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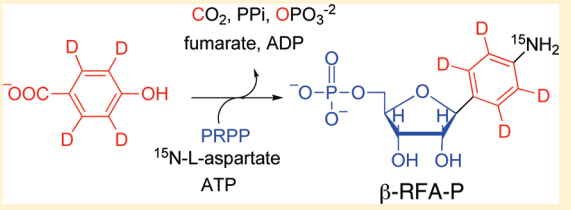
The Conversion of a Phenol to an Aniline Occurs in the Biochemical Formation of the 1-(4-Aminophenyl)-1-deoxy-D-ribitol Moiety in Methanopterin

Robert H. White*

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

ABSTRACT: Recent work has demonstrated that 4-hydroxybenzoic acid is the in vivo precursor to the 1-(4-aminophenyl)-1-deoxy-D-ribitol (APDR) moiety present in the C_1 carrier coenzyme methanopterin present in the methanogenic archaea. For this transformation to occur, the hydroxyl group of the 4-hydroxybenzoic acid must be replaced with an amino group at some point in the biosynthetic pathway. Using stable isotopically labeled precursors and liquid chromatography with electrospray-ionization mass spectroscopy, the first step of this transformation in *Methanocaldococcus jannaschii* occurs by the reaction of 4-hydroxybenzoic acid with phosphoribosyl pyrophosphate (PRPP) to form 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate (β-RAH-P). The β-RAH-P then condenses with L-aspartate in the presence of ATP to form 4-(β-D-ribofuranosyl)-*N*-succinylaminobenzene 5′-phosphate (β-RFSA-P). Elimination of fumarate from β-RFSA-P produces 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P), the known precursor to the APDR moiety of methanopterin [White, R. H. (1996) *Biochemistry* 35, 3447−3456]. This work represents the first biochemical example of the conversion of a phenol to an aniline.



Tetrahydromethanopterin (H_4MPT) and tetrahydrofolate (H_4folate) represent the functional forms of two different coenzymes that function as biological C_1 carriers (Figure 1). These coenzymes were both identified and characterized in their oxidized forms, methanopterin (MPT) and folate, respectively. MPT was first identified as a coenzyme in the methanogenic archaea[1] and was later identified in bacteria.[2] Early experiments showed that the pterin portion of both of these coenzymes arose from GTP[3−5] and that the arylamine moieties in both of the structures could arise from 4-aminobenzoic acid (AB).[5,6] These early observations indicated that these coenzymes could be modified versions of each other. However, despite their similar structures at the C_1 binding sites,[7] our current data on the biosynthesis of these cofactors indicate that they are not related.[8]

Despite the fact that the 1-(4-aminophenyl)-1-deoxy-D-ribitol (APDR) of methanopterin was derived from AB when supplied to growing cultures of methanogens, no evidence has ever been obtained that AB is biosynthesized by the methanogens. This is consistent with the absence in archaeal genomes of the *pabA*, *pabB*, and *pabC* genes known to be involved in AB biosynthesis in the bacteria.[9−11] Although the first two steps in the shikimic acid pathway are modified in the archaeal 6-deoxy-5-ketofructose 1-phosphate (DKFP) pathway,[12] the pathway still produces chorismate, the metabolite used to produce AB using these gene products. A deletion mutant of AroA′, which encodes the first step in the DKFP pathway, in *Methanococcus maripaludis* was found to require both the aromatic amino acids and AB for growth,[13] yet a 3-dehydroquinate dehydratase (AroD) deletion mutant did not require AB and incorporated labeled hydroxyphenylacetic acid into tyrosine but not into the APDR moiety of MPT.[14] These observations led to the conclusion that 3-dehydroquinate (DHQ), not chorismate, was the precursor to AB. A possible alternate pathway for the biosynthesis of AB from DHQ is shown in Figure 2. Attempts to confirm the operation of this pathway by incubating cell extracts with L-asparatate semialdehyde and 6-deoxy-5-ketofructose 1-phosphate, DHQ, and 4-amino-5-hydroxy-1,3-cyclohexadiene-1-carboxylic acid and assaying for an increase in the amount of AB were all proven negative. One idea not considered in this earlier work was that chorismate-derived 4-hydroxybenzoic acid (HB) normally serves as a precursor to APDR and that HB could also be derived from DHQ. We have recently found that the protein product of the MJ0807 gene is a chorismate lyase and produces HB but not AB from chorismate (L. Grochowski and R. H. White, unpublished results). This finding and the observed incorporation of the aromatic portion of the HB molecule into the phenyl portion of the APDR moiety of MPT indicated that HB is the true in vivo precursor to the APDR in MPT. Here I show that the first step in this process occurs by the previously observed condensation of PRPP with HB to produce 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate (β-RFH-P)[15] catalyzed by the product of the MJ1427 gene. The phenol in the β-RFH-P is then converted to an aniline to generate β-RFA-P. Here I present evidence of how this occurs.

