**Thermotoga maritima** 3-Deoxy-d-arabino-heptulosonate 7-Phosphate (DAHP) Synthase

THE ANCESTRAL EUBACTERIAL DAHP SYNTHASE?

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The gene encoding the 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) synthase from the thermophilic microorganism *Thermotoga maritima* was cloned, and the enzyme was overexpressed in *Escherichia coli*. The purified DAHP synthase displays a homotetrameric structure and exhibits maximal activity at 90 °C. The enzyme is extremely thermostable, with 50% of its initial activity retained after incubation for ~5 h at 80 °C, 21 h at 70 °C, and 86 h at 60 °C. The enzyme appears to follow Michaelis-Menten kinetics with *Km* for phosphoenolpyruvate = 9.3–13 μM, *Km* for d-erythrose 4-phosphate = 57.3–350.1 μM, and *kat* = 2.3–7.6 s⁻¹ between 50 °C and 70 °C. Metal analysis indicates that DAHP synthase as isolated contains Zn²⁺, and the enzyme is inactivated by treatment with EDTA. The apo-enzyme is partially reactivated by a variety of divalent metals including Zn²⁺, Cd²⁺, Mn²⁺, Cu²⁺, Co²⁺, and Ni²⁺.

These observations suggest that *T. maritima* DAHP synthase is a metalloenzyme. The activity of *T. maritima* DAHP synthase is inhibited by two of the three aromatic amino acids (L-Phe and L-Tyr) formed in the Shikimate pathway. This report is the first description of a thermostable eubacterial DAHP synthase.

The enzyme 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP)³ synthase (EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form DAHP and inorganic phosphate. The formation of DAHP is the first committed step in the Shikimate pathway. This pathway is responsible for the biosynthesis of the intermediate compounds, chorismate and prephenate, which are precursors to the aromatic amino acids (Phe, Tyr, and Trp), catechols, and p-aminobenzoic acid (folic acid biosynthesis) as well as a number of other highly important microbial compounds (1).

DAHP synthases exist in most microorganisms and plants. Based on phylogenetic analysis, this protein family has been separated into two classes, Class I and Class II, by Birck and Woodard (2). Alternatively, Jensen and co-workers (3, 4) classified DAHP synthases into two distinct homology families (aroA and araoA). The AroA family was defined as “plant-like” DAHP synthases that included the higher plant proteins and a cluster of microbial proteins (5). The AroA family was further divided into subfamilies AroA and AroA₂, which correspond to Class II and Class I, respectively (4). *Escherichia coli* expresses three DAHP synthase isoenzymes that are representative of Class I or the AroA₂ family, and require a divalent metal for activity (6). Each of the isoenzymes is specifically feedback-inhibited by only one of the three aromatic amino acids, Phe, Tyr, or Trp (7). The *Bacillus subtilis* DAHP synthase, which is representative of Class I or the AroA₂ family, is inhibited by the intermediates prephenate and chorismate in the Shikimate pathway and has been reported by Jensen and Nester (8, 9) to be insensitive to EDTA treatment and was thus proposed as a non-metalloenzyme (2).

Based on the total lack of any information of a DAHP synthase from a thermophile, an investigation of a DAHP synthase from a thermophilic eubacterium was initiated in order to provide further insight into the biochemical reason for the bifurcation in the DAHP synthase phylogenetic tree. The extreme thermophile *Thermotoga maritima* DAHP synthase, which belongs to Class I (or the AroA₂ subfamily), was chosen for this study. Evolutionary studies have placed this bacterium in one of the deepest and most slowly evolving branches of the domain *Bacteria* (10, 11). Thus, studies on the DAHP synthase from this bacterium should provide a better understanding of the divergence of the two classes of DAHP synthase and the evolution of the aldol-like condensation catalyzed by this enzyme family.

Herein the cloning, overexpression, purification, and biochemical characterization of the *T. maritima* DAHP synthase are reported. The metal requirements and feedback inhibition profile of the enzyme are also reported. This is the first description of a thermostable eubacterial DAHP synthase.

