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Bacillus subtilis 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase revisited: resolution of two long-standing enigmas

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The mono/bifunctional and metallo/non-metallo properties of *Bacillus subtilis* DAHPS (3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase) have been controversial for several decades. The present study investigated the DAHPSs from both the *B. subtilis* parent Marburg strain and the derivative strain 168 in detail and clarified the above two long-standing questions. The DAHPSs from the parent and the derivative 168 strains have identical sequence and are both bifunctional enzymes with a CM (chorismate mutase) activity and a DAHPS activity. The parent strain expresses a second independent monofunctional CM, encoded by *aroH*, that is highly active, while the 168 strain expresses an aroH containing a single residue mutation (A112V) that is significantly less active thus leading to previous confusion regarding the mono/bifunctionality of DAHPS. Metal analysis showed that *B. subtilis* DAHPS as isolated contained iron and zinc and is inactivated by dipicolinic acid; the inactive apoenzyme can be reactivated by bivalent metal ions, indicating that the enzyme is a metalloenzyme. The enzyme-bound metal is insensitive to EDTA treatment, leading to the previous conclusion that this DAHPS does not require a metal. The enzyme displays a homotetrameric structure in solution and appears to follow Michaelis–Menten kinetics with $K_m^{PEP} = 139 \pm 11.4\ \mu M$ for phosphoenolpyruvate, $K_m^{E4P} = 1760 \pm 110\ \mu M$ for D-erythrose 4-phosphate, $k_{cat} = 4.6 \pm 0.1\ s^{-1}$ for DAHPS activity and $K_m^{chorismate} = 850 \pm 97\ \mu M$, $k_{cat} = 0.41 \pm 0.01\ s^{-1}$ for CM activity. *B. subtilis* DAHPS is inhibited by the Shikimate pathway intermediates prephenate and chorismate.

Key words: *Bacillus subtilis*, bifunctional enzyme, chorismate mutase, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHPS), Marburg strain, metalloenzyme.

INTRODUCTION

The Shikimate pathway is responsible for the biosynthesis of aromatic amino acids as well as folates and isoprenoid quinones in bacteria, fungi and plants [1]. PEP (phosphoenolpyruvate) and E4P (D-erythrose 4-phosphate) are condensed to form DAHP via an aldol-type reaction by DAHPS (3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; EC 2.5.1.54) in the first committed enzymatic step in this pathway (Scheme 1). DAHP, via six enzyme steps, is converted into chorismate, the last common or branch point intermediate in the pathway. Chorismate is then rearranged to prephenate by CM (chorismate mutase; EC 5.4.99.5) or converted into anthranilate in subsequent Phe/Tyr or Trp branch biosyntheses respectively. The enzymes of monofunctional DAHPSs and CMs have been extensively investigated [2–8].

An unusual non-sequential CM-DAHPS bifunctional enzyme has been found in *Bacillus subtilis* 168 [9], a commonly used laboratory strain originally created by X-ray mutagenesis of the wild-type *B. subtilis* Marburg [10]. Previously, biochemical and genetic evidence indicated that the DAHPS and CM activities were inseparable and existed in one single peptide in Marburg strain 168 [9,11]. Later it was reported that DAHPS from the parent Marburg strain was a monofunctional enzyme since the DAHPS and CM activities were for the most part separable by standard chromatographic techniques [12]. It was further suggested that the bifunctional enzyme in strain 168 arose from the mutation of the native monofunctional enzyme in the parent Marburg strain. However, more recent studies demonstrated that the DAHPS from the parent Marburg strain (aroA^{Marburg}, DAHPS from *B. subtilis* Marburg) and the CM-DAHPS from Marburg strain 168 [aroA(G)^{168} was a misnomer, aroA(Q)^{168} (DAHPS from *B. subtilis* 168) is more correct] were identical in amino acid sequence, deduced from DNA sequencing [13], and suggested that the biochemical differences reported by previous workers [12] for monofunctional aroA^{Marburg} versus bifunctional aroA(Q)^{168} were due to 'particularities of the multi-enzyme complex formed in these two strains . . . '. To date, no definitive conclusion on this apparent discordance has been reached. It would be of general biological interest to clarify these issues.

A second question surrounding the *B. subtilis* CM-DAHPS is its metal requirement. Based on phylogenetic analysis, DAHPSs have been separated into two classes (class I and class II) [14], or into two distinct homology families (AroAI and AroAII) [15,16]. The AroAII family was defined as 'plant-like' DAHPSs that included the higher plant proteins and a cluster of microbial proteins [17]. The AroAI family was further divided into subfamilies AroAI$_\alpha$ and AroAI$_\beta$ which correspond to class II and class I above, respectively. Metal requirement was previously suggested to be one property distinguishing the two classes or subfamilies [14] since CM-DAHPS from *B. subtilis* (a member of class I or AroAI$_\beta$) have been reported to be insensitive to EDTA treatment [18] while the three *Escherichia coli* iso-DAHPSs (a member of class II or AroAI$_\alpha$) were reported to utilize a metal cofactor [19]. However, in a recent report [20], *Thermotoga maritima* DAHPS (class I or AroAI$_\beta$) has been reported to be metallo. Sequence alignment of various DAHPS, including DAHPS from *T. maritima* and

Abbreviations used: aroA(Q)^{168}, DAHPS from *B. subtilis* 168; aroA^{Marburg}, DAHPS from *B. subtilis* Marburg; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; CM, chorismate mutase; aroH^{168}, CM from *B. subtilis* 168; aroH^{Marburg}, CM from *B. subtilis* Marburg; CM-DAHPS^{Bacillus}, DAHPSs from both *B. subtilis* Marburg and 168 strain; DAHPS, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; DPA, dipicolinic acid; E4P, D-erythrose 4-phosphate; ORF, open reading frame; PEP, phosphoenolpyruvate.

