L-Aspartate Semialdehyde and a 6-Deoxy-5-ketohexose 1-Phosphate Are the Precursors to the Aromatic Amino Acids in *Methanocaldococcus jannaschii*†

Robert H. White*

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308

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ABSTRACT: No orthologs are present in the genomes of the archaea encoding genes for the first two steps in the biosynthesis of the aromatic amino acids leading to 3-dehydroquinate (DHQ). The absence of these genes prompted me to examine the nature of the reactions involved in the archaeal pathway leading to DHQ in *Methanocaldococcus jannaschii.* Here I report that 6-deoxy-5-ketofructose 1-phosphate and L-aspartate semialdehyde are precursors to DHQ. The sugar, which is derived from glucose 6-P, supplies a "hydroxyacetone" fragment, which, via a transaldolase reaction, undergoes an aldol condensation with the L-aspartate semialdehyde to form 2-amino-3,7-dideoxy-D-*threo*-hept-6-ulosonic acid. Despite the fact that both hydroxyacetone and hydroxyacetone-P were measured in the cell extracts and confirmed to arise from glucose 6-P, neither compound was found to serve as a precursor to DHQ. This amino sugar then undergoes a NAD dependent oxidative deamination to produce 3,7-dideoxy-D-*threo*-hept-2,6-diulosonic acid which cyclizes to 3-dehydroquinate. The protein product of the *M. jannaschii* MJ0400 gene catalyzes the transaldolase reaction and the protein product of the MJ1249 gene catalyzes the oxidative deamination and the cyclization reactions. The DHQ is readily converted into dehydroshikimate and shikimate in *M. jannaschii* cell extracts, consistent with the remaining steps and genes in the pathway being the same as in the established shikimate pathway.

The pathway leading to the biosynthesis of the aromatic amino acids is well established in bacteria and eukarya where the genes/enzymes/reactions for each of the steps have been well studied (*1*). Analysis of archaeal genomes has revealed several examples where essential enzymes in biosynthetic pathways appear "invisible" based on sequence searches (*2*). One pathway where this has been previously noted is the shikimic acid pathway leading to the aromatic amino acids (*3*−*5*) where the genes for the first two steps are "invisible". The first step in the established shikimic acid pathway for the biosynthesis of aromatic amino acids involves an aldol-like condensation of erythrose 4-phosphate with phosphoenolpyruvate (PEP)[1] to form 3-deoxy-D-*arabino*-2-heptulosonate 7-phosphate (DAHP) (Figure 1). The reaction is catalyzed by 2-dehydro-3-deoxyphosphoheptonate aldolase (AroA, AroG, or AroH). The DAHP is then converted into 3-dehydroquinate (DHQ) by 3-dehydroquinate synthase (AroB). Genes for these two steps are either "invisible" as a result of being replaced by nonorthologous genes (*4*) or are not present in most archaeal genomes (*5*). If the latter is the case, the reactions must be different. Biochemical and labeling experiments (*6*−*8*) suggested that a noncanonical series of reactions is responsible for either the biosynthesis of 3-dehydroquinate or for the biosynthesis of the precursor molecule erythrose-4-phosphate. Here I show that the former is the case, with DHQ arising from L-aspartate semialdehyde and 6-deoxy-5-ketofructose 1-phosphate (DKFP).

MATERIALS AND METHODS

Materials. *O*-(4-Nitrobenzyl)hydroxylamine HCl, glucose 6-phosphate, *E. coli* alkaline phosphatase ammonium sulfate suspension 10 units/mg protein, glucokinase from *Bacillus stearothermophilus* (300 units/mg protein), hydroxyacetone, methyl glyoxal (40% solution), aldolase from rabbit muscle, chloroacetone, glycerol kinase from *Bacillus stearothermophilus*, fructose 1,6-bisP, L-allylglycine, [$^2H_5$]glycerol, [$^{13}C_3$]-glycerol, [1-^{2}H]glucose, [$^{13}C_6$]glucose, [6,6′-$^2H_2$]glucose, [1,2,3,4,5,6,6′-$^2H_7$]glucose, protocatechuic acid, phosphoenolpyruvate, sodium pyruvate, sodium oxalacetate, shikimic acid, quinic acid, L-homoserine, dihydroxyacetone, NAD, NADP, and dihydroxyacetone phosphate were obtained from Sigma/Aldrich. Sodium [$^{13}C_3$]pyruvate was obtained from Cambridge Isotopes.

3-Dehydroquinate, 3-dehydroshikimate, and 3-deoxy-D-*arabino*-2-heptulosonate-7-phosphate were a gift from John W. Frost, Michigan State University. 3-Deoxy-D-*ribo*-2-heptulosonate-7-phosphate (DRHP) was prepared by the condensation of pyruvate with erythrose 4-phosphate catalyzed by the MJ0400 derived aldolase (R. H. White, unpublished results). [2-^{2}H]Shikimic acid, [4-^{2}H]shikimic acid, and [6-^{2}H]shikimic acid were a gift from Heinz Floss,

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* Corresponding author. Mailing address: Department of Biochemistry (0308), Virginia Polytechnic Institute and State University, Blacksburg, VA 24061. Phone: (540) 231−6605. Fax: (540) 231−9070. E-mail: rhwhite@vt.edu

[1] Abbreviations: DHQ, 3-dehydroquinate; DHS, dehydroshikimate; DAHP, 3-deoxy-D-*arabino*-2-heptulosonate 7-phosphate; DRHP, 3-deoxy-D-*ribo*-2-heptulosonate-7-phosphate; DKFP, 6-deoxy-5-ketofructose 1-phosphate; PEP, phosphoenolpyruvate; TMS, trimethylsilyl; TFA, trifluoroacetyl; DHB, 3,4-dihydroxybenzoic acid; L-aspartate semialdehyde (ASA); GC-MS, gas chromatography−mass spectrometry; AATase, amino acid transferase.

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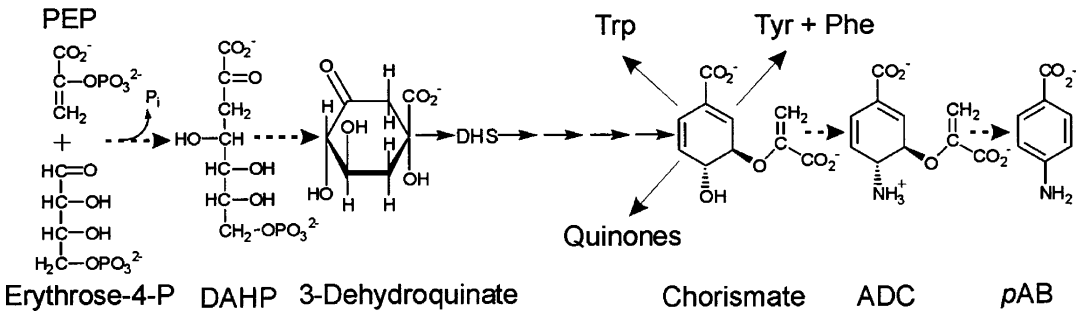

FIGURE 1: Established pathway for the biosynthesis of aromatic amino acids. Abbreviations: DAHP, 3-deoxy-D-*arabino*-2-heptulosonate 7-phosphate; ADC, 4-amino-4-deoxychorismate; *p*AB, 4-aminobenzoic acid.

