Supporting Information

Conversion of *Aquifex aeolicus* 3-Deoxy-D-*manno*-octulosonate 8-phosphate Synthase, a Metalloenzyme, into a Non-metalloenzyme

Preparation of Mutant KDO 8-P Synthases. The 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (kdsA) mutants were prepared utilizing the QuikChange site-directed mutagenesis kit marketed by Stratagene. Two oligonucleotide primers (Table 1) each containing the desired mutagenic replacement codon, and wild-type plasmid DNA (pT7-7/*kds*A - miniprep) as template were temperature-cycled 16 times with high-fidelity *Vent* DNA polymerase in the presence of buffer and dNTPs through the following cycle program: 95 °C for 30 sec, 55 °C for 1 min, and 72 °C for 6.5 min. The thermal-cycled reaction mixture containing the mutated plasmid with staggered nicks was treated with *Dpn*I to digest the parental pT7- 7/*kdsA* DNA template. The *Dpn*I digestion reaction mixture, containing the nicked mutagenic DNA, was used to transform "super-competent" *E. coli* XL1-Blue cells. Plasmid DNAs were isolated and purified from each of the clones and initially characterized by restriction digestion and then DNA sequencing. DNA containing the proper mutagenic sequence was used to transform chemically competent *E. coli* BL21 (DE3).

Isolation of KDO 8-P Synthases. The *E. coli* BL21 (DE3) cells harboring the pT7- 7/*kdsA* were grown in 2xYT medium containing ampicillin at 37ºC with shaking. When the culture had reached and absorbance of 1.5 at 600 nm, IPTG was added to a final concentration of 0.4 mM. The culture was grown for an additional 4 h at 37ºC with shaking, after which the cells were collected by centrifugation (18000 x g, 20min, 4 ºC) and the cells were suspended in 20 mM Tris (pH 7.5). The cell suspension was subjected to sonication on ice and then clarified by centrifugation (18000 x g, 40min, 4 ºC) to produce the cell extract. Solid sodium chloride was added to the cell extract to a final concentration of 0.1M and the solution was heated in a boiling water bath for 1.5 min and then at 80 ºC for 10 min with continuous swirling. The suspension was allowed to cool to room temperature and then placed on ice for 15 min. Precipitated protein was removed by centrifugation (18000 x g, 20min, 4 ºC) and the supernatant was dialyzed against 20mM Tris (pH7.5) (buffer A). The protein was applied to a Mono Q column previously equilibrated with buffer A. The column was developed at a flow rate of 2 ml/min using a linear gradient to 0.3 M potassium chloride in the same buffer (over 40 min). kdsA activity resolved into a single peak, and the active fractions containing kdsA were pooled, and solid ammonium sulfate was added to a final concentration of 20%. The sample was filtered (0.22 μm) and applied to a phenyl-Superose column equilibrated with 20% ammonium sulfate in buffer A. The column was developed with a linear gradient from 20% to 0% ammonium sulfate in buffer A (over 60 min). Fractions from successive runs were pooled, dialyzed against 20 mM Tris (pH 7.5), aliquoted and the aliquots frozen with dry ice with acetone and stored at -80 ºC.

Target Amino Acid	Primers 5' - 3'	Resulting Amino Acid
C11	GTGATAGCTGGACCCAATGCGATAGAGAGCGAGG CCTCGCTCTCTATCGCATTGGGTCCAGCTATCAC	N
C11	GTGATAGCTGGACCCTCGGCGATAGAGAGCGAGG CCTCGCTCTCTATCGCCGAGGGTCCAGCTATCAC	S
C11	GTGATAGCTGGACCTAAGGCGATAGAGAGCGAGG CCTCGCTCTCTATCGCCTTAGGTCCAGCTATCAC	K
C11	GTGATAGCTGGACCCGGGGCGATAGAGAGCGAGG CCTCGCTCTCTATCGCCCCGGGTCCAGCTATCAC	G
N26	CGTACTGTTGGGCGGTATGTGCGTGTTGGAATCTC GAGATTCCAACACGCACATACCGCCCAACAGTA	C

Table 1. Oligonucleotides Used for the Mutagenesis of KDO8PS.

Kinetic parameters. A continuous spectrophotometric method for the measurement of the disappearance of the α,β-unsaturated carbonyl absorbance of PEP was used to determine kinetic parameters. The standard assay mixture contained PEP, A5P, 100 mM Tris-acetate (pH 7.5), and KDO8PS in 1 mL total volume. The first three reagents were mixed and incubated at 60 °C for 2 min. The assay, initiated by the addition of the KDO8PS, was monitored for 3 min at λ = 232 nm for a decrease in absorption. (ε = 2840 $M^{-1}cm^{-1}$ for the disappearance of the double bond.) K_m and V_{max} values were determined from a nonlinear regression of (substrate concentration, initial velocity) data pairs fit to the Michaelis-Menten equation using KaleidaGraph 3.08d.