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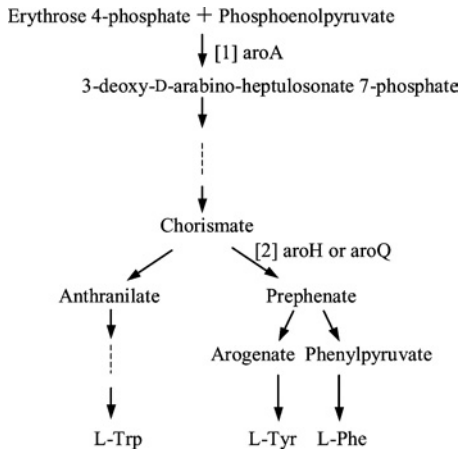

Scheme 1 Shikimate pathway

(1) DAHPS (encoded by *aroA*) and (2) CM (encoded by *aroH* or *aroQ*).

CM-DAHPS, demonstrated that the four metal-chelating residues in Phe-sensitive *E. coli* DAHPS (Cys^{61}, His^{268}, Glu^{302} and Asp^{326} ligands to Pb^{2+} or Mn^{2+}) [21] are invariable in all DAHPS families leading to the hypothesis that all the DAHPSs are metallo [20]. Therefore whether *B. subtilis* DAHPS is a metallo- or non-metalloenzyme becomes critical in the phylogenetic studies of DAHPSs. If the enzyme is indeed a non-metallo enzyme, it could suggest a new class of DAHPS potentially functioning via an entirely new mechanism. Thus the metal requirement of *B. subtilis* CM-DAHPS requires clarification.

In the present study, the DAHPSs from *B. subtilis* parent Marburg and the derivative strain 168 were thoroughly investigated and the results presented here finally clarified the above decades-old questions.

EXPERIMENTAL

Materials

B. subtilis Marburg (ATCC 6051) was purchased from A.T.C.C. (Manassas, VA, U.S.A.). All nucleic acid manipulations were performed according to standard procedures [22]. Restriction enzymes and T_4 DNA ligase were purchased from New England Biolabs (Beverly, MA, U.S.A.). The Promega Wizard DNA purification kit was utilized for plasmid isolation and purification. Epicurian Coli™ XL1-Blue cells and *E. coli* BL21(DE3) cells were obtained from Stratagene Cloning System (La Jolla, CA, U.S.A.) and Novagen (Madison, WI, U.S.A.) respectively. Primers were synthesized by Invitrogen (Carlsbad, CA, U.S.A.) and are listed in Table 1. DNA sequencing and primer syntheses were performed by the University of Michigan Biomedical Resources Core Facility. The PEP mono(cyclohexylammonium) salt, A5P (D-arabinose 5-phosphate) disodium salt, R5P (D-ribose 5-phosphate) disodium salt, E4P (D-erythrose 4-phosphate) sodium salt and DPA (dipicolinic acid) were obtained from Sigma (St. Louis, MO, U.S.A.). Puratronic grade $NiCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$, $CdCl_2$, $FeSO_4$, $CuSO_4$, $ZnSO_4$ and HCl (99.999 %, metal basis) were purchased from Alfa Aesar (Ward Hill, MA, U.S.A.). The BTP {1,3-bis[tris(hydroxymethyl)methylamino]propane} was purchased from Research Organics (Cleveland, OH, 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 kDa molecular mass cut-off and metal-free) was obtained from VWR (St. Louis, MO, U.S.A.). The Mono Q (HR 10/10), Phenyl Superose (HR 10/10), Superose 12 (HR 10/30) and FAST Desalting (HR 10/10) chromatography columns were from Amersham Biosciences.

Table 1 Oligonucleotide primers used in the present study

Name	Sequence
P1	CATGTAGGATAGCTTCATAGATTACTTTATCAC
P2	GTCGTTTATGATTTGTAAGCGTAAGTCAGTAGTGTAC
P3	GATTCTAGAATTCATATGAGCAACACAGAGTTAGAGC
P4	GATTCTGAATTCGGATCCGATTAAGCGTTGACTTTCACC
P5	GTTTCTCGTTGCTGTTTCGTATGTTCTGATTCCG
P6	AACCCGTGGCGGGAAGATTCAGT
P7	TCAGCTCAACTAAACTCATTCTCTGC
P8	CTAGATTCTAGAATTCATATGATGATTCGCGGAATTC
P9	GATTATGAATTCGGATCCTTACAATTCAGTATTTTTTGTCAATG
P10	CAGACATGTATATCTCGAGAAAGTTGTCGTATTGAGG
P11	CCTCAATACGACAACTTTCTCGAGATATACATGTCTG

Construction of plasmids

pT7-7/aro$A^{Marburg}$ was constructed as follows. A DNA fragment containing the potential *aroA*^{Marburg} was first obtained by standard PCR procedure using *B. subtilis* Marburg (ATCC 6051) genomic DNA as template and the forward (P1) and reverse (P2) primer (Table 1). P1 and P2 were designed to complement the approx. 150 bp up- and downstream of *aroA*(*Q*)^{168} ORF (open reading frame) [gene access number for *aroA*(*Q*)^{168}: AF008220 region: 132601–133677] from the *B. subtilis* 168 genome database at NCBI website. The sequence of *aroA*^{Marburg} ORF was obtained within this DNA fragment. Three primers were constructed to clone *aroA*^{Marburg} since it contains an internal NdeI restriction site. The forward primer P3 and the reverse primer P4 correspond to the 5′ and 3′ terminus of the *aroA*^{Marburg} ORF respectively. P3 contains an NdeI site (underlined) and P4 contains a BamHI site (underlined). P5 was designed to remove the internal NdeI site in an amino acid silent fashion. A two-step PCR procedure was utilized. In the first PCR, the 5′-terminal part of the *aroA*^{Marburg} (802 bp) was amplified from the *B. subtilis* Marburg (ATCC 6051) genomic DNA with P3 and P5 primers. In the second PCR, the full-length *aroA*^{Marburg} ORF was amplified using the first PCR product and P4 as primers. The second amplification product was isolated, restricted with NdeI and BamHI, and ligated into the similarly restricted vector, pT7-7. The ligation mixture was used to transform Epicurian Coli™ XL1-Blue cells. The presence of plasmids containing the desired gene from several transformants was verified by restriction analysis and the gene sequence was confirmed by DNA sequencing. One plasmid with the correct sequence, pT7-7/aro$A^{Marburg}$, was used to transform chemically competent *E. coli* BL21(DE3) cells.