University of Washington (*9*). [3-^{2}H]Shikimic acid was prepared by reduction of 3-dehydroshikimate with $NaBH_4$. [4,4′-$^2H_2$] L-Homoserine was prepared as previously described (*10*). Hydroxyacetone-P was prepared from chloroacetone as described by Pratt (*11*). The carbon bound hydrogens of the hydroxyacetone-P were exchanged-labeled by incubation of a 10 mM solution of hydroxyacetone-P in 0.2 M NaO^2H for 3 h at room temperature followed by neutralization with 1 M $^2H_3PO_4$ in ^2H_2O. A portion of the resulting [1,1′,3, 3′, 3″-$^2H_5$] hydroxyacetone-P was passed through a Dowex 50W-X8-pyridinium column (2- × 10-mm), and the resulting dried salt was converted into the TMS derivative and assayed by GC-MS to show a $M^+ - 15 =$ 303 *m*/*z* ion with 86% of the molecules containing five deuteriums. L-Aspartate semialdehyde was prepared by the ozonolysis of L-allylglycine in 1 M HCl (*12*). The resulting L-aspartate semialdehyde solution was diluted to 0.1 M in 20 mM anaerobic phosphate/K^+ buffer and adjusted to pH 7.0 with 1 M NaOH prior to use. [$^2H_5$]-α-L-Glycerol-P was prepared by phosphorylation of [$^2H_5$]glycerol with ATP catalyzed by the *B. stearothermophilus* glycerol kinase. The product was expected to have an enantiomeric purity of > 97% based on previous work (*13*). [$^2H_5$]-α-DL-Glycerol-P was prepared by heating a equal molar mixture of [$^2H_5$]-glycerol and 85% phosphoric acid for 4 h at 140 °C followed by TLC purification of the product. The glycerol-P samples were checked for chemical and isotopic purity by GC-MS analysis of their TMS derivatives ($M^+ - 15 = 435$ *m*/*z*). Precursors were placed in anaerobic TES buffer (50 mM TES/K^+ 10 mM $MgCl_2$ pH 7.5) at a concentration of 0.1 M and the resulting samples were added to the anaerobic incubation mixtures to give the indicated initial concentrations.

Formation of TMS Derivatives. Samples to be assayed as TMS derivatives were dried by evaporation with a stream of nitrogen gas while being held at 100 °C in a water bath and reacted with 20 μL of a mixture of pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1 v/v/v) for 2 min at 100 °C.

Preparation of Labeled Glucose 6-Phosphates. The labeled glucose 6-Ps were prepared by phosphorylation of glucose with ATP catalyzed by the *B. stearothermophilus* glucokinase. Thus, to a solution of the labeled glucose (10 μmol) in 0.5 mL of 10 mM tris/HCl pH 8.5 buffer, containing 10 mM $MgCl_2$, was added 5.5 mg of solid disodium ATP (10 μmol), and the pH of the solution was adjusted to 8.5 by the addition of 1 M NaOH solution. Glucokinase (20 units) was added, and the sample was incubated for 1 h at 37 °C. The pH was held at 8.5 by the addition of NaOH, as required. Nucleotides and free glucose were removed from the incubation mixtures by passing through a column (5 × 20 mm) consisting of a 1:1 mixture of norite and Celite. The samples were then concentrated and deoxygenated with a stream of argon to volume of 0.1 mL. TLC analysis showed the complete absence of glucose and nucleotides.

Preparation of 6-Deoxy-5-ketofructose 1-Phosphate. This sugar phosphate was prepared by the aldolase-catalyzed condensation of methyl glyoxal and dihydroxyacetone phosphate as previously described (*14*). The 6-deoxy-5-ketofructose 1-phosphate was purified by preparative TLC in solvent system 1 consisting of acetonitrile−water−formic acid (88%), 80:20:10 v/v/v, where it has an R_f of 0.29. ^{1}H NMR of the product was as previously reported (*14*). A 20-mM solution of the compound (20 μL) was heated at 70 °C for 15 min with an equal volume of 40 mM *O*-(4-nitrobenzyl)hydroxylamine to produce the dioxime derivative, which had an R_f of 0.19 in the TLC solvent acetonitrile−formic acid (88%), 95:5 v/v. Removal of the phosphate with alkaline phosphatase gave the dephosphorylated product that had an R_f 0.73 in acetonitrile-formic acid (88%), 95:5 v/v. The $(TMS)_3$ derivative of the dephosphorylated product showed a molecular ion at 678 *m*/*z* and an $M^+ - 15$ at 663 *m*/*z* confirming that both carbonyl groups were modified as the oxime with *O*-(4-nitrobenzyl)hydroxylamine. The mass spectra also contained intense fragments at *m*/*z* 295 and *m*/*z* 383, confirming the positions of the keto groups.

Preparation of M. jannaschii Cell Extracts. A cell extract of *M. jannaschii* was prepared by sonication of 4.67 g of frozen cells suspended in 10 mL of TES buffer (50 mM TES/K^+ 10 mM $MgCl_2$ pH 7.5) under Ar for 5 min at 3 °C. *M. jannaschii* cells were grown as previously described (*15*). The resulting mixture was centrifuged under Ar (27 000*g*, 10 min) and stored frozen at −20 °C until used. The protein concentration of the *M. jannaschii* extract was 38 mg/mL. Protein concentrations were measured using the BCA total protein assay (Pierce) with bovine serum albumin as a standard.

Incubation of M. jannaschii Cell Extracts with Precursors, Isolation, and Derivatization of Products. Two separate procedures were used for sample incubation and processing depending on whether the products of the incubations were to be assayed as the TMS or methyl TFA derivatives. In incubations where TMS derivatives were to be used, 5-μL amounts of 0.1 M solutions of the desired substrates or precursors were added to a 50-μL portion of the cell extract and the samples were incubated for 1 h at 70 °C under Ar

and the proteins precipitated by the addition of 80 μL of methanol. After centrifugation (14 000*g*, 10 min), the clear extract was separated and evaporated to dryness with a stream of nitrogen gas, dissolved in 100 μL of water, and passed through a Dowex 50W-X8-pyridinium column (2- × 10-mm). After evaporation to dryness the samples were converted into their TMS derivatives by reaction with 20 μL of TMS reagent at 100 °C for 2 min prior to GC-MS analysis.

For samples where a phosphorylated compound was the expected product, the phosphate was removed by treatment with alkaline phosphatase prior to the addition of methanol and formation of the TMS derivatives. This was accomplished by adding 50 μL of 0.1 M glycine buffer, pH 10.4, with 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ to the sample and 1 μL of the *E. coli* alkaline phosphatase suspension in ammonium sulfate and incubating for 1 h at 37 °C. The samples were then processed as described in the preceding paragraph.

For the analysis of 3-dehydroshikimate and shikimate as the methyl ester TFA derivative, *M. jannaschii* cell extracts (100 μL) were incubated for 30 min at 70 °C under argon with the indicated precursors. The samples were then cooled to room temperature and 10 μL of a 5.3 M solution of $NaBH_4$ in water was added. After incubation for 15 min at room temperature, 100 μL of 1 M HCl was slowly added and the precipitated proteins were removed by centrifugation (14 000*g*, 10 min). The resulting sample was evaporated to dryness with stream of nitrogen gas and repeatedly (3 times) evaporated from 0.5 mL of methanol to remove the borate. After the samples were dissolved in 200 μL of water and passed through a Dowex 50W-X8 H^+ column (2- × 5-mm) they were evaporated to dryness and treated for 12 h with 0.5 mL of 1 M HCl in methanol at room temperature. After evaporation of the methanol/HCl with a stream of nitrogen, the entire sample was purified by preparative TLC using a TLC solvent consisting of acetonitrile−water−formic acid (88%), 19:2:1 v/v/v. In this solvent system, the methyl ester of (3*R*, 4*S*, 5*R*) shikimate and its (3*S*, 4*S*, 5*R*) isomer (3-*epi*-shikimate), formed by reduction of the 3-dehydroshikimate, both had an R_f of 0.59. The area of the plate containing these compounds was removed and eluted with 70 μL of 70% methanol, which, after evaporation to dryness, was reacted with 100 μL of a 50% v/v solution of trifluoroacetic anhydride in methylene chloride for 2 h. After evaporation of the solvent and solution in 20 μL of methyl acetate, the samples were ready for GC-MS analysis.