**EXPERIMENTAL PROCEDURES**

Materials and General Methods—Genomic *T. maritima* DNA was purchased from ATCC (ATCC 43589D). The Promega Wizard DNA purification kit was utilized for plasmid isolation and purification. The *E. coli* cells, 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. Thermal cycling was performed using an MJR Research Thermal Cycler. DNA sequencing and primer syntheses were performed by the University of Michigan Biomedical Resources Core Facility. The PEP monocyclohexylammonium) salt, E4P sodium salt, d-arabinose 5-phosphate disodium salt, 2-deoxyribose 5-phosphate sodium salt, d-ribose 5-phosphate disodium salt, reagent grade chorismate, shikimate, and prephenate were obtained from Sigma. Puratronic grade NiCl₂, MgCl₂, CoCl₂, MnCl₂, CdCl₂, FeSO₄, CuSO₄, ZnSO₄, and HCl (99.999%, metal basis) were purchased from Alfa Aesar. The 1,3-bis(tris(hydroxymethyl)methylamino)propylamine (BTP) was purchased from Research Organics. The EDTA disodium salt was obtained from Mallinckrodt. High grade Spectra/Por7 dialysis tubing (M, 10,000 cutoff and metal-free) was ob-

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tained from VWR Scientific. The Mono Q (HR10/10), Phenyl Superose (HR10/10), Superose 12 (HR10/50), and FAST Desalting (HR10/10) chromatography columns were from Amersham Biosciences.

Protein concentrations were determined using the Bio-Rad Protein Assay Reagent, with bovine serum albumin (Sigma) serving as the standard. Optical spectroscopy was performed using a HP 8453 UV-visible spectrophotometer. Unless otherwise stated, the pH of all buffers was measured at 25 °C, and all purification steps were performed at 25 °C.

Construction of Plasmids—Standard polymerase chain reaction methodologies were used to amplify the araG-like gene (gi:7448834) from T. maritima genomic DNA. The forward primer was GATTCT-GAACATGATATATGGCGAAAGC, and the reverse primer was GTTGAATTCGGATCCTTGAG.

The amplification product was isolated, restricted with NdeI and BamHI (underlined), and ligated into the similarly restricted expression vector, pT7-T. The ligation mixture was used to transform E. 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-araG, was used to transform chemically competent E. coli BL21(DE3) cells.

Overexpression and Purification of DAHP Synthase—The E. coli BL21(DE3) cells harboring the pT7-araG plasmid were grown in 2×YT medium containing ampicillin (100 μg/ml) at 37 °C with shaking (220 rpm). Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.4 mM when the culture reached an absorbance of 1.5. The cells were harvested 4 h after induction by centrifugation (29,000 × g, 20 min, 4 °C), the pellet was suspended in 20 mM Tris-HCl (pH 7.5), and the suspension was 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, 30 min, 4 °C).

Solid sodium chloride was added to the supernatant (typically, a solution of 40 ml containing 400 mg of total protein is obtained from a 1.0-liter culture) to a final concentration of 0.1 M, and the solution was heated at 80 °C for 1.5 min and then heated at 60 °C for 10 min with gentle continuous hand swirling. The suspension was allowed to cool to 25 °C, and ice for 15 min, the precipitated protein was removed by centrifugation (29,000 × g, 20 min, 4 °C). The supernatant (32 ml, 64 mg of total protein) was dialyzed against 1 liter of buffer A (20 mM Tris-HCl, pH 7.5) overnight at 4 °C and then applied to a Mono Q column (HR 10/10) pre-equilibrated with buffer A. The column was developed at a flow rate of 1.0 ml/min using a linear gradient from 0 to 0.5 M potassium chloride in the same buffer over 50 min. The fractions containing DAHP synthase activity as determined by the discontinuous assay at 50 °C, 60 °C, and 70 °C in 100 mM Tris acetate (pH 7.5) and 50 mM MnCl₂. The concentration of one substrate was held constant (10 × K_m), whereas the other was varied over the range of 0.1–10 × K_m.