B. subtilis 168 has a monofunctional CM encoded by *aroH*. The *aroH*^{Marburg} (CM from *B. subtilis* Marburg) ORF sequence was obtained using the same method as described above for obtaining *aroA*^{Marburg} ORF. A DNA fragment was first obtained using primers P6 and P7, which were designed to complement the approx. 150 bp up- and downstream *aroH*^{168} (CM from *B. subtilis* 168) ORF sequence (gene access number for *aroH*^{168}: M80245 region: 7732–8115). The sequence of *aroH*^{Marburg} was obtained by searching the ORF within this DNA fragment. P8 and P9, designed to correspond to the 5′ and 3′ termini of the *aroH*^{Marburg} ORF, were used to amplify *aroH*^{Marburg}. The PCR product was digested with NdeI and BamHI, purified, and ligated into the similarly treated pT7-7. The ligation mixture was used to transform competent

Epicurian Coli™ XL1-Blue cells. One plasmid, pT7-7/aroH^{Marburg}, from these clones containing the correct insert, confirmed by sequencing, was transformed into the expression cells, *E. coli* BL21(DE3). The pT7-7/aroH^{Marburg}V112A was prepared by site-directed mutagenesis using pT7-7/aroH^{Marburg} as template and P10 and P11 as primers according to the manufacturer's instructions.

Protein overexpression and purification

All proteins were expressed and purified using the following method. The *E. coli* BL21(DE3) cells harbouring the desired plasmid were grown in LB (Luria–Bertani) medium containing ampicillin (100 mg/l) at 37 °C with shaking (250 rev./min). When the culture reached D_{600} of ~1.5, it was allowed to cool to 25 °C and induced with isopropyl-β-D-thiogalactoside at a final concentration of 0.4 mM. The cells were harvested after 16 h of additional growth at 25 °C and stored at − 20 °C.

The cells were suspended in 10 mM BTP (pH 7.5) (buffer A), subjected to sonication on ice (30 s pulses with a 2 min rest between pulses, five times) and centrifuged to remove cell debris (40 000 ***g***, 30 min, 4 °C). The clarified supernatant was loaded on to a Q-Sepharose anion exchange column pre-equilibrated with buffer A and the column eluted with a gradient of 0–0.4 M KCl in buffer A. The fractions containing the desired protein with the expected molecular mass as confirmed by SDS/PAGE were pooled. Solid $(NH_4)_2SO_4$ was slowly added to the pooled fractions with gentle stirring on ice to a final concentration of 20 % (w/v). The solution was filtered (0.22 μm, Millipore) and loaded on to a Phenyl Superose column (HR 10/10) pre-equilibrated with 20 % $(NH_4)_2SO_4$ in buffer A. A reverse gradient from 20 to 0 % $(NH_4)_2SO_4$ in buffer A was applied at a flow rate of 1.0 ml/min for 60 min. The fractions containing the desired protein were pooled and dialysed against 2 litres of 10 mM BTP (pH 7.5) overnight. The purified enzymes were aliquoted, frozen on ethanol/solid CO_2, and stored at − 80 °C.

Enzyme assay

The DAHPS activity was determined by either discontinuous colorimetric assay or continuous spectrophotometric assay. One unit of enzyme activity is defined as the production of 1 μmol of DAHP or the disappearance of 1 μmol of PEP per min. The standard discontinuous colorimetric assay was measured in a final volume of 50 μl containing PEP (3 mM), DAHPS (varying amounts) and BTP buffer (100 mM, pH 7.5). The assay solution was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of E4P to a final concentration of 6 mM. After the appropriate incubation time, the reaction was quenched by the addition of 50 μl of 10 % (w/v) ice-cold trichloroacetic acid. The amount of DAHP produced was quantified using a modified Aminoff periodate-thiobarbituric acid assay [23]. The continuous spectrophotometric assay measures the disappearance of the α,β-unsaturated carbonyl absorbance ($\lambda = 232$ nm, $\varepsilon = 2840\ M^{-1} \cdot cm^{-1}$) of PEP in an assay mixture containing 100 mM BTP buffer (pH 7.5), 600 μM PEP, 1 mM E4P and 20–80 nM DAHPS.

The CM activity was determined by the previously reported fixed point assay [24] with the following modification. The standard assay was measured with 3 mM of chorismate in BTP buffer (100 mM, pH 7.5) in a final volume of 100 μl. The assay solution was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of the enzyme. The reaction was allowed to proceed for 2 min, and quenched by the addition of 100 μl of 1 M HCl. The entire reaction mixture was incubated at 37 °C for 15 min to convert the enzymatically formed prephenic acid into phenylpyruvic acid, and then neutralized by adding 500 μl of 2 M NaOH. The concentration of phenylpyruvic acid was determined immediately at 320 nm ($\varepsilon_{320} = 17\,500\ M^{-1} \cdot cm^{-1}$). One unit of enzyme activity is defined as 1 μmol of phenylpyruvic acid produced per min at 37 °C.

Molecular mass determinations of aroA^{Marburg}

The subunit molecular mass of the enzyme was determined by SDS/PAGE. The native molecular mass of aroA^{Marburg} was determined by gel filtration utilizing a Superose 12 column (HR 10/30) according to the manufacturer's instructions (Sigma). The elution volume was determined in triplicate for all samples and standards.