Identification of Possible Genes Involved in Aromatic Amino Acid Biosynthesis. The identification of the *M. jannaschii* MJ0400 and MJ1249 genes as being involved in aromatic amino acid biosynthesis was based on their association with other aromatic amino acid biosynthetic genes in other archaeal genomes (*4*).

Cloning Expression and Purification of the MJ0400 and MJ1249 Proteins in E. coli. The *M. jannaschii* genes with Swiss-Prot accession number Q57843 for MJ0400 and Q58646 for MJ1249, were amplified by PCR from genomic DNA using oligonucleotide primers synthesized by Invitrogen: MJ0400-Fwd, (5′-GGTCATATGGAATTATTTAAAGAC-3′) and MJ0400-Rev, (5′-GATGGATCCTTATTTCTTCCTAATC-3′); MJ1249-Fwd, (5′-GGTCATATGAAATTTGGATG-3′) and MJ1249-Rev, (5′-GATGGATCCTCACTTTTCAATAATCG-3′). PCR were performed as described previously (*16*), using a 45 °C annealing temperature for MJ0400 and 50 °C annealing temperature for MJ1249. The primers introduced an *Nde*I restriction site at the 5′-end and a *Bam*HI site at the 3′-end of the amplified DNA. The amplified PCR products were purified by QIAQuick spin column (Invitrogen) and digested with restriction enzymes *Nde*I and *Bam*HI then ligated into the compatible sites individually in plasmid pT 7-7(USB) by bacteriophage T4 DNA ligase (Invitrogen) to make the recombinant plasmids pMJ0400 and pMJ1249. DNA sequences were verified by dye-terminator sequencing at the University of Iowa DNA facility. The resulting plasmids pMJ0400 and pMJ1249 were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) cells. The transformed cells were grown in Luria-Bertani medium (200 mL; Difco) supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an absorbance at 600 nm of 1.0. The recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional culture of 2 h with shaking at 37 °C, the cells were harvested by centrifugation (4000*g*, 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% T, 4% C acrylamide, using a Tris/glycine buffer system) analysis of total cellular proteins. This analysis showed the recombinant proteins to be the major cellular protein in each case. Proteins were recovered from the *E. coli* cells by sonication and purified by heat treatment as previously described (*17*). The MJ0400 protein was purified by chromatography on Mono Q and the MJ1249 by Sephadex G-25 chromatography (*17*). SDS-PAGE analysis showed each enzyme was essentially pure, except that the MJ0400 derived protein contained a small amount of the coexpressed chloramphenicol acetyltransferase.

Incubation of Recombinant Enzymes with Precursors, Isolation, and Derivatization of Product. Mixtures of the recombinant proteins were incubated with precursors under the conditions specified in Table 2. After incubation, samples 1−6 were mixed with a known amount of quinic acid, to serve as the internal standard, passed through Dowex 50W-X8 H^+ column (2-× 5-mm), and converted into their TMS derivatives and analyzed by GC-MS for DHQ and 3,4-dihydroxybenzoic acid (DHB). The DHQ generated in samples 7−11 was reduced with $NaBH_4$ to quinic acid and shikimate was added as an internal standard and the samples were assayed by GC-MS as the TMS derivatives.

GC-MS Analysis of Intermediates. GC-MS analysis of the samples was obtained using a VG-70-70EHF gas chromatography-mass spectrometer operating at 70 eV and equipped with an HP-5 column (0.32-mm by 30-m) programmed from 95 to 280 °C at 10 °C/min. Under the GC-MS conditions used, the indicated derivatives of the following compounds had the following retention times (min/sec) and mass spectral data (molecular weight, *base peak*, the most abundant ions with masses over 200 *m/z* listed in order of decreasing intensities): methyl shikimate tri-trifluoroacetyl derivative (8:37.8) [476, *127*, 248, 303, 205, 445, 444, 330, 331, 362]; methyl 3-epi-shikimate tri-trifluoroacetyl derivative (9:1.3) [476, *127*, 248, 303, 207, 331]; 3,4-dihydroxybenzoic acid (DHB, protocatechuic acid) $(TMS)_3$ derivative (14:11) [370, *193*, 370, 333, 311, 289, 223]; DHS $(TMS)_3$ derivative (14:26) [388, *73*, 204, 373, 283, 298, 388]; shikimic acid $(TMS)_4$ derivative (14:36) [462, *204*, 255, 357, 372, 462,

447]; quinic acid $(TMS)_5$ derivative (15:31) [552, *345*, 255, 435, 204, 537]; DHQ $(TMS)_4$ derivative (15:42) [478, *73*, 388, 333, 373, 463].

Quantitation and Identification of Hydroxyacetone and Hydroxyacetone-P Produced in M. jannaschii Cell Extracts. To 50-μL portions of *M. jannaschii* cell extracts were added 10-μL portions of anaerobic solutions of either 0.1 M DL-glycerol-P or 0.1 M glucose 6-P and the samples were incubated for 30 min at 70 °C under argon. An extract with no additions served as a control. The samples were then cooled to room temperature, 80 μL of methanol and 10 μL of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine were added, and the precipitated proteins were removed by centrifugation (14 000*g*, 5 min). The resulting separated clear liquid was concentrated to 20 μL by evaporation and heated 30 min at 70 °C and separated by preparative TLC using the TLC solvent 1. Spots were visualized by exposing the plates to UV light. In this solvent, the hydroxyacetone *O*-(4-nitrobenzyl)hydroxylamine derivative, *O*-(4-nitrobenzyl)hydroxylamine, and the hydroxyacetone-P *O*-(4-nitrobenzyl)-hydroxylamine derivative had R_f's of 0.80, 0.72, and 0.38, respectively. The desired bands were removed from the TLC plate and eluted with 100 μL of 70% methanol in water. After evaporation of the solvent, the samples were dissolved in water and adjusted to pH 6.0 for HPLC analysis. Analyses of compounds were performed on a Shimadzu SCL-6B HPLC using a C-18 reversed-phase column (AXXI Chrom octadecyl silane column, 5-μm, 4.6-mm $\times$ 25-cm) eluted with methanol in 25 mM sodium acetate buffer (pH 6.0, 0.02% NaN_3) at a flow rate of 0.5 mL/min. The separation was monitored at 275 nm. With the HPLC system used, the *O*-(4-nitrobenzyl)hydroxylamine derivatives of both hydroxyacetone-P and hydroxyacetone produced two peaks corresponding to the syn and anti oxime isomers. The hydroxyacetone-P derivatives were separated with 40% methanol, and the two isomers had retention times of 10.5 and 11.2 min. The hydroxyacetone derivatives were separated with 60% methanol, and the two isomers had retention times of 10.6 and 11.4 min.

Quantitation of the derivatives was accomplished by comparison to samples of known concentration generated from the known compounds that were purified by preparative TLC. Concentrations were established using the $\epsilon = 9490$ M^{-1} cm^{-1} for *O*-(4-nitrobenzyl)hydroxylamine. The sum of the areas of these two peaks was used for the quantitation of each compound.