Feedback Inhibition—Feedback inhibition of DAHP synthase was determined by incubating 0.5 μM enzyme and 3 mM PEP with a fixed concentration of possible inhibitor (1 mM) in 100 mM Tris acetate (pH 7.5) containing DAHP synthase to a final concentration of 0.5 mM. The kinetic activity was measured by incubating the purified enzyme (5.3 μM) in 100 mM Tris acetate (pH 7.5) at 60 °C, 70 °C, or 80 °C. At various times, aliquots of enzymes were removed, centrifuged, and assayed for residual activity at the respective incubation temperatures by the discontinuous colorimetric assay.

Kinetic Studies—Reactions were carried out separately by continuous assay at 50 °C, 60 °C, and 70 °C in 100 mM Tris acetate (pH 7.5) and 50 mM MnCl₂. The concentration of one substrate was held constant (10 × K_m), whereas the other was varied over the range of 0.1–10 × K_m.

Preparation of Apo-DAHP Synthase—The DAHP synthase used for metal studies was purified by a modification of the method described above. The cells were suspended and sonicated in 10 mM BTP buffer (pH 7.0) instead of Tris-HCl buffer. The heat-treated supernatant containing the enzyme was diluted 2-fold with buffer B (10 mM BTP buffer, pH 7.0) containing 1 mM PEP and applied to a Mono Q column (HR 10/10) equilibrated with buffer B. The column was developed at a flow rate of 1 ml/min using a linear gradient from 0 to 0.5 M potassium chloride in buffer B over 80 min. PEP was added immediately to the fractions containing DAHP synthase to a final concentration of 0.5 mM. The pooled fractions were concentrated by ultrafiltration (Centriprep YM-10 concentrator) to 8 mg/ml and used immediately in the following studies.

The freshly purified DAHP synthase was treated with 10 mM EDTA in buffer B containing 0.5 mM PEP for 2 h at 25 °C and then dialyzed against 500 ml of buffer C (metal-free 10 mM BTP, pH 7.0, 0.5 mM PEP) for 24 h at 25 °C with two buffer changes. The metal-free BTP buffer was prepared from Chelex 100 resin-treated 1 M BTP, metal-free HCl, and distilled deionized water (PURELAB Plus System).
was collected. The protein concentration and enzymatic activity of each protein-metal complex were determined immediately. The identity and quantity of metals in the enzyme preparations and buffer solutions were determined by high-resolution inductively coupled plasma mass spectrometry on a Finnigan MAT ELEMENT instrument at the W. M. Keck Elemental Geochemistry Laboratory (Department of Geology, University of Michigan) by Dr. Ted Huston. In a separate experiment, the apo-enzyme was incubated with a mixture of the metal salts containing 1 molar equivalent of each metal per enzyme subunit (see Table IV), desalted, and analyzed as described above.

To determine the metal content of the enzyme directly after purification, the enzyme as isolated was dialyzed against 1 liter of buffer B overnight at 25 °C. In another experiment, the enzyme as isolated was treated with a 4-fold molar excess of Zn\(^{2+}\) per subunit for 2 h at 25 °C and then dialyzed against 2 liters of buffer B overnight. The dialyzed enzyme samples were assayed for protein concentration and enzymatic activity and subjected to metal analysis as described above. As a control, the metal content of the dialysis buffer after dialysis was analyzed.

The time dependence of Cu\(^{2+}\) binding to T. maritima apo-DAHP synthase was determined by adding CuSO\(_4\) (400 µM, final concentration) to 100 µM apo-enzyme in buffer C that had been preincubated for 5 min at 37 °C. Spectra were taken 5, 10, and 30 min after addition of Cu\(^{2+}\) at 37 °C.

Sequence Alignments—Sequences were aligned using Clustal W (13).

RESULTS

Overexpression and Purification of the Enzyme—The T. maritima DAHP synthase was highly overexpressed in E. coli BL21(DE3) cells harboring the pT7-aroG plasmid. The purification procedure was developed by taking advantage of the expected thermal stability of the protein. Heat treatment of the cell extract resulted in substantial precipitation of the host cell proteins, whereas negligible loss of total enzymatic activity was observed. After two chromatographic steps, the recombinant protein was determined to be homogeneous by SDS-PAGE. The typical yield of purified protein was 30–40 mg/liter cell culture.