Characterization of the products of aroA^{Marburg} catalysed reactions

To obtain the condensation products by aroA^{Marburg}, the enzyme (2 mg, 53 nmoles) was added to a 10 ml plastic centrifuge tube containing the following: PEP mono(cyclohexylammonium) salt (7 mg, 0.026 mmol), E4P (13.0 mg, 0.065 mmol), BTP buffer (pH 6.8; 0.075 mmol) and water in a final volume of 1.5 ml. This reaction mixture was incubated at 37 °C for 2 h. The reaction mixture was quenched by adding 0.3 ml of 10 % (w/v) trichloroacetic acid, centrifuged to remove precipitated protein and immediately loaded on to a 5 ml Econo-Pac High Q Anion Ion Exchange Catridge (chloride form) which had been pre-equilibrated with water. After the sample was loaded, the column was washed with 20 ml of water at a flow rate of 1 ml/min and then eluted with a linear gradient of 0–500 mM LiCl (for 60 min at 1 ml/min, total volume 60 ml). Fractions (2 ml) were collected. One major periodate-TBA positive peak, corresponding to DAHP, was obtained at 200 mM LiCl. The fractions containing the DAHP were pooled, freeze-dried, reconstituted in 0.5 ml of ^2H_2O and the ^{1}H-, ^{13}C- and ^{31}P-NMR measured on a Bruker Advance DRX 500 NMR utilizing the WALTZ water suppression pulse sequence (Supplementary Figure 1, http://www.BiochemJ.org/bj/390/bj3900583add.htm).

To obtain the products due to the CM activity of aroA^{Marburg}, the enzyme (2 mg, 53 nmol) was added to a 10 ml plastic centrifuge tube containing the following: chorismic acid (10 mg, 0.04 mmol), sodium phosphate buffer (pH 6.0; 0.10 mmol) and water in a final volume of 1 ml. This reaction mixture was incubated for 2 h at 37 °C. The reaction mixture was freeze-dried, reconstituted in 0.5 ml of ^2H_2O and the ^{13}C-NMR measured on a Bruker Advance DRX 500 NMR. The NMR sample was then directly treated with 50 μl of DCl and heated for 1 h at 40 °C. The ^{13}C-NMR spectrum was measured again as above (Supplementary Figure 2, http://www.BiochemJ.org/bj/390/bj3900583add.htm).

Metal requirement of DAHPS

To test the effect of metal chelators on DAHPS activity for recombinant aroA^{Marburg}, an enzyme sample (0.6 μM) was incubated with various concentrations of metal chelators (EDTA or DPA) in 100 mM BTP (pH 7.5) at 25 °C for 10 min. PEP (3 mM) was added to the enzyme/chelator solution and the mixture was incubated at 37 °C for 2 min before initiating the reaction with E4P (6 mM). The reaction time was 2 min. The DAHPS activity was measured in triplicate using the discontinuous colorimetric assay.

To determine the metal content of recombinant enzyme directly following purification, samples of enzyme were dialysed against 1 litre of 20 mM Tris/HCl (pH 7.5) overnight. The protein concentration and enzymatic activity of the dialysed enzymes were determined and the samples were subjected to metal analysis by high-resolution inductively coupled plasma MS on a Finnigan

Table 2 Metal analysis of aroA^{Marburg}

DPA-treated apo-aroA^{Marburg} (100 μM) was incubated with each individual metal or a cocktail of metal salts (100 μM each) in 20 mM Tris/HCl (pH 7.5) at 25 °C for 2 h and then applied to a FAST desalting column to remove excess metal salts from the protein–metal complex. The protein fraction was subjected to enzyme assay, protein assay and metal analysis.

	Metal (mol)/enzyme (mol) subunit								
Sample	Zinc	Cadmium	Cobalt	Manganese	Copper	Magnesium	Iron	Nickel	Activity (units/mg)
As isolated*	0.30	–†	–	–	–	0.03	0.15	–	4.9
Apoenzyme‡	0.02	–	–	0.02	0.01	0.02	0.02	–	< 0.1
$ZnSO_4$	0.63	–	–	–	–	0.04	0.04	–	3.3
$CdCl_2$	0.03	0.47	–	–	–	0.05	0.04	–	6.1
$CoCl_2$	0.05	–	0.24	–	–	0.01	0.02	–	0.3
$MnCl_2$	0.02	–	–	0.1	–	0.01	0.01	–	0.2
$CuSO_4$	0.01	–	–	–	0.52	0.01	0.01	–	< 0.1
$MgCl_2$	0.05	–	–	–	–	0.3	0.03	–	< 0.1
FeSO4	0.06	–	–	–	–	0.04	0.4	–	< 0.1
$NiCl_2$	0.02	–	–	–	–	0.05	0.01	0.2	< 0.1
All§	0.37	0.18	0.04	0.06	0.34	0.02	0.34	0.05	6.8
Blank‖	0.03	–	0.02	0.01	–	0.01	0.01	–	

* Enzyme as isolated without DPA treatment.
† Value < 0.01.
‡ DPA-treated apoenzyme without metal reconstitution.
§ DPA-treated apoenzyme was incubated with 1 mol of a cocktail of metal salts and desalted as described above.
‖ A solution, without enzyme, was treated under identical conditions as the 'All' sample. Values represent metal concentration divided by the protein concentration determined for the 'All' sample.

MAT ELEMENT instrument at the W. M. Keck Elemental Geochemistry Laboratory (Department of Geology, University of Michigan) by Dr T. Huston. As a control, the metal content of the dialysis buffer post dialysis was also analysed.

Apo-aroA^{Marburg} was obtained by treating the enzyme (6–7 mg/ml) with 10 mM DPA in 20 mM Tris/HCl (pH 7.5) at 25 °C for 2 h and then dialysed against 1 litre of 20 mM metal-free Tris/HCl (pH 7.5) at 4 °C for 24 h with two buffer changes. The metal-free Tris/HCl buffer was prepared directly by using metal-free water (PURELAB plus system), ultra pure Trizma base and HCl (metal-free).

Apo-aroA^{Marburg} was incubated with a mixture of metal salts containing 1 mol of each metal (Table 2) at 25 °C for 2 h and then applied to a Fast Desalting column (HR 10/10) to remove excess metal salts from the potential protein–metal complex. The column was equilibrated with 20 mM Tris/HCl (pH 7.5) and developed at a flow rate of 0.75 ml/min. The fraction eluting between 1.5 and 3.5 min containing the entire protein fraction was collected. The protein concentration and enzymatic activity of each protein–metal complex were determined immediately without freeze–thawing. The samples were subjected to metal analysis.