Aminoff assay. Enzyme activity was measured in a final volume of 50 μl containing PEP (3mM), A5P (3mM), Tris-acetate buffer (100mM, pH 7.5) and using thin-walled PCR tubes as the reaction vessel. The assay solution was pre-incubated at a specified temperature (2 min) and the reaction was initiated with the addition of enzyme (5 μg) and incubated at the desired temperature. At specified time, the reactions were stopped with the addition of ice-cold TCA (to a final concentration of 5%) and then centrifuged to remove protein. To this reaction solution was added 0.2 ml 0.025 M $NaIO_4$ in 0.125 M H_2SO_4 and the mixture incubated at room temperature for 10 min, then 0.4 ml of 2% $NaAsO_2$ in 0.5 M HCl was added followed by 1 ml of thiobarbituric acid (0.36%, pH 9.0), heated at 100 ºC for 10min. The amount of KDO8P produced was determined by measuring the absorption at λ = 549 nm (ε =1.03 x 10^5 $M^{-1}cm^{-1}$).

Temperature optima. The temperature optima was determined by measuring the activity between 30 and 100 ºC using the Aminoff assay and 0.65 μM purified enzyme. Reactions were incubated at the respective temperatures for 5 min.

Thermostability. Purified enzyme (10 μM) in 100 mM Tris-acetate (pH 7.5) was constantly maintained at 90 ºC in thin-walled PCR tubes. At various times, aliquots of enzyme (10 μl) were removed and subjected to the Aminoff assay. Reactions were incubated for 5 min at 90 ºC.

	Organism	Partial sequence				Accession No.
Class I	*E. coli*	22 FGGMNV 27	201 THALQC 206	237 FIEAHP 242	249 CDGPSA 254	P17579
	P. aeruginosa	22 FGGMNV 27	199 THALQM 204	235 FLEAHP 240	247 CDGPCA 252	AAG07024
	V. cholerae	22 FAGMNV 27	201 THSLGM 206	237 FLEAHP 242	249 CDGPSA 254	Q9KQ29
	S. typhimurium	22 FGGMNV 27	201 THALQC 206	237 FLESHP 242	249 CDGPSA 254	Q8XGR9
	Y. pestis	22 FGGMNV 27	201 THALQC 206	237 FLEAHP 242	249 CDGPSA 254	CAC90835
	H. influenzae	22 FGGMNV 27	201 THSLQC 206	237 FLEAHP 242	249 CDGPSA 254	P45251
Class II	*A. aeolicus*	7 IAGPCA 12	184 THSVQL 189	220 FMETHP 225	232 SDASTQ 237	O66496
	C. jejuni	7 IAGPCV 12	194 THSVQM 199	230 FFETHI 235	242 CDGPNM 247	Q9PIB8
	H. pylori	14 IAGPCV 19	203 THSVQM 208	239 FAETHI 244	251 SDGANM 256	Q9ZN55
	C. psittaci	9 IAGPCV 14	188 THSVQL 193	224 FIETHM 229	236 SDAASM 241	Q46225
	R. prowazekii	20 IAGPCQ 25	196 THSVQQ 201	234 YMEVHQ 239	246 SDGPCM 251	Q92JH7
	C. tepidum	9 IAGPCV 14	188 THSVQL 193	224 FIETHT 229	236 SDAASM 241	Q9Z7I4

Figure 1. Sequence alignment of KDO8PS. Sequences were aligned using Clustal W.[9] Invariant residues identified as metal-binding ligands are shaded (C^{11}, H^{185}, E^{222}, and D^{233} based on amino acid sequence for *A. aeolicus* KDO8PS). The conserved N residues in the Class I are shaded in black. The sequences are followed by their NCBI accession number.

Metal Salt	Sp. Activity (units/mg)
Apo (no metal)	1.62
$MgCl_2$ (0.1 mM)	1.54
$ZnSO_4$ (0.1 mM)	0.38
$FeSO_4$ (0.1 mM)	1.47
$CoCl_2$ (0.1 mM)	1.38
$NiCl_2$ (0.1 mM)	1.50
$MnCl_2$ (0.1 mM)	1.45
$MnCl_2$ (0.5 mM)	1.36
$MnCl_2$ (1 mM)	1.24
$MnCl_2$ (3 mM)	1.15

Table 2. C11N specific activity after incubation with different metals. C11N apo-enzyme was incubated with individual metal salts (different concentrations) listed in 20 mM Tris buffer (pH 7.5) at 25ºC for 15 mins, and then the specific activities were measured at 60ºC using the Aminoff assay.