Physical Properties—The molecular weight of the purified enzyme as determined by SDS-PAGE was 38,000. The molecular weight of the native enzyme determined by analytical gel filtration chromatography was 134,000, 3.5 times the molecular weight determined by SDS-PAGE. For comparison, the Phe-sensitive E. coli DAHP synthase (David L. Howe, this laboratory) was subjected to similar analysis. The molecular weight of the native E. coli enzyme is 3.4 times that of its monomer. Based on its crystal structure, E. coli DAHP synthase (Phe-sensitive) has been assumed to be that of a tetramer (14). Therefore, T. maritima enzyme is likely to adopt a tetrameric structure.

pH Optimum—The purified enzyme exhibited the highest enzymatic activity (>90% of maximum) between pH 6.0 and 7.0, with an optimum of pH 6.3 at 60 °C (Fig. 1).

Temperature Optimum and Thermostability—During preliminary experiments, it was determined that the substrate PEP is about 10 times more stable at higher temperatures than the other substrate, E4P (data not shown). Based on these findings, the enzyme assays were carried out by preincubation of PEP and enzyme at a desired temperature. The reaction was then initiated by the addition of E4P, and the substrates were allowed to react for 30 s after the addition of E4P. The reaction rates were measured under initial rate conditions between 30 °C and 80 °C (data not shown). Enzyme activities at higher temperatures could not be measured accurately due to the thermal instability of E4P. Under these restricting reaction conditions, the temperature optimum was found to be 90 °C (Fig. 2). An Arrhenius plot of the data (Fig. 2, inset) showed a transition point at 60 °C, resulting in activation energy values.
of 62 kJ/mol between 30 °C and 60 °C and 51 kJ/mol between 60 °C and 80 °C.

The thermostability of the purified DAHP synthase was determined at 60 °C, 70 °C, and 80 °C (Fig. 3). After 9 h of incubation at 60 °C, maximum DAHP synthase activity was reached as compared with that of the enzyme as isolated when assayed at 60 °C. Further incubation at 60 °C resulted in diminished activity. No significant gain or loss of enzyme activity was observed for the first 5 h at 70 °C; however, the enzyme activity appeared to decrease exponentially after 5 h. When incubated at 80 °C, a simple exponential decay was observed. As shown in Fig. 3, 50% of the enzyme activity was retained after −5 h (80 °C), 21 h (70 °C), and 86 h (60 °C) of incubation.

Feedback Inhibition—The data in Table II illustrate the effects of possible feedback inhibitors on DAHP synthase activity. The results showed that L-Phe and L-Tyr significantly inhibited T. maritima DAHP synthase with only 32% and 23% of activity remaining, respectively. The compounds L-Trp, chorismate, shikimate, prephenate, D-Phe, and L-His had no effect on enzymatic activity under the experimental conditions.

Metal Requirement—The results from the metal analysis studies demonstrated that the enzyme as isolated contains 0.20 mol equivalent of Zn^{2+} per subunit followed by the removal of free metal by gel filtration. The protein-metal fraction was analyzed by gel filtration. The protein-metal fraction was analyzed by adding a divalent metal ion directly to the assay mixture (Table III). The results from these experiments demonstrated that Mn^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, and Cu^{2+} restored the enzymatic activity to 3–5 units/mg, whereas Fe^{2+} and Mg^{2+} had little effect on enzyme activity.

In order to determine the stoichiometry of the enzyme-metal complex, the apo-enzyme was incubated with a 4-fold molar excess of metal salt per subunit followed by the removal of free metal by gel filtration. The protein-metal fraction was analyzed for specific activity and metal content. As can be seen from Table IV, the DAHP synthase binds twice as much Cd^{2+} and Zn^{2+} as Mn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}. When apo-enzyme was incubated with a mixture of metals (1 mol equivalent of each of the above metals per subunit), the DAHP synthase bound mainly Zn^{2+} and Cd^{2+} as well as a trace amount of the other metals. As can also be seen in Table IV, DAHP synthase activity was only partially restored. Furthermore, the Zn^{2+} reconstituted apo-enzyme could bind twice as much Zn^{2+} but only regained 72% of the specific activity as compared with the original enzyme.