Identification of the Precursor of Hydroxyacetone and Hydroxyacetone-P in Cell Extracts. An *M. jannaschii* cell extract (100 μL) was incubated with 9.1 mM [U-$^{13}C_6$]glucose 6-P or [$^2H_7$]glucose 6-P for 30 min at 70 °C under argon, after which 20 μL of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine was added, and the heating was continued for an additional 25 min at 70 °C. The samples were then cooled to room temperature, 180 μL of methanol was added, and the proteins were removed by centrifugation (14 000*g*, 10 min). The clear yellow liquid was separated and evaporated to dryness with a stream of nitrogen gas and dissolved in 50 μL of water and 50 μL of 0.1 M glycine buffer, pH 10.4, containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, and 0.17 units of *E. coli* alkaline phosphatase was added. After incubation for 2 h at 37 °C, the sample was extracted twice with 0.5 mL of methyl acetate, and after evaporation of the solvent, the sample was purified by preparative TLC using the TLC solvent methylene chloride−methyl acetate, 1:1, v/v. In this solvent system, the dihydroxyacetone derivative had an R_f of 0.25, and the hydroxyacetone derivative had an R_f of 0.48. The areas of the plate corresponding to these compounds were removed and eluted with methyl acetate, evaporated, and reacted with TMS for GC-MS analysis.

Testing the Lack of Metabolism of Expected Precursors to DHQ or DHS. M. jannaschii cell extracts (100 μL) were incubated with 10 mM E-4-P and 10 mM pyruvate, 10 mM OAA, or 10 mM PEP, 3-deoxy-D-*ribo*-2-heptulosonate-7-phosphate, or 3-deoxy-D-*arabino*-2-heptulosonate-7-phosphate. Each failed to generate detectable amounts of DHQ or DHS as measured by GC-MS of the TMS derivative.

Establishing the Conversion of 5-Dehydroquinate to 5-Dehydroshikimate. A cell extract of *M. jannaschii* (100 μL) was incubated with 10 mM DHQ at 60 °C for 30 min, and the resulting DHQ and DHS were assayed by GC-MS of their TMS derivatives. A control carried out under identical conditions without the cell extract showed no formation of DHS.

RESULTS AND DISCUSSION

Quantitation and Identification of Hydroxyacetone and Hydroxyacetone-P Present in and Produced by M. jannaschii Cell Extracts. The 50-μL control and 50 μL sample incubated with DL-glycerol-P contained 4.4 nmol of hydroxyacetone-P and 2.4 nmol of hydroxyacetone and the 50 μL sample incubated with glucose 6-phosphate contained 9.2 nmol of hydroxyacetone-P and 5.2 nmol of hydroxyacetone, showing that only incubation with glucose 6-P increased the amount of both hydroxyacetone and hydroxyacetone-P.

Identification of the Precursor of Hydroxyacetone and Hydroxyacetone-P in Cell Extracts. Hydroxyacetone and hydroxyacetone-P present in cell extracts incubated with labeled glucose 6-P's were found to contain 80% of the molecules with an intact $^{13}C_3$ unit in the [$^{13}C_6$]glucose 6-P experiment and 23% no deuterium, 24% one deuterium and 52% two deuterium in the [$^2H_7$]glucose 6-P experiment. The *m*/*z* 103 fragment (CH_2OTMS) of the hydroxyacetone derivative was increased by one *m*/*z* in the [$^{13}C_6$]glucose 6-P experiment and contained no detectable deuterium in the [$^2H_7$]glucose 6-P experiment. These results show that the hydroxyacetone methyl is derived from C-6 of the glucose 6-P and the C-4 and C-5 hydrogens of the glucose 6-P are lost in the formation of hydroxyacetone. This is exactly as was observed for the glucose 6-P-derived fragment incorporated into the shikimate (see below).

Aldol Reactions Catalyzed by MJ0400. Incubation of the MJ0400 protein with 10 mM E-4-P and 10 mM pyruvate, OAA, or PEP produced a condensation product only in the case of the incubation with E-4-P and pyruvate. The compound produced was a different stereoisomer from 3-deoxy-D-*arabino*-2-heptulosonate-7-phosphate (DAHP). This conclusion was based on the observation that the set of four isomers of the TMS derivative of the dephosphorylated sugar were different from the set of four isomers of the TMS derivative of the dephosphorylated sugar produced from an authentic sample of DAHP. The differences in their retention times, the relative amounts of each isomer, and the similarity of their mass spectra upon GC-MS analysis confirmed this

occurrence of different isomers. On the basis of these data, it was concluded that the enzymatically generated compound was the 2-dehydro-3-deoxy-D-*ribo*-2-heptulosonate-7-phosphate (DRHP) isomer, with the new anomeric center at C-4 being established by the enzyme. The formation of four isomers from each sugar resulted from the cyclization of the sugars to the different anomeric pyranose and furanose rings.

Incubation of the MJ0400 protein with 2 mM DKFP and 2 mM L-aspartate semialdehyde followed by borohydride reduction of the product led to the identification of four isomers of 4,5-dihydroxy-6-methylpipecolinic acid (Figure 3), which were separated by GC-MS as their methyl tri-trifluoroacetyl derivatives. The mass spectrum of each isomer was similar showing a molecular ion at 477 *m*/*z*, with fragments at $M^+ - 31 = 446$ *m*/*z*, $M^+ - 59 = 418$ *m*/*z*, and additional intense ions at 190, 276, and 336 *m*/*z*. It was assumed that the different isomers resulted from racemization at C-5 and C-6 during the preparation of the derivative.

Incubations with MJ0400 and MJ1249. Incubation of the MJ0400 and MJ1249 proteins with 10 mM E-4-P and 10 mM pyruvate, OAA or PEP in the presence and absence of different mixtures of F_{420}, Zn^{2+}, and NAD failed to produce any DHQ or DHS.

Testing Known Precursors to DHQ and DHS. Cell extracts were incubated with erythrose-P and PEP, oxalacetate or [$^{13}C_3$]pyruvate each at a concentration of 10 mM. In these experiments, GC-MS of the TMS derivatives of shikimate, DHQ, and DHS were used to detect their production. Quinic acid was added as an internal standard to quantitate the amounts produced since its TMS derivative eluted close to those of DHQ, DHS, and shikimate. No increase in the amount of shikimate was found over that present in the cell extracts, and no DHQ or DHS was ever detected. In the experiment with labeled pyruvate, no incorporation of label was observed in the shikimate.

Metabolism of DAHP and DRHP by Cell Extracts of M. jannaschii. Incubation of cell extracts with 10 mM DAHP or 10 mM DRHP, produced by incubation of erythrose-P and pyruvate with the MJ0400 enzyme, failed to show any production of DHQ or DHS. This result established that the other isomer of DAHP was also not an intermediate in the pathway. These results indicate that the canonical pathway to the aromatic amino acids is not functioning in *M. jannaschii*.

Confirming Subsequent Steps in the Shikimate Pathway. Genomic data indicate that all of the remaining enzymes in the pathway to the aromatic amino acids are present in *M. jannaschii* (*5*). To specifically test for the presence for the third enzyme in the pathway, 3-dehydroquinate dehydratase, I measured the conversion of DHQ to DHS in cell extracts. Thus, DHQ incubated at a concentration of 10 mM with a cell extract at 70 °C for 30 min was quantitatively converted into DHS. Establishing this conversion indicated that first steps in the biosynthesis, what ever they were, likely led to the formation of DHQ.

Considering the rapid enzymatic conversion of DHQ to DHS, the chemical instability of DHS (*18*), and the stability of shikimate, it was decided to use borohydride to reduce the DHS prior to GC-MS analysis. This reduction produces the isomer of shikimate with the opposite stereochemistry at C-3 as the major product (3-*epi*-shikimate), and since this product separates from shikimate in the GC-MS analysis, it allowed for the extent of labeling of both of these compounds to be measured in one GC-MS run.