Kinetic studies of aroA^{Marburg}

Reactions were performed at 37 °C in 100 mM BTP (pH 7.5) using the continuous assay for DAHPS activity and the discontinuous assay for CM activity. Substrate concentrations typically ranged from 0.1 to 5 or 10K_m. For DAHPS activity, initial velocities were determined by varying the concentration of one substrate while holding the concentration of the other substrate constant (> 5K_m). Best fits of K_m and V_{max} were determined by fitting the initial velocity versus substrate concentration using a non-linear least squares fit to the standard Michaelis–Menten equation.

pH dependence of DAHPS activity

The pH dependence of the enzyme activity was measured between pH 4.5 and pH 10.0 at 37 °C by the discontinuous assay described above using 3 mM PEP and 6 mM E4P in 100 mM succinic acid/sodium tetraborate (pH 4.5–5.5), 2-(*N*-morpholino)ethanesulphonic acid (pH 5.5–6.5), BTP (pH 6.5–9.5) or glycine (pH 9.5–10.0) buffers.

Table 3 Feedback inhibition of DAHPS

The enzyme (0.5 μM) was incubated with 3 mM PEP and a fixed concentration of possible inhibitor (1 mM) in 100 mM BTP (pH 7.5) on ice for 15 min. The reaction was initiated by the addition of 6 mM E4P at 37 °C. Activities were measured by the discontinuous assay. The results are the averages of triplicate assays.

Compound	Activity remaining after treatment (%)
None*	100
Prephenate	3 ± 1
Chorismate	50 ± 4
Shikimate	97 ± 3
L-Phe	101 ± 4
L-Tyr	99 ± 6
L-Trp	98 ± 5

* Addition of the inhibitor post enzymatic reaction but pre-assay served as control for potential interference of the inhibitor with the colorimetric assay.

Feedback inhibition of DAHPS

The feedback regulation of DAHPS activity was determined by incubating 0.5 μM enzyme and 3 mM PEP with a fixed concentration of potential inhibitor (1 mM) (Table 3) in 100 mM BTP (pH 7.5) on ice for 15 min. The reaction was initiated by the addition of E4P (6 mM) and allowed to continue for 2 min. The activity of the enzyme was determined using the discontinuous assay. Addition of the inhibitor post enzymatic reaction but pre-assay served as a control for potential interference of the inhibitor with the Aminoff colorimetric assay [20].

Substrate specificity of DAHPS

A 50 μl reaction mixture containing PEP (3 mM), a phosphorylated monosaccharide (3 mM A5P or R5P) and BTP buffer (100 mM, pH 7.5) was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of recombinant aroA^{Marburg} (11 μM) for 10 min. The reaction was quenched by the

addition 50 μl of 10 % (w/v) trichloroacetic acid. The amount of potential monosaccharide produced was determined by a modified Aminoff periodate-thiobarbituric acid assay in which the oxidization step was carried out at 60 °C instead of 25 °C to ensure the complete oxidation of the potential monosaccharide products.

Miscellaneous methods

Protein concentrations were determined using the Bio-Rad Protein Assay Reagent with BSA (Sigma) serving as the standard. SDS/PAGE was performed under reducing conditions on a 12 % (w/v) polyacrylamide gel with a Mini-PROTEAN II electrophoresis unit (Bio-Rad) and visualized with 0.25 % Coomassie Brilliant Blue R250 stain. Optical spectroscopy was performed using an HP 8453 UV–visible spectrophotometer.

RESULTS

Cloning, overexpression and purification of aroA^{Marburg}

In order to prevent any potential artifact of 'identical protein sequence' between aroA^{Marburg} and aroA(Q)^{168} reported previously [13], the gene sequence of *aroA*^{Marburg} was re-examined since the gene sequence of *aroA*(*Q*)^{168} is available in the NCBI database. Given that the genome sequences of *B. subtilis* Marburg and strain 168 should have very high identity since the latter was derived from the former by X-ray irradiation [10], a genomic fragment containing *aroA*^{Marburg} ORF was obtained by the standard PCR methodology using the primer complementary to the up- and downstream of *aroA*(*Q*)^{168} ORF from *B. subtilis* 168 genome database. Two separate PCR experiments were performed at different dates and both purified PCR products were subjected to DNA sequencing in order to prevent potential error caused during PCR by DNA polymerase or DNA sequencing. The sequences of the 'two batches' of PCR products were identical and an ORF was located within the genomic fragment. The sequence of this ORF was identical with the sequence of ORF for *aroA*(*Q*)^{168} from the NCBI database, suggesting that the protein sequence of aroA^{Marburg} is identical with that of aroA(Q)^{168}. The *aroA*^{Marburg} gene was cloned into the expression vector pT7-7 and the protein was overexpressed in *E. coli* BL21(DE3) cells. The homogeneous recombinant protein exhibited both DAHPS and CM activity.

Cloning, overexpression and purification of aroH^{Marburg} and aroH^{168}

B. subtilis 168 has a monofunctional CM encoded by *aroH*^{168}. The sequence of *aroH*^{Marburg} ORF from *B. subtilis* Marburg was obtained by the same methodology, described above, utilized for obtaining the sequence of *aroA*^{Marburg}. The genomic fragment containing *aroH*^{Marburg} ORF was first obtained by PCR using the primers complementary to the up- and downstream of *aroH*^{168} ORF from *B. subtilis* 168 genome database. The *aroH*^{Marburg} ORF was obtained within this sequenced genomic fragment and compared with the *aroH*^{168} ORF from the NCBI database. A single base difference, 335T in *aroH*^{Marburg} versus 335C in *aroH*^{168}, was observed. This difference results in a single amino acid change in the protein sequences with Ala^{112} in aroH^{Marburg} versus Val^{112} in aroH^{168}. The *aroH*^{Marburg} gene was cloned into the expression vector pT7-7. The construction of the plasmid containing aroH^{168} gene, and pT7-7/aroH^{168} was carried out by site-directed mutagenesis of aroH^{Marburg} Ala^{112} into Val^{112} using pT7-7/aroH^{Marburg} as template. Both aroH^{Marburg} and aroH^{168} were overexpressed and purified to homogeneity. The specific activity of aroH^{168} and aroH^{Marburg} was 1.2 and 254 units/mg respectively.