Because Cu^{2+} has been shown to bind to a number of DAHP synthases, leading to a spectral signature that provided insight into the nature/identity of the enzyme’s metal ligands (6, 15, 16), the effect of Cu^{2+} on the UV-visible spectra of T. maritima apo-DAHP synthase was examined. The addition of Cu^{2+} to apo-enzyme resulted in the time-dependent appearance of a new peak at ~365 nm (Fig. 4), consistent with previous reports that assigned this peak to be a ligand-to-metal charge transfer interaction with an active site thiolate and/or imidazole ion(s) of the enzyme (6).
DAHP Synthase from T. maritima

TABLE IV
Metal analysis of DAHP synthase

| Incubation metal salt | Zinc | Cadmium | Manganese | Copper | Cobalt | Nickel | Magnesium | Iron | Specific activity units/mg |
|-----------------------|------|---------|-----------|--------|--------|--------|------------|------|---------------------------|
| As isolated           | 0.20 | —       | —         | —      | —      | —      | —          | —    | —                         |
| Zn$^{2+}$ as isolated | 0.34 | —       | —         | —      | —      | —      | —          | —    | —                         |
| Apo-enzyme            | 0.02 | —       | —         | —      | —      | —      | —          | —    | —                         |
| ZnSO$_4$              | 0.40 | —       | —         | —      | —      | —      | —          | —    | —                         |
| CdCl$_2$              | 0.03 | 0.43    | —         | —      | —      | —      | —          | —    | —                         |
| MnCl$_2$              | 0.06 | —       | 0.21      | —      | —      | —      | —          | —    | —                         |
| CuSO$_4$              | 0.02 | —       | 0.23      | —      | —      | —      | —          | —    | —                         |
| CoCl$_2$              | 0.06 | —       | —         | —      | 0.25   | 0.04   | 0.02       | 0.02 | 2.2                       |
| NiCl$_2$              | 0.03 | —       | —         | —      | —      | 0.16   | 0.01       | 0.02 | 1.5                       |
| MgCl$_2$              | 0.02 | —       | —         | —      | —      | —      | 0.03       | 0.02 | 0.5                       |
| All$^a$               | 0.14 | 0.21    | 0.02      | 0.04   | 0.06   | 0.01   | 0.02       | 0.02 | 5.0                       |
| Blank$^b$             | 0.01 | —       | —         | —      | —      | —      | 0.01       | 0.01 | —                         |

$^a$ Value < 0.01.
$^b$ Enzyme as isolated was incubated with 4-fold molar excess of Zn$^{2+}$ and dialyzed.
$^c$ Apo-enzyme was incubated with 1 mol equivalent each of a mixture of metal salts and desalted as described above.
$^d$ A solution, without enzyme, comprising all of the metal salts (400 μM each), was subjected to gel filtration under conditions identical to those used for the enzyme-containing samples. The fraction corresponding to the elution volume of protein samples was collected for analysis. Values represent metal concentration divided by the average protein concentration determined for all other samples.

$^a$ Molar equivalent metal/enzyme subunit

$^b$ M$^b$ M

$^c$ H$^c$ H

$^d$ H$^d$ H

**DISCUSSION**

These results indicate that *T. maritima* DAHP synthase has a native molecular weight of ~134,000 and appears to consist of four identical subunits of $M^b$ 38,600. As expected for a thermophilic enzyme, *T. maritima* DAHP synthase reached maximum activity at 90 °C, was virtually inactive at 37 °C, and showed a high thermostability (19). DAHP synthase from *T. maritima* appears to follow Michaelis-Menten kinetics. The $K_m$ value for E4P increases with increasing temperature, whereas the $K_m$ for PEP is not greatly affected by temperature (Table I). These $K_m$ values compare favorably with those reported from other microorganisms (20–23). Unlike the three isoforms of DAHP synthases from *E. coli* (aroG, aroF, and aroH, each of which is specifically feedback-inhibited by only one of the three aromatic amino acids, Phe, Tyr, and Trp, respectively) (7), *T. maritima* DAHP synthase is inhibited by both Phe and Tyr, but not by Trp (Table II).