Fragmentation of the Tri-Trifluoroacetyl Derivative of Methyl Shikimate. A problem with the mass spectral analysis of the TMS derivative of shikimate was that it was not possible to establish the position at which the label was incorporated into the molecule from the different precursors. Examination of the tri-trifluoroacetyl derivative of methyl shikimate showed that it was a more suitable derivative for such a determination, since it underwent electron impact elimination reactions that removed specific hydrogens from the molecule. This was confirmed by the examination of the mass spectra of this derivative produced from a series of known monodeuterated shikimates. These results are summarized in Figure 2, which is a composite of data from all the individual monodeuterated shikimates. The loss or retention of deuterium in the M^+- $HOCH_3$ 444 *m*/*z* fragment ion specifically measured the extent of deuteration at C-3. The difference between the deuterium in the *m*/*z* 330 and *m*/*z* 331 specifically measured the extent of deuteration at C-4. Retention of deuterium in all of the fragments in the figure showed that a deuterium resided at C-2, whereas loss of deuterium in the 248, 217, and 189 ions indicates a deuterium resided at C-5. Finally the partial loss of deuterium from the 248, 217, and 189 ions showed deuterium incorporation at C-6. This partial loss of deuterium was a result of having two positions at C-6 for the single deuterium to reside. Loss of ^{13}C in the *m*/*z* 303 fragment specifically measured labeled carbon in the carboxylic portion of the molecule.

Defining Alternate Substrates Leading to DHQ and Shikimate. Incubation of cell extracts with dihydroxyacetone or fructose 1,6-bisP and [$^{13}C_3$]pyruvate led to only a small increase in the amount of shikimate and DHS over that found in the cell extract alone (Table 1, Experiments 1, 2, and 3). No detectable amount of ^{13}C was found to be incorporated, indicating that pyruvate was not the precursor. Considering that the C_4 sugar erythrose-P and the C_3 unit of pyruvate or PEP were not the precursors, I next considered that a C_3 fragment derived from glucose 6-P and a C_4 unit were possible precursors.

To test this idea, cell extracts were incubated with [U-$^{13}C_6$]-glucose 6-P, L-homoserine and NAD. The NAD was required to produce L-aspartate semialdehyde from L-homoserine catalyzed by homoserine dehydrogenase. This incubation greatly increased the amount of shikimate and DHS produced, with both being derived from a $^{13}C_3$ unit of the glucose (Table 1, Experiment 4). A similar incubation with [4,4′-$^2H_2$]-L-homoserine incorporated a single deuterium, indicating oxidation of the homoserine to aspartate semialdehyde before condensation with a $^{13}C_3$ unit derived from the glucose (Table 1, Experiment 5). Analysis of the fragmentation of the labeled shikimate derivative showed that this single incorporated deuterium resided at C-5 and that no portion of the $^{13}C_3$ unit was incorporated in the carboxylate carbon. This indicated that the L-homoserine supplied the carboxylate carbon and carbons 1, 6, and 5 of the product. [1-^2H]Glucose 6-P, [6, 6′-$^2H_2$]glucose 6-P, and [1, 2, 3, 4, 5, 6, 6′-$^2H_7$]glucose 6-P each labeled the shikimate with a single deuterium (Table 1, Experiments 6, 7, and 8), which, based on the fragmentation pattern of the known labeled shikimates resided exclusively on C-2. Of these three labeled

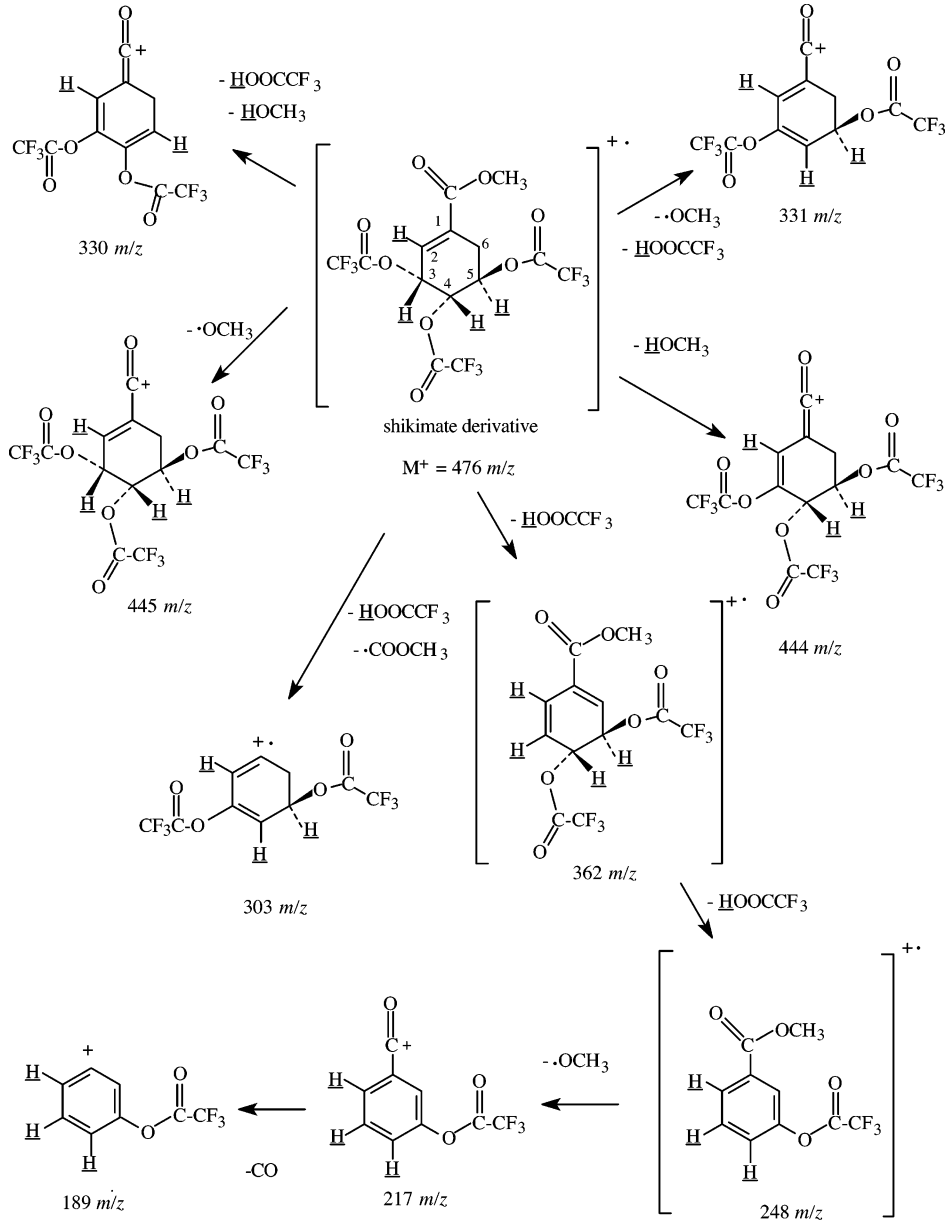

FIGURE 2: Fragmentation of the methyl ester tri-trifluoroacetyl derivative of shikimate. A similar fragmentation pattern was observed with *epi*-shikimate. H indicates the loss of deuterium from the specifically monodeuterated methyl shikimate derivative.

glucoses, the [1-^{2}H]glucose 6-P labeled the products to the lowest extent. These data are consistent with a partial equilibration of the C-1 and C-6 position of the glucose 6-P prior to incorporation into DHQ and the incorporation of the C-6 of the glucose 6-P at C-2 and the C_3 unit at carbons 2, 3, and 4 of DHQ. The partial equilibration of the label from C-1 with C-6 could be due to equilibration of the glucose 6-P through fructose 6-P, fructose 1,6-bisP, glyceraldehyde-3P, and dihydroxyacetone-P (top of Figure 4) or the presence of a dehydrogenase oxidizing and reducing C-2 and C-5 of fructose 1,6-bisP, as shown in Figure 4. Also, the deuterium on C-4 of the glucose is lost during the incorporation. This loss of the C-4 hydrogen could have occurred during oxidation of the C-4 carbon to a carbonyl, as discussed below, since proton exchange from this position would not be expected during an aldolase or transaldolase reaction (*19*).