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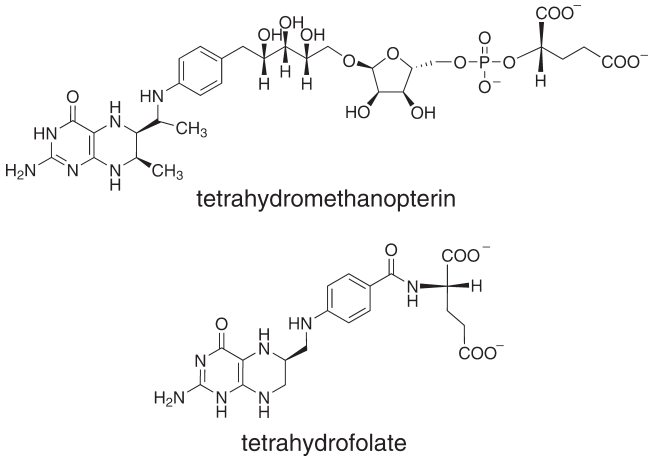

Figure 1. Chemical structures of the biologically active forms of the coenzymes tetrahydromethanopterin and tetrahydrofolate.

MATERIALS AND METHODS

Chemicals. [2,3,5,6-$^2H_4$]-4-Hydroxybenzoic acid (minimum of 98 atom % ^2H), [^{15}N]ammonium chloride (minimum of 98 atom % ^{15}N), [*amide*-^{15}N]glutamine (minimum of 98 atom % ^{15}N), [^{15}N]-L-aspartic acid (minimum of 98 atom % ^{15}N), 4-aminobenzoic acid, 4-aminophenethyl alcohol, adenylosuccinic acid, (±)-bromosuccinic acid, and phosphoribosyl pyrophosphate (PRPP) were obtained from Sigma-Aldrich. Water (minimum of 95 atom % ^{18}O) was obtained from Monsanto Research Corp. β-RFA-P was prepared enzymatically from the reaction of AB with PRPP catalyzed by the MJ1427 gene product. APDR-P was prepared by phosphorylation of APDR as previously described.[16] APDR was either isolated from cell extracts or prepared synthetically.[17]

Testing the Chemical Conversion of 4-Hydroxybenzyl Alcohol to an Aniline. Two separate experiments were conducted to test this idea. The first measured the exchange of the oxygen(s) of 4-hydroxybenzyl alcohol with ^{18}O-labeled water, and the second tested for the replacement of the hydroxyl groups(s) in 4-hydroxybenzyl alcohol with ammonia. In the first experiment, 4-hydroxybenzyl alcohol (0.5 mg) was heated for 12 h at 100 °C with 30 μL of 95% ^{18}O-enriched water. The water was evaporated, and the sample reacted with trifluoroacetic anhydride to form the ditrifluoroacetyl derivative of 4-hydroxybenzyl alcohol that was then examined by GC−MS to determine the extent of oxygen exchange. In the second experiment, 15 mg of 4-hydroxybenzyl alcohol was dissolved in 2 mL of 16 M ammonia and heated at 100 °C in a sealed bottle for 24 h. After cooling, the sample was evaporated to dryness with a stream of nitrogen gas, and the resulting residue was extracted with dilute HCl. The amino-containing compounds were isolated on a Dowex 50 50W-X8 H^+ column and analyzed by GC−MS as their ditrifluoroacetyl derivatives.