The recombinant enzyme as isolated from *E. coli* contained only 0.20 Zn$^{2+}$ ion/enzyme subunit (Table IV). Additional Zn$^{2+}$ (0.14 Zn$^{2+}$/subunit; total, 0.34 Zn$^{2+}$/subunit) bound to the enzyme as isolated without further increase in specific activity. Zn$^{2+}$ (0.28 and 0.29 Zn$^{2+}$/subunit, respectively) has also been found to bind to the Trp- and Tyr-sensitive DAHP synthases from *E. coli* (6) in combination with 0.26 and 0.19 Fe$^{2+}$/subunit, respectively. Only trace quantities of Zn$^{2+}$ were found in the *E. coli* Phe-sensitive form (6). The recombinant 3-deoxy-o-manno-octulosonate 8-phosphate (KDO8P) synthase from *Aquifex aeolicus* (24) also contains both Zn$^{2+}$ and Fe$^{2+}$ (0.42 Zn$^{2+}$/subunit and 0.31 Fe$^{2+}$/subunit). In addition, the recombinant KDO8P synthase from *Helio bacter pylori* (25) was reported to contain 1 Zn$^{2+}$/subunit. KDO8P synthase and DAHP synthase are structurally and mechanistically related and probably originated from a common ancestor (2, 14, 26–28). It is possible that any Fe$^{2+}$ present in the original *T. maritima* DAHP synthase was oxidized to Fe$^{3+}$ that can bind in the Fe$^{2+}$ site, thus allowing more Zn$^{2+}$ to bind. Crystal structures suggest that the active site metal binds to the four same residues in *A. aeolicus* KDO8P synthase (Cys$^{11}$, His$^{185}$, Glu$^{222}$, and Asp$^{235}$ ligands to Cd$^{2+}$) and *E. coli* Phe-sensitive DAHP synthase (Cys$^{81}$, His$^{268}$, Glu$^{302}$, and Asp$^{306}$ ligands to Pb$^{2+}$ or Mn$^{2+}$) (14, 28, 29) in an octahedral geometry. These same active site residues are absolutely conserved in *T. maritima* and *E. coli* DAHP synthases as well as *H. pylori* KDO8P synthase. At this time, however, there is no direct evidence that the Zn$^{2+}$ is bound to the same conserved active site amino acid residues. Whereas Zn$^{2+}$ normally occupies the center of a tet-

**FIG. 4. Absorption spectra of *T. maritima* DAHP synthase.** A solution of CuSO$_4$ (400 μM, final concentration) was added to 100 μM apo-enzyme in 10 mM BTP (pH 7.0) containing 0.5 mM PEP that had been preincubated for 5 min at 37 °C. Spectra were taken 5, 10, and 30 min after addition of Cu$^{2+}$. The dashed curve is that of apo-DAHP synthase.
DAHP Synthase from T. maritima

| Organism      | Partial sequence | Accession No. |
|---------------|------------------|---------------|
| T. maritima   | IAGFQEVU   108  | 266 LLVDFELR  | E27238 |
| C. pneumonae  | 43 IAGFYLET  59  | 203 VIYDFQRA  | H11001 |
| C. thermotolerans | 34 IAGFYLET  46  | 205 VIYDFQRA  | H11001 |
| P. abyssii    | 30 MAGFSEQ   40  | 198 IVYDFQRA  | H11001 |
| P. furiosus   | 27 IAGFYLET  37  | 195 IVYDFQRA  | H11001 |
| E. coli       | 96 MAGFSEQ   108 | 204 IVYDFQRA  | H11001 |
| B. subtilis   | 122 MAGFSEQ   150 | 195 IVYDFQRA  | H11001 |
| S. pyogenes   | 19 IAGFYLET  29  | 187 IVYDFQRA  | H11001 |
| C. acetoxyrticus | 96 IAGFYLET  108 | 204 IVYDFQRA  | H11001 |
| A. pernis     | 42 IAGFYLET  52  | 210 IVYDFQRA  | H11001 |

**Fig. 5. Sequence alignment of DAHP synthases.** Sequences were aligned using Clustal W (13). Invariant residues that are putative metal-binding sites are shaded (Cys$^{325}$, His$^{326}$, Glu$^{327}$, and Asp$^{328}$ based on amino acid sequence for E. coli Phe-sensitive DAHP synthase) (14, 27). The sequences are followed by their NCBI accession numbers.

rahedral or trigonal bipyramidal arrangement (30), it is possible that it may bind to the conserved amino acid residues in a non-octahedral arrangement.