A logical first step in the assembly of DHQ would then consist of the condensation of a C_3 unit from C-4, 5, 6 of a glucose 6P-derived hexose-P with L-aspartate semialdehyde, as shown in Figure 3. A logical choice for the C_3 unit could be either hydroxyacetone or hydroxyacetone-P, both of which I have shown to be produced from glucose 6-P by the cell extracts. One possible pathway for the production of hydroxyacetone-P would be reduction of dihydroxyacetone-P to L-glycerol-P, the major glycerol-P isomer in the archaea (*20*), followed by elimination of water (*21*). Incubation of a cell extract with labeled [$^2H_5$]-L-glycerol-P and L-aspartate semialdehyde, generated from homoserine and NAD, still led to the production DHQ and shikimate, but neither contained deuterium (Table 1, Experiment 10). The same results were obtained using [$^2H_5$]-DL-glycerol-P (Table 1, Experiment 11), proving that neither glycerol phosphate isomer was the precursor. Since it was also possible for both hydroxyacetone and hydroxyacetone-P to be generated directly from glucose 6-P, [$^2H_5$]hydroxyacetone-P was prepared and incubated with a cell extract, and again no label incorporation was observed (Table 1, Experiment 12).

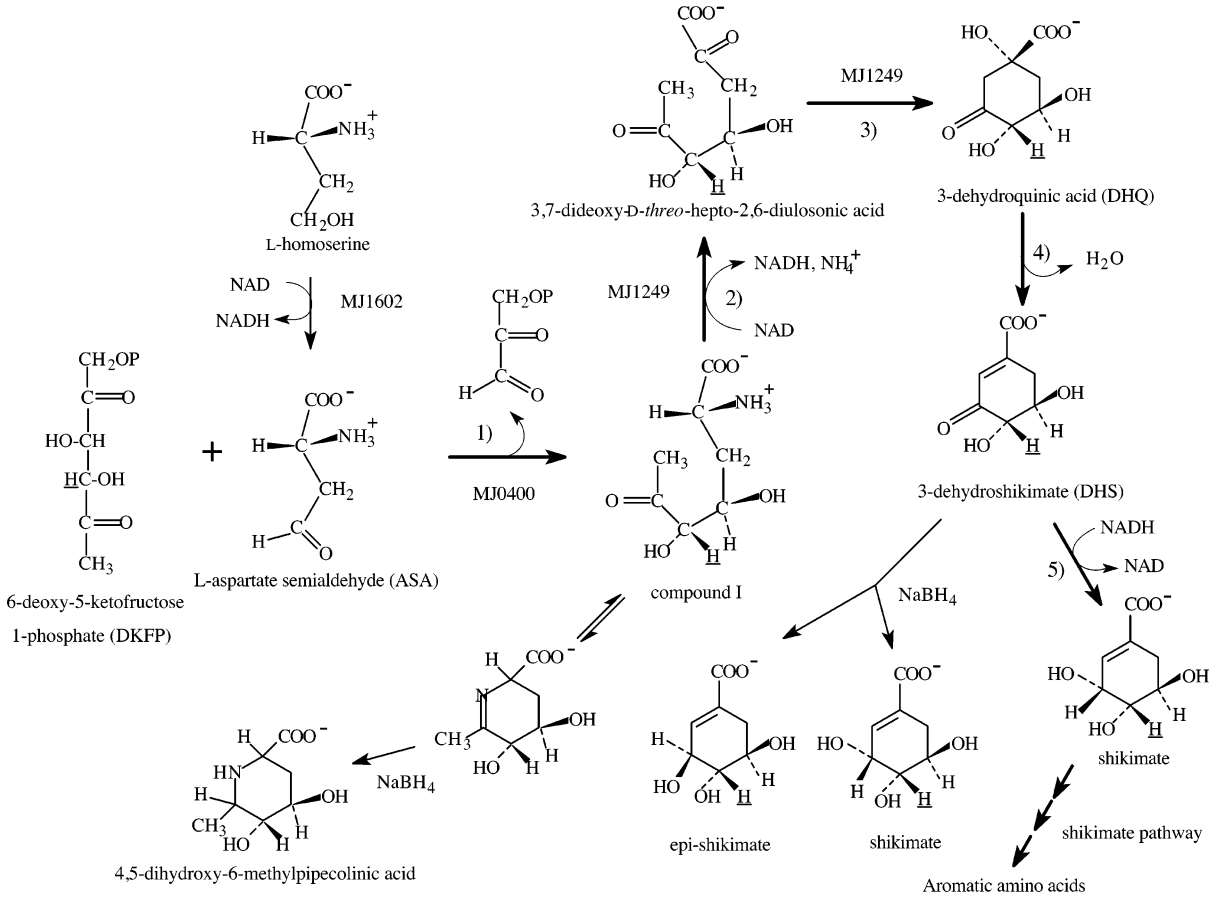

FIGURE 3: Biosynthetic pathway for aromatic biosynthesis in *M. jannaschii* and the chemical routes for the generation of assayable intermediates.

Table 1: Quantitation of Shikimate and Dehydroshikimate Generated in Cell Free Incubations

experiment[a]	shikimate (nmol)	*epi*-shikimate (DHS) (nmol)	total (nmol)
1. cell extract	0.001	0.0007	0.002
2. dihydroxyacetone	0.004	0.007	0.011
3. fructose-1,6 diP + [$^{13}C_3$]pyruvate	0.005 (<1% excess ^{13}C)	0.01 (<1% excess ^{13}C)	0.015
4. [$^{13}C_6$]glucose 6-P + L-homoserine	0.08 (92% $^{13}C_3$)	0.22 (85% $^{13}C_3$)	0.3
5. [$^{13}C_6$]glucose 6-P + [4,4′-$^2H_2$]-L-homoserine	0.15[b]	0.15[c]	0.3
6. [1-$^2H_1$]glucose 6-P + L-homoserine	0.51 (20% $^2H_1$)	0.31 (21% $^2H_1$)	0.82
7. [6,6-$^2H_2$]glucose 6-P + L-homoserine	0.8 (67% $^2H_1$)	0.1 (65% $^2H_1$)	0.9
8. [1,2,3,4,5,6,6′-$^2H_7$]glucose 6-P + L-homoserine	0.8 (67% $^2H_1$)	0.1 (65% $^2H_1$)	0.9
9. fructose 1,6-bisP + L-homoserine	0.4	0.1	0.5
10. [$^2H_5$]-L-glycerol-P + L-homoserine	0.15 (<0.5% ^2H)	0.36 (<0.5% ^2H)	0.51
11. [$^2H_5$]-DL-α-glycerol-P + L-homoserine	0.24 (<0.5% ^2H)	0.125 (<0.5% ^2H)	0.37
12. [$^2H_5$]hydroxyacetone-P + L-homoserine	0.9 (<0.5% ^2H)	0.04 (<0.5% ^2H)	1.0
13. hydroxyacetone-P + aspartate semialdehyde	0.4	0.056	0.46
14. hydroxyacetone + [4,4′-$^2H_2$]-L-homoserine	0.12 (22% $^2H_1$)	0.16 (30% $^2H_1$)	0.28
15. hydroxyacetone-P + L-homoserine	0.9	0.04	1.0
16. [$^{13}C_6$]glucose 6-P + L-homoserine + 6-deoxy-5-ketofructose 1-phosphate	0.57 (79% $^{13}C_3$)	0.22 (82% $^{13}C_3$)	0.63

