J. C., Wooten, E. W. and Woodard, R. W. (1995) Overproduction and one-step purification of *Escherichia coli* 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase and oxygen transfer studies during catalysis using isotopic-shifted heteronuclear NMR. *J. Biol. Chem.* **270**, 13698–13705
- 28 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
- 29 Edwards, R. A., Jacobson, A. L. and Huber, R. E. (1990) Thermal denaturation of β -galactosidase and of two site-specific mutants. *Biochemistry* **29**, 11001–11008
- 30 Edge, V., Allewell, N. M. and Sturtevant, J. M. (1985) High-resolution differential scanning calorimetric analysis of the subunits of *Escherichia coli* aspartate transcarbamoylase. *Biochemistry* **24**, 5899–5906
- 31 Fukada, H., Sturtevant, J. M. and Quioco, F. A. (1983) Thermodynamics of the binding of L-arabinose and of D-galactose to the L-arabinose-binding protein of *Escherichia coli*. *J. Biol. Chem.* **258**, 13193–13198
- 32 Duewel, H. S. and Woodard, R. W. (2000) A metal bridge between two enzyme families. 3-Deoxy-D-manno-octulosonate-8-phosphate synthase from *Aquifex aeolicus* requires a divalent metal for activity. *J. Biol. Chem.* **275**, 22824–22831
- 33 Birck, M. R., Holler, T. P. and Woodard, R. W. (2000) Identification of a slow tight-binding inhibitor of 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase. *J. Am. Chem. Soc.* **122**, 9334–9335
- 34 Vitali, J., Carroll, D., Chaudhry, R. G. and Hackert, M. L. (1999) Three-dimensional structure of the Gly121Tyr dimeric form of ornithine decarboxylase from *Lactobacillus* 30a. *Acta Crystallogr. D* **55**, 1978–1985
- 35 Howe, D. L., Sundaram, A. K., Wu, J., Gatti, D. L. and Woodard, R. W. (2003) Mechanistic insight into 3-deoxy-D-manno-octulosonate-8-phosphate synthase and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase utilizing phosphorylated monosaccharide analogues. *Biochemistry* **42**, 4843–4854
- 36 Jensen, R. A., Xie, G., Calhoun, D. H. and Bonner, C. A. (2002) The correct phylogenetic relationship of KdsA (3-deoxy-D-manno-octulosonate 8-phosphate synthase) with one of two independently evolved classes of AroA (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase). *J. Mol. Evol.* **54**, 416–423
- 37 Labedan, B., Xu, Y., Naumoff, D. G. and Glansdorff, N. (2004) Using quaternary structures to assess the evolutionary history of proteins: the case of the aspartate carbamoyltransferase. *Mol. Biol. Evol.* **21**, 364–373
- 38 Shumilin, I. A., Kretsinger, R. H. and Bauerle, R. H. (1999) Crystal structure of phenylalanine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Escherichia coli*. *Structure Fold. Des.* **7**, 865–875
- 39 Meredith, T. C. and Woodard, R. W. (2003) *Escherichia coli* YrbH is a D-arabinose 5-phosphate isomerase. *J. Biol. Chem.* **278**, 32771–32777
- 40 Wu, J. and Woodard, R. W. (2003) *Escherichia coli* YrbI is 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase. *J. Biol. Chem.* **278**, 18117–18123
- 41 Matsuura, K., Miyagawa, I., Kobayashi, M., Ohta, D. and Matoh, T. (2003) *Arabidopsis* 3-deoxy-D-manno-oct-2-ulosonate-8-phosphate synthase: cDNA cloning and expression analyses. *J. Exp. Bot.* **54**, 1785–1787
- 42 Delmas, F., Petit, J., Joubes, J., Seveno, M., Paccalet, T., Hernould, M., Lerouge, P., Mouras, A. and Chevalier, C. (2003) The gene expression and enzyme activity of plant 3-deoxy-D-manno-2-octulosonic acid-8-phosphate synthase are preferentially associated with cell division in a cell cycle-dependent manner. *Plant Physiol.* **133**, 348–360
- 43 Sheflyan, G. Y., Duewel, H. S., Chen, G. and Woodard, R. W. (1999) Identification of essential histidine residues in 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase: analysis by chemical modification with diethyl pyrocarbonate and site-directed mutagenesis. *Biochemistry* **38**, 14320–14329

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