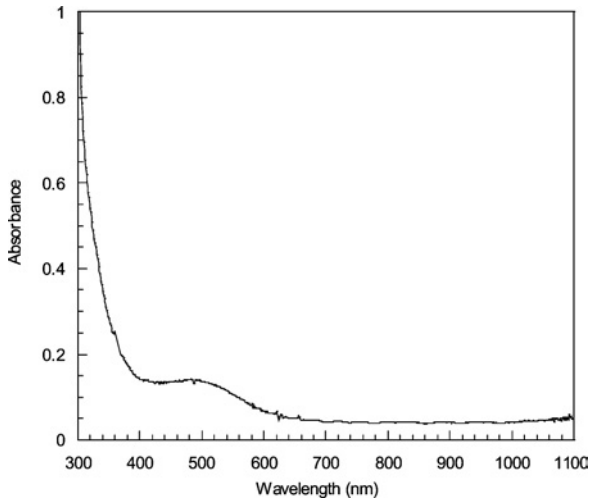

Figure 1 Absorption spectrum of aroA^{Marburg} as isolated

Physical properties of aroA^{Marburg}

The molecular mass of the purified enzyme as determined by SDS/PAGE was 40 kDa. The molecular mass of the native enzyme determined by analytical gel-filtration chromatography was 156 kDa, 3.9 times the molecular mass determined by SDS/PAGE. Therefore *B. subtilis* DAHPS is likely to adopt a tetrameric structure in solution.

Characterization of the products of aroA^{Marburg} catalysed reactions

The ^{1}H-NMR spectra (Supplementary Figure 1, http://www.BiochemJ.org/bj/390/bj3900583add.htm) of the monosaccharide product generated from DAHPS activity of *aroA*^{Marburg} (PEP and E4P as substrates) are consistent with the previously reported spectra for DAHP generated from *E. coli* DAHPS (Phe-sensitive) condensation [7]. The prephenate product generated from CM activity of *aroA*^{Marburg} was chemically converted into phenylpyruvic acid. The presence of prephenic and phenylpyruvic acid was verified by NMR spectroscopy (Supplementary Figure 2, http://www.BiochemJ.org/bj/390/bj3900583add.htm).

Metal requirement of DAHPS

During the purification of aroA^{Marburg}, a light pink colour was observed in the fractions containing DAHPS activity. The concentrated homogeneous aroA^{Marburg}, as isolated, was subjected to UV–visible spectral scanning. A peak was observed at approx. 480 nm (Figure 1), which is consistent with previous reports for an iron-containing enzyme [19]. The identity of the metal was confirmed by metal analysis. The recombinant aroA^{Marburg}, as isolated, contained approx. 0.15 mol of iron and 0.3 mol of zinc per subunit (Table 2). To determine if the metal associated with the purified aroA^{Marburg} is important to DAHPS activity, the separate proteins were treated individually with metal chelators, either EDTA or DPA. It was found that the DAHPS activity in aroA^{Marburg} was effectively eliminated in a concentration-dependent manner by treatment with DPA but not by EDTA at concentrations as high as 10 mM (Figure 2).

To investigate further the role of metal(s) in the aroA^{Marburg}, the enzyme was first treated with 10 mM DPA followed by extensive dialysis against metal-free buffer. The DAHPS activity of

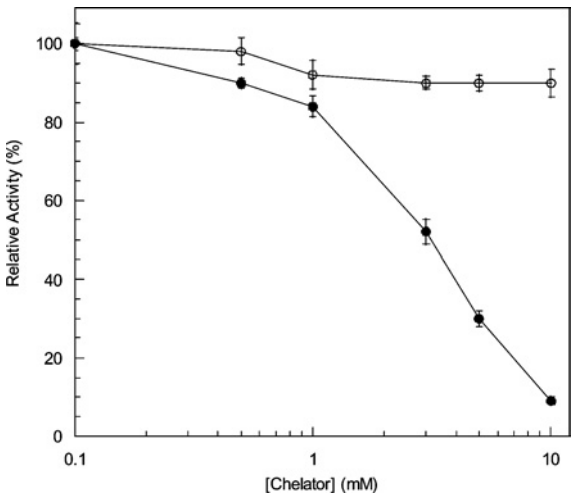

Figure 2 Effect of metal chelators on DAHPS activity

The enzymes were incubated with various concentrations of EDTA (○) or DPA (●) in 100 mM BTP (pH 7.5) at 25 °C for 10 min and then subjected to activity assay by the discontinuous method. 'Error bars' correspond to the S.D. for three determinations.

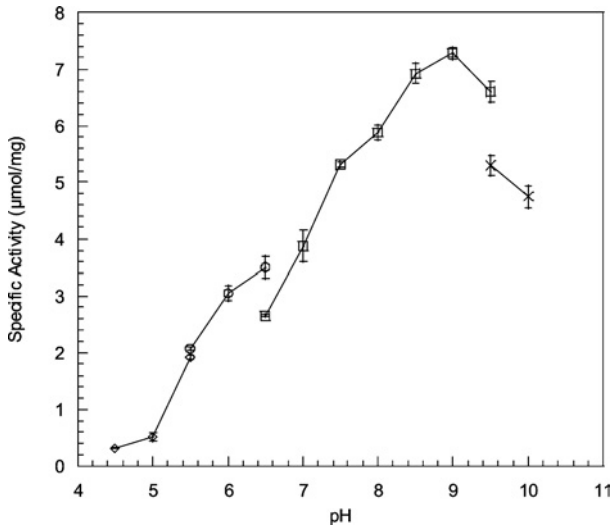

Figure 3 Optimal pH of DAHPS

Enzymatic activity was measured using 3 mM PEP, 6 mM E4P in 100 mM succinic acid/sodium tetraborate (pH 4.5–5.5; ◇), 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 assay. 'Error bars' correspond to S.D. for three determinations.