Treatment of the enzyme as isolated with EDTA resulted in inactive enzyme that did not contain any metal ions (Table IV), thus the enzyme requires a metal ion for activity. The reconstituted apo-DAHP synthase bound twice as much Zn$^{2+}$ as isolated, yet the reconstituted enzyme exhibited only 72% of the specific activity of the enzyme as isolated (Table IV). No other metal ion or combination of metal ions, at any concentration tested, could restore the activity of the apo-enzyme to that of the enzyme as isolated. This could be due to the instability of the apo-enzyme. Park and Bauerle (31) reported that E. coli apo-DAHP synthase (Phe-sensitive) is unstable due to an oxidation process; however, inclusion of PEP in the presence of BTP buffer was found to stabilize the apo-DAHP synthase against this inactivation. The authors proposed that this stabilization resulted from the ability of PEP to protect the active site residue, Cys$^{328}$, from oxidation (31). The activity of the apo-DAHP synthase from T. maritima, in the present study, could not be restored to that of the enzyme as isolated with the addition of PEP in BTP buffer during purification and/or manipulation. It should be noted that Cys$^{328}$ of the Phe-sensitive E. coli DAHP synthase is not conserved in the T. maritima enzyme, thus the lack of stabilization by PEP was not unexpected. Similarly, Baasov and Knowles (15) observed that E. coli DAHP synthase (Tyr-sensitive) as isolated contained 0.5 Cu$^{2+}$/enzyme subunit; however, copper-reconstituted apo-enzyme, which contained 1.0 Cu$^{2+}$/enzyme subunit, exhibited only 70% of the specific activity of enzyme as isolated, even in the presence of PEP. As seen with E. coli DAHP synthase (Tyr-sensitive), the apo-DAHP synthase from T. maritima could be reconstituted by Cu$^{2+}$ and showed the characteristic spectrum for copper binding (Fig. 4), and the activity with Cu$^{2+}$ was less than that of the enzyme as isolated. Unlike the E. coli DAHP synthase (Tyr-sensitive), the reconstituted DAHP synthase from T. maritima contained only 0.23 Cu$^{2+}$/subunit (Table IV). Thus, overall, the metal requirements of the T. maritima DAHP synthase are quite similar to those reported for DAHP synthase from other microbial sources, except it contains less metal, and the apo-enzyme seems more unstable and thus less able to rebind metals in a catalytically competent manner.

In summary, DAHP synthase from the hyperthermophile T. maritima is (i) a thermostable enzyme, (ii) inhibited by both Phe and Tyr, and (iii) a metalloenzyme, although the endogeneous metal cofactor remains to be defined.

Birck and Woodard (2) separated DAHP and KDO8P synthases individually into two classes: Class I and Class II. The difference between Class I and Class II KDO8P synthases has been shown to be their metal requirements. The difference between Class I and Class II DAHP synthases was proposed by Birck and Woodard (2) to be their metal requirements. This hypothesis was based on the facts that the DAHP synthase from B. subtilis Marburg 168, which is ascribed to Class I, was insensitive to EDTA treatment according to Jensen and Nester (8) and three of the E. coli isoenzymes, which are ascribed to Class II, were known to require divalent metal ions for activity according to Stephens and Bauerle (6).

An alternative phylogenetic analysis suggested by Jensen and co-workers (3, 4) divided DAHP synthases into two families (AroA$_{1}$ and AroA$_{2}$). The AroA$_{1}$ family was further divided into two subfamilies, AroA$_{1a}$ (exemplified by the E. coli DAHP synthases) corresponding to Class I and AroA$_{2a}$ (exemplified by the B. subtilis DAHP synthase) corresponding to Class II. KDO8P synthases were assigned as a single second group in the I$_{p}$ clade portion of AroA$_{1a}$ subgroup. It was suggested that the division between the two subfamilies could possibly be due to substrate specificity (4).