[a] The following concentrations of substrates were used in these experiments: glucose 6-P, fructose 1,6-bisP, L-α-glycerol-P, DL-α-glycerol-phosphate, L-homoserine, aspartate semialdehyde, and hydroxyacetone, 8.1 mM; hydroxyacetone-P, 1.8 mM; 6-deoxy-5-ketofructose 1-phosphate, 1 mM. All of the experiments but the first three had NAD and NADP added at a concentration of 1.6 mM to facilitate the formation of L-aspartate semialdehyde from L-homoserine. In each case, the precursors were added as anaerobic solutions to 100 μL of cell extract, and the mixture was incubated for 30 min at 70 °C under argon and processed as described in the methods section. The extent of labeling is expressed as the mole % of the molecules containing the indicated stable isotopes. [b] (12% no label, 6% $^2H_1$, 31% $^{13}C_3$, 51% $^{13}C_3$ + $^2H_1$). [c] (12% no label, 6% $^2H_1$, 31% $^{13}C_3$, 51% $^{13}C_3$ + $^2H_1$).

Incubation of cell extracts with aspartate semialdehyde and hydroxyacetone-P also failed to increase the amount of product (Table 1, Experiment 13). Incubation with hydroxyacetone and [4, 4′-$^2H_2$]-L-homoserine incorporated a single deuterium at C-5, but failed to increase the amount of product (Table 1, Experiment 14), indicating that hydroxyacetone is not a precursor. Also, the fact that the labeled glucose 6-Ps were incorporated so efficiently (90%), even with the

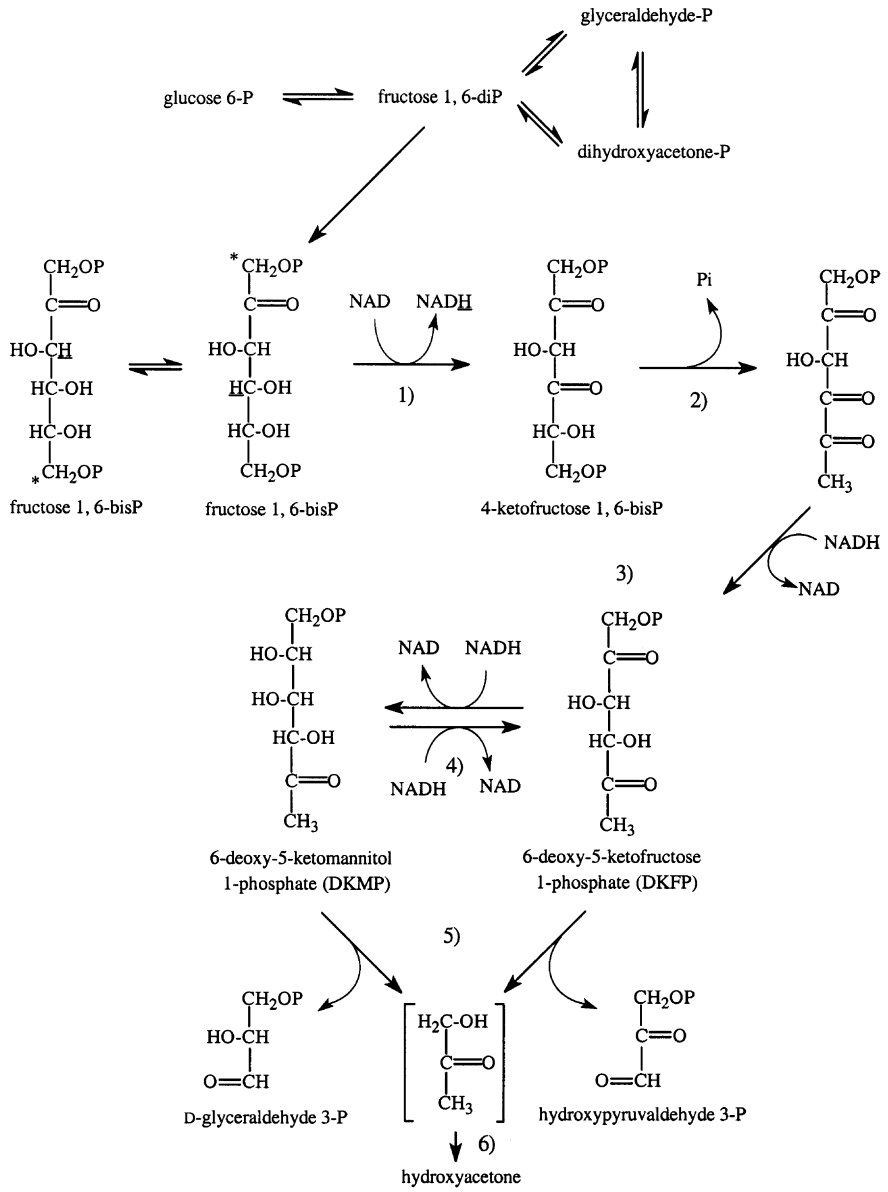

FIGURE 4: A proposed route for the generation of 6-deoxy-5-ketofructose 1-phosphate, hydroxyacetone, and hydroxyacetone-P

hydroxyacetone and hydroxyacetone-P present in the cell extracts, further indicate that hydroxyacetone or hydroxyacetone-P are not precursors.

It thus appeared likely that the terminal three-carbon unit of the glucose 6-P is derived by direct transfer from a sugar precursor. A possible choice of this sugar would be 6-deoxy-5-ketofructose 1-phosphate (DKFP) which, after a transaldolase reaction with ASA, would produce hydroxypyruvaldehyde phosphate as the other product (*22*). DKFP was prepared, and when incubated with extracts containing [$^{13}C_6$]-glucose 6-P, was found to only slightly decrease the extent of label incorporation into the products (Table 1, Experiment 16). This result would indicate that DKFP may not be the preferred precursor to Compound 1. Reduction of DKFP to 6-deoxy-5-ketomannitol 1-phosphate would produce a hexose phosphate that after the transaldolase reaction would produce D-glyceraldehyde 3-P, a central metabolite. Incubation of aspartate semialdehyde and 6-deoxy-5-ketofructose 1-phosphate with protein product derived from MJ0400, followed by reduction of the product with $NaBH_4$, produced four isomers of dihydroxy-6-methylpipecolinic acid, which were assayed as the methyl tri-trifluoroacetyl derivative of 4,5 dihydroxy-6-methylpipecolinic acid ($M^+ = 477$ *m*/*z*). These products were presumably generated by cyclization of compound I to the imine followed by its reduction, as shown in Figure 3.