apo-aroA^{Marburg} was reduced to 1 % of that of the untreated enzyme. The DPA-treated apo-aroA^{Marburg} was incubated with individual metal salts (4-fold molar excess per subunit) or a cocktail of the same metal salts (1 mol of each of the metals per subunit), respectively (Table 2), and the excess free metals were removed by gel filtration. The protein–metal fraction was analysed for specific activity and metal content. The apo-aroA^{Marburg} was found to be able to bind a variety of bivalent metals including Zn^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} when reconstituted with individual metal salts. However, only the enzymes incubated with cadmium and zinc showed activity, which was 124 and 67 % of that of the as-isolated enzyme respectively. When the apo-enzyme was incubated with a cocktail of metals (1 mol of each of the above metals per subunit), it bound mainly Zn^{2+}, Cd^{2+}, Co^{2+} and iron as well as a trace amount of other metals. The specific activity of the cocktail reconstituted apo-aroA^{Marburg} was approx. 120 % of that of the as-isolated enzyme (Table 2).

Kinetic studies of aroA^{Marburg}

The kinetic constants of the enzymes were determined at 37 °C in 100 mM BTP (pH 7.5) using a continuous assay for DAHPS activity and a discontinuous assay for CM activity. The enzymes showed both DAHPS and CM activity. The substrate appears to exhibit Michaelis–Menten kinetics with $K_m^{PEP} = 139 \pm 11.4\ \mu M$, $K_m^{E4P} = 1760 \pm 110\ \mu M$, $k_{cat} = 4.6 \pm 0.1\ s^{-1}$ for DAHPS activity, and $K_m^{chorismate} = 850 \pm 97\ \mu M$ and $k_{cat} = 0.41 \pm 0.01\ s^{-1}$ for CM activity.

pH dependence of DAHPS activity

The purified enzyme exhibited highest enzymatic activity (> 85 % of maximum) between pH 8.0 and 9.5, with an optimum of pH at 9 (Figure 3).

Feedback inhibition of DAHPS activity

Of the possible feedback regulators tested (Table 3), prephenate and chorismate inhibited the DAHPS activity of aroA^{Marburg} with 3 and 50 % of activity remaining, respectively. The compounds L-Phe (L stands for the stereochemical configuration of the amino acid), L-Tyr, L-Trp and shikimate had no effect on enzymatic activity under the experimental conditions.

Substrate specificity of DAHPS

Neither A5P nor R5P served as substrate as determined by a modified Aminoff periodate-thiobarbituric acid assay in which the oxidation of the potential monosaccharide product was enhanced to ensure the detection of lower level products.

DISCUSSION

Two of the long-standing debates concerning the biochemical properties of the DAHPSs from *B. subtilis* Marburg, aroA^{Marburg} and aroA(Q)^{168}, have been solved. Both enzymes are bifunctional and both require a metal for catalysis.

It has been previously reported that aroA^{Marburg} is a monofunctional enzyme while aroA(Q)^{168} is a bifunctional enzyme [11,12]. aroA^{Marburg} was concluded to be a monofunctional enzyme by Llewellyn et al. [12] based on the findings that the activity of CM was always separable from the DAHPS in Marburg strain by standard chromatographic techniques and that CM activity was always associated with the DAHPS in 168 strain. This conclusion was further supported by their observation that the activity ratio of DAHPS/CM in Marburg strain (ATCC 6051) increased drastically during purification whereas this ratio remained almost constant in strain 168 (WB 2802) whose DAHPS was believed to be bifunctional according to earlier biochemical and genetic evidence [9,11]. Nevertheless, the present study and a previous report [13] demonstrated that aroA^{Marburg} shares identical amino acid sequence with aroA(Q)^{168}. An NMR analysis of the reaction products of recombinant aroA^{Marburg} in this study confirmed that this enzyme exhibits both DAHPS and CM activity. One possible explanation for the previous separable versus inseparable CM and DAHPS activity is that there is another enzyme capable of catalysing the chorismate rearrangement reaction in the parent Marburg strain, and this enzyme does not exist or exists only in a less active/mutated form in strain 168. It is noteworthy that in

Organism	Partial sequence	Accession No.
B. subtilis 168	IRHVYLEKVVVLRPDLSL 121	NP_390150
B. subtilis Marburg	IRHVYLEKAVVLRPDLSL 121	present study
B. halodurans	IHHVYLNEAVKLRPDLQL 121	BAB05377
G. stearothermophilus	ICHVYLKDAAMLRPDLSL 120	AF139534
O. iheyensis	INHVFHNNAVKLRPDL-L 120	BAC13739
S. avermitilis	IAHVYLGAAGALRKDIAQ 120	BAC74231
S. coelicolor	IAHVYLGAAAALRKDIAQ 120	CAB52826
T. fusca	LHHVYLRGAQVLRLDIAQ 120	ZP_00057584
T. tengcongensis	VKHVYLKEAKNLRPDLA- 88	AAM24571
T. thermophilus	VRHVYLREAVRLRPDLES 120	AAO40746
D. hafniense	IQPVYLRDAVNLRRDLVE 121	ZP_00098421
N. punctiforme	IHHIYLRNAASLRPDWSL 122	ZP_00106828
T. erythraeum	IVHTYLREAKNLRPDLVN 147	ZP_00074755
T. elongatus	IYHAYLRHAQSLRPDLAM 128	BAC09355
G. violaceus	IQHVYLRGARGLRPDRV- 123	BAC90646
C. thermocellum	IKHVYLNGAKVLRPDLT- 118	ZP_00061130
L. innocua]	INHVYLHGAKVLRPDLVK 120	CAC97270
C. tetani	IKYVYLGKAKKLRDDLIE 117	AAO35880

Figure 4 Sequence alignment of aroH

Sequences were aligned using Clustal W [39]. Invariant residues corresponding to the Ala^{112} in aroA^{Marburg} are shaded in black. The sequences are followed by their NCBI accession numbers.