Phylogenetic analyses have placed the two hyperthermophiles, A. aeolicus and T. maritima, basal to all other bacteria (10, 11, 32). However, no DAHP synthase gene has been found in A. aeolicus based on annotated genome and homology searches. Therefore, we believed that the information obtained from the investigation of the biochemical properties of the DAHP synthase from T. maritima, which represents the earliest known diverging bacterial DAHP synthase for which a sequence is known, would help clarify what biochemical properties account for the differences among the various subdivisions.

As a Class I DAHP synthase, the T. maritima enzyme would be predicted to be a non-metalloenzyme if metal requirements were indeed the property that distinguishes Class I and II DAHP synthases. However, in the present study, T. maritima DAHP synthase is identified as a metalloenzyme, which provides the first direct evidence for the existence of a metalloenzyme in Class I. This was not unexpected because sequence alignment of various DAHP synthases demonstrates that the four metal-chelating residues in E. coli DAHP synthase (Phe-
sensitive) are absolutely conserved in both Class I and Class II enzymes (Fig. 5). The insensitivity of *B. subtilis* DAHP synthase (Class I) to EDTA treatment to remove any bound metal, originally reported by Jensen and Nester (8), does not necessarily rule out a metal requirement for enzymatic activity. It has been reported for other metalloenzymes that EDTA is not effective at removing tightly bound metals (25). Studies on the metal requirements of other Class I DAHP synthases as well as a reinvestigation of the *B. subtilis* DAHP synthase metal requirements should provide a better understanding on this issue.

Other than the metal requirement, allosteric effects have been postulated as a possible difference between the two classes of DAHP synthases (2). A broad diversity of feedback inhibition patterns has been observed for this enzyme family. In Class I, the *B. subtilis* enzyme is sensitive to the downstream intermediates in the Shikimate pathway (8, 9), chorismate and prephenate. *T. maritima* DAHP synthase (Class I), as discussed above, is feedback-inhibited by two of the three aromatic amino acids, Phe and Tyr. In Class II, each of the three *E. coli* isoenzymes is feedback-regulated by only one of the aromatic amino acids (7), whereas the DAHP synthase from *Corynebacterium glutamicum* (Class II) has been shown to be feedback-inhibited by both Phe and Tyr (33). The difference between Class I and Class II DAHP synthases, therefore, does not appear to be due to differences in allosteric effectors.

Finally, Jensen *et al.* (4) suggested that the division between the two subfamilies could be due to substrate specificity. According to this hypothesis, ancient DAHP synthase generally had broad substrate specificities and evolved to have differentially narrow substrate specificities. This implies that the more ancient AroA*II* subfamily would have broad substrate specificities, and the more recent AroA*III* subfamily would have narrow substrate specificities. However, it has been shown that the AroA*III* DAHP synthase (Phe-sensitive) from *E. coli* can utilize d-arabinose 5-phosphate, d-ribose 5-phosphate, and 2-deoxyribose 5-phosphate as alternate substrates (12) and that the AroA*III* DAHP synthase from *Neisseria gonorrhoeae* (a subclass of the AroA*III* class) can utilize d-arabinose 5-phosphate as an alternate substrate (34). On the other hand, neither d-arabinose 5-phosphate nor d-ribose 5-phosphate can be used as an alternate substrate for the AroA*II* DAHP synthase from *T. maritima* (data not shown) or *B. subtilis*. These experimental results do not support the hypothesis of Jensen *et al.* Therefore, it is likely that other biochemical properties must account for the phylogenetic distribution observed among DAHP synthases. Only after further experimentation with purified DAHP synthases from multiple sources from each subfamily will the true property(ies) that leads to the division be known.

In summary, *T. maritima* DAHP synthase is the first thermostable eubacterial DAHP synthase to be characterized. The enzyme exhibits maximum activity at 90 °C but displays functional characteristics similar to its mesophilic counterparts. This could be the earliest known diverging bacterial DAHP synthase and provides a model to study the structural/functional features and the evolution of the DAHP synthase family. Results obtained from this study have allowed us to test the various hypotheses on the division of this enzyme family. Additional studies will attempt to characterize the DAHP synthase from a member of the Archaea family, which will provide deeper insight into the evolution of this enzyme family. Efforts are currently under way to obtain an x-ray crystal structure and assign the metal binding ligands for the zinc ion.

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