Final support of the proposed pathway has been the demonstration that a mixture of the MJ0400 and MJ1249 derived proteins catalyzed the formation of DHQ from L-aspartate semialdehyde and 6-deoxy-5-ketofructose 1-phosphate. The identification of these two genes as being involved in aromatic amino acid biosynthesis was based on their association with other aromatic amino acid biosynthetic genes in other archaeal genomes. Thus, incubation of only the mixture of the two proteins produced DHQ, whereas the individual proteins produced no product (<0.012 nmol) (Experiments 1−3, Table 2). Considering that a transamination reaction could be responsible for the removal of the ammonia from compound I, I did the incubations with the possible amino acceptors pyruvate and α-ketoglutarate along with recombinant PLP dependent enzymes identified in the *M. jannaschii* genome that could possibly catalyze the

Table 2: Quantitation of DHQ Generated with Purified Recombinant Enzymes Incubated with Precursors

experiment[a]	DHQ produced (nmol)
1. MJ0400	<0.012
2. MJ1249	<0.012
3. MJ0400, MJ1249	7
4. MJ0400, MJ1249, MJ0959, α-ketoglutarate	12
5. MJ0400, MJ1249, MJ0959, + transaminases α-ketoglutarate, pyruvate	1
6. MJ0400, MJ1249, hydroxyacetone	7
7. MJ0400, MJ1249 + FMN and FAD	5
8. MJ0400, MJ1249 + F_{420}−3	1.2
9. MJ0400, MJ1249 + NAD and NADP	330
10. MJ0400, MJ1249 + NAD	330
11. MJ0400, MJ1249 + NADP	150

[a] Experiments 1−5 were conducted in 145-μL reaction volumes containing 9.7 mM phosphate/K^+ buffer, pH 7.2, 1.9 mM 6-deoxy-5-ketofructose 1-phosphate, 1.4 mM aspartate semialdehyde. α-Ketoglutarate and pyruvate were added to Experiments 4 and 5 each at a concentration of 6.9 mM. Both DHQ and dihydroxybenzoic acid (DHB) were observed as products of these reactions with the 4,5-dihydroxybenzoic acid formed via a phosphate ion catalyzed decomposition of DHS (*18*). The amount of DHQ reported is the sum of the DHQ and DHB. Quinic acid was used as an internal standard for quantitation. In experiment 6, the buffer concentration was 11.7 mM potassium phosphate, pH 7.2, and the ASA and hydroxyacetone concentrations were 1.7 mM and 8.3 mM, respectively. Incubations 7−11 were conducted in a 125-μL volume containing 28 mM tris pH 7.5, 5.6 mM $MgCl_2$, 1.6 mM ASA and 2.2 mM DKFP and were incubated for 30 min at 60 °C in air. At the completion of the incubation, the DHQ was reduced with $NaBH_4$ to quinic acid, and the value reported was the amount of quinic acid observed. Shikimate was used as an internal standard. The enzymes used and amount (μg) included: MJ0400 (12.8); MJ1249 (35.7); MJ0959 (5.6). The transaminases used were a mixture of a pyruvate AATase; MJ0684 (15), a subclass 1 AATase; MJ1008 (29), a branched chain AATase, MJ1391 (13); MJ1479 (7.5), subclass 1 AATases; and MJ1678 (18), a protein predicted to fold like cystathionine synthase (White, R. H., unpublished results). All samples were incubated for 30 min at 60 °C under argon.

transamination reaction. As can be seen from the data in Table 2 (Experiments 4 and 5), the addition of these enzymes did not stimulate the production of DHQ. In addition, Experiment 6 again demonstrated that hydroxyacetone was not a precursor as confirmed with the cell extracts. Likewise, the addition of FMN, FAD, and F_{420}-3 isolated from *M. jannaschii* (*23*) did not stimulate the production of DHQ (Experiments 7 and 8) whereas a mixture of NAD and NADP greatly stimulated the reaction by generating 330 nmol of product (Experiment 9). Of these two pyridine nucleotides, NAD was better, and based on the 330 nmol of product generated and the amount NAD added, all of the NAD would have been completely reduced during the reaction. From the data presented, the specific activity for the MJ0400 enzyme would be > 860 nmol/min/mg protein.

It is interesting to note that the transaldolase reaction catalyzed by the MJ0400 gene product produces the same absolute stereochemistry at C-4 and C-5 of compound I as occurs in other aldolase and transaldolase enzymatic reactions (*24*). The same absolute stereochemistry observed at C-5 of compound I is also observed at C-4 of the DRHP produced by the enzymatic condensation of pyruvate with erythrose-4-P catalyzed by this enzyme.

Possible Origins of 6-Deoxy-5-Ketofructose 1-Phosphate. The exact biochemical origin of DFKP remains to be established. Several enzymes are annonated in the *M. jannaschii* genome that could be involved in the formation of 6-deoxy-5-ketofructose 1-phosphate. One possibility would be an enzyme related to UDP-glucose 4,6-dehydratases and UDP-glucose 4-epimerases, which are members of the short-chain dehydrogenases/reductases superfamily (*25*). Some of these enzymes catalyze the elimination of water from C-5, 6 of a sugar after the NAD dependent oxidation at C-4. Subsequent reduction can occur at C-6 or C-4, depending on whether the enzyme is a 4,6-dehydratase or a 4-epimerase (*26*). *M. jannaschii* has two genes, MJ0211 and MJ1055, both annotated as nucleoside-diphosphate-sugar epimerases that could encode enzymes responsible for this series of reactions. However, these enzymes all contain a tightly bound NAD that does not exchange the transferred hydrogen with solvent (*27*), and we have shown that the derived hydroxyacetone has lost this C-4 hydrogen. Therefore, if the hydroxyacetone comes from the 6-deoxy-5-ketofructose 1-phosphate, then these enzymes are not likely to be involved in the reaction. A simpler scheme not involving NDP-sugars is shown in Figure 4. Here, fructose 1,6-bisP, derived from glucose 6-P, is oxidized at C-4 (step 1), to 4-ketofructose 1,6-bisP which undergoes elimination of phosphate to form the enol that rearranges to the 6-deoxysugar (step 2). Reduction at C-4 (step 3) using a different NADH produces 6-deoxy-5-ketofructose 1-phosphate. Steps 1−3 would be like that seen in dehydroquinate synthase. Reduction of 6-deoxy-5-ketofructose 1-phosphate would produce 6-deoxy-5-ketomannitol 1-phosphate (step 4). Either 6-deoxy-5-ketofructose 1-phosphate or 6-deoxy-5-ketomannitol 1-phosphate could do the required transaldolase reaction to generate the required C_3 unit (step 5). An aldolase cleavage of either precursor would produce hydroxyacetone (step 6).

It should be noted here that based on the promiscuous nature of the MJ0400 enzyme, it is possible that the true sugar precursor is not 6-deoxy-5-ketofructose 1-phosphate but is in fact its C-2 reduced material, 6-deoxy-5-ketomannitol 1-P (Figure 4). This precursor has the advantage that the other product of the reaction would be D-glyceraldehyde 3-P, which would be readily metabolized by the cells. Attempts to identify either 6-deoxy-5-ketofructose 1-phosphate or 6-deoxy-5-ketomannitol 1-phosphate in cell extracts have repeatedly failed, indicating that neither compound is likely to be a free intermediate in the cells (R. H. White, unpublished results) This finding would indicate that either of these compounds may be formed and used while bound to an enzyme or enzyme complex.

In total, the overall series of reaction postulated for the formation of DHQ closely follows those seen in the normal pathway catalyzed by DHQ synthase (*28*−*31*), which involves the NAD-dependent oxidation of a sugar phosphate followed by elimination of phosphate and rearrangement to a methyl ketone. The enol of the ketone then intramolecularly adds to the α-keto acid to form DHQ. Our reaction route, however, is made more complex since the different parts of the reaction are separated and more than one enzyme and substrate is involved in carrying out the required series of reactions, which is readily accomplished by the multistep catalysis of dehydroquinate synthase operating on DAHP (*32*).

It is very ironic that the cyclization of 3,7-dideoxy-D-*threo*-hepto-2,6-diulosonic acid proposed 40 years ago by Sprinson

to be involved in aromatic amino acid biosynthesis in *E. coli* has now been demonstrated to function in the Archaea (*33*).

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