Llewellyn et al.'s purification [12], the CM activity in the extract from the parent Marburg strain is much higher than that from the derivative strain 168. This phenomenon partly supports the proposed explanation and prompted us to investigate the nature of the CM enzyme(s) in both the parent and the derivative strains.

In prokaryotes, both aroQ and aroH have been found to exhibit CM activity although they share no sequence similarity with each other [25]. The aroQ protein, an all-helix bundle protein structure [26], is widely distributed and exists either as a monofunctional enzyme or as a 'CM domain' in a bifunctional fusion enzyme. The aroH protein, a trimeric pseudo-α/β-barrel structure [8], is limitedly distributed and represents a monofunctional CM. The *B. subtilis* 168 genome contains both the *aroQ* and *aroH* sequences. The *aroQ* is fused with the *aroA* gene, while the *aroH* gene is located near a number of genes that encode other enzymes in the Shikimate pathway. In the present study, the amino acid sequence of the aroH^{Marburg} was found to differ by only one amino acid from aroH^{168} (Ala^{112} in aroH^{Marburg} and Val^{112} in aroH^{168}). The CM activity of aroH^{168} is only 0.5 % of that of aroH^{Marburg}. Sequence alignment showed that Ala^{112} (numbering in aroH^{Marburg}) is highly conserved among the aroH family (Figure 4). Crystallography and mutagenesis studies have suggested that Ala^{112} plays a potential role in the binding of the substrate and stabilization of the transition state [27].

Based on the facts and analyses presented above, it is intriguing to propose that during purification [12], the 'dead' aroH^{168} in the initial extract did not contribute to the activity ratio (DAHPS versus CM) while the highly active form of aroH^{Marburg} caused a higher ratio of enzyme activities. When the aroH^{Marburg} was separated from DAHPS, a large activity ratio change was observed, which no doubt led to the confusion concerning the mono/bifunctional properties of the two *Bacillus* DAHPSs. Therefore, during the X-ray mutation, there were no amino acid changes in aroA^{Marburg} (purported to be monofunctional) that led to an aroA(Q)^{168} which was assumed to be a bifunctional enzyme. Rather, in the aroH^{Marburg}, one amino acid was mutated, resulting in a relatively inactive mutant aroH^{168} as compared with the highly active parent aroH^{Marburg}. Mutations in other enzymes could also have occurred.

The second question on CM-DAHPS^{Bacillus} (the abbreviation CM-DAHPS^{Bacillus} will now be used for both aroA^{Marburg} and aroA(Q)^{168} since they have identical sequences) regarding the metal requirement has also been answered in our present study. CM-DAHPS^{Bacillus}, as are all other DAHPSs studied to date, is unequivocally a metallo enzyme based on the following: (i) the purified enzyme as isolated contains bound metal and is inactivated by metal chelating agent, and (ii) the inactive apoenzyme can be reactivated by bivalent metal ions.

The recombinant CM-DAHPS^{Bacillus} as isolated contains both zinc and iron ions. Similar metal contents have been observed with the *E. coli* Trp- and Tyr-sensitive DAHPS [19] as well as the *Aquifex aeolicus* 3-deoxy-D-manno-octulosonate 8-phosphate synthase [28] which is evolutionally and mechanistically related to DAHPS [14,21,29–31]. When the isolated aroA^{Marburg} was assayed after the addition of various concentrations of zinc or iron to the assay mixture, no stimulation of the enzymatic activity was observed (results not shown). This suggests that the enzyme as isolated was incapable of binding additional metals (iron or zinc) in an enzymatically productive manner. These results are similar to the results reported for DAHPS from *T. maritima* [20].

Unlike previously studied DAHPSs, EDTA could not remove the tightly bound metal in the CM-DAHPS^{Bacillus} (Figure 2); however, DPA, a stronger metal chelator [32], was able to inactivate the CM-DAHPS^{Bacillus} by removing the enzyme-bound metal (Table 2). Thus the inability of EDTA to affect the DAHPS activity by removing the metal led to the decades-old misconception that the DAHPS from *B. subtilis* does not need a metal for catalytic activity. Since all DAHPSs, putative or fully characterized, contain the same four absolutely conserved metal binding residues, identified by X-ray crystal studies, it has been proposed that all the DAHPSs are metallo enzymes [20]. The confirmation of CM-DAHPS^{Bacillus} as a metallo enzyme further supports this hypothesis.

In addition to its unusually high metal binding affinity, the recombinant CM-DAHPS^{Bacillus} demonstrates significantly higher K_m for PEP and especially high K_m for E4P as compared with those reported from other bacterial DAHPSs [20,33–35]. While other reported bacterial DAHPSs (both class I and II) are feedback-inhibited by the end-products (Phe, Tyr or Trp [20,36,37]) in the Shikimate pathway, CM-DAHPS^{Bacillus} is feedback inhibited by the branch point intermediates prephenate and chorismate, with prephenate being a much better inhibitor than chorismate (Table 2). The kinetic properties and feedback inhibition pattern exhibited in recombinant CM-DAHPS^{Bacillus} are consistent with previous reports on CM-DAHPS directly isolated from *B. subtilis* 168 [18,38]. In addition, recombinant CM-DAHPS^{Bacillus} exhibits tetrameric solution structure and strict substrate specificity, which are in agreement with the results reported for another class II DAHPS from *T. maritima* [20].

In summary, two of the long-standing questions surrounding the DAHPSs from *B. subtilis* Marburg, aroA^{Marburg} and aroA(Q)^{168}, have been solved. The DAHPSs from *B. subtilis* parent Marburg strain and its derivative strain 168 are identical in sequence, are metallo enzymes as are all the other DAHPSs studied to date, and both are bifunctional enzymes consisting of a CM activity and a DAHPS activity.

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