Cell Extracts of *Methanocaldococcus jannaschii*. Cell extracts of *M. jannaschii* were prepared by sonication under argon and stored under argon at −20 °C at a protein concentration of ~30 mg/mL.[12] The buffer used in the extraction consisted of 50 mM TES/K^+, 10 mM $MgCl_2$, and 20 mM DTT (pH 7.5).

Cloning, Overexpression, and Purification of the *M. jannaschii* M1427 Gene in *Escherichia coli*. The MJ1427 gene (Swiss-Prot accession number Q58822) was amplified by polymerase chain reaction (PCR) from genomic DNA using oligonucleotide primers MJ1427-Fwd (5′-GGTCATATGATAATTCAAACACCATCG-3′) and MJ1427-Rev (5′-GCTGGATCCTCACCAAATTTTATG-3′). PCR amplification was performed as described previously[8] using an annealing temperature of 55 °C. Purified PCR product was digested with *Nde*I and *Bam*HI restriction enzymes and ligated into compatible sites in plasmid pT7-7. The sequence of the resulting plasmid pMJ1427 was verified by DNA sequencing. pMJ1427 was transformed into *E. coli* strain BL21-CodonPlus(DE3)-RiL (Stratagene). The transformed cells were grown in Luria-Bertani medium (200 mL) supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an OD_{600} of 1.0. Production of the recombinant protein was induced by addition of lactose to a final concentration of 28 mM.[8] After being cultured for an additional 2 h, the cells were harvested by centrifugation (4000*g* for 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis of total cellular proteins. The frozen *E. coli* cell pellet (~0.4 g wet weight from 200 mL of medium) was suspended in 3 mL of extraction buffer and lysed by sonication. The samples were then centrifuged (16000*g* for 10 min) to remove cell debris, and the resulting cell extract was heated for 10 min at 70 °C followed by centrifugation (16000*g* for 10 min). This process allowed for the purification of the protein from the majority of *E. coli* proteins, which denature and precipitate under these conditions. The next step of purification was performed by anion-exchange chromatography of the 70 °C soluble fractions on a MonoQ HR column (1 cm × 8 cm; Amersham Bioscience) using a linear gradient from 0 to 1 M NaCl in 25 mM TES buffer (pH 7.5), over 55 min at a flow rate of 1 mL/min and collecting 1 mL fractions. Protein concentrations were determined by Bradford analysis.[18]

Enzymatic Synthesis of 4-(β-D-Ribofuranosyl)hydroxybenzene 5′-Phosphate (β-RAH-P). To 130 μL of extraction buffer containing 7.8 mM HB and 7.8 mM phosphoribosyl pyrophosphate (PRPP) was added 12 μg of recombinant MJ1427 protein, and the mixture was incubated at 70 °C for 0.5 h. After the sample had cooled to room temperature, 8 μL of 2 M trichloroacetic acid (TCA) was added and the sample centrifuged (16000*g* for 5 min) to remove the precipitated protein. The TCA was neutralized with 1 M NaOH, and HPLC analysis of a 20 μL portion of the sample was performed. Chromatographic separation and analysis were preformed on a Shimadzu HPLC system equipped with a diode array, a fluorescence detector, and a C18 reverse phase column (Varian PursuitXRs, 250 mm × 4.6 mm, 5 μm particle size). The solvent system consisted of 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% methanol for 5 min followed by a linear gradient to 20% sodium acetate buffer and 80% methanol over 40 min at a rate of 0.5 mL/min. The fluorescence intensity was measured with excitation at 284 nm and emission at 310 nm. The fluorescence data were quantitated using a known sample of 4-hydroxybenzyl alcohol separated under the same conditions used for the reference. It was assumed that the quantum yield of fluorescence for both compounds is the same.

Enzymatic Synthesis of 4-(β-D-Ribofuranosyl)aminobenzene 5′-Phosphate (β-RFA-P). To 60 μL of extraction buffer without DTT containing 0.02 M PRPP and 0.02 M μmol of AB was added 12 μg of recombinantly produced MJ1427 protein, and the samples were incubated at 70 °C for 1 h. After the addition of 150 μL of water to the samples, the azo dye derivatives were prepared as previously described.[19,20] Because of the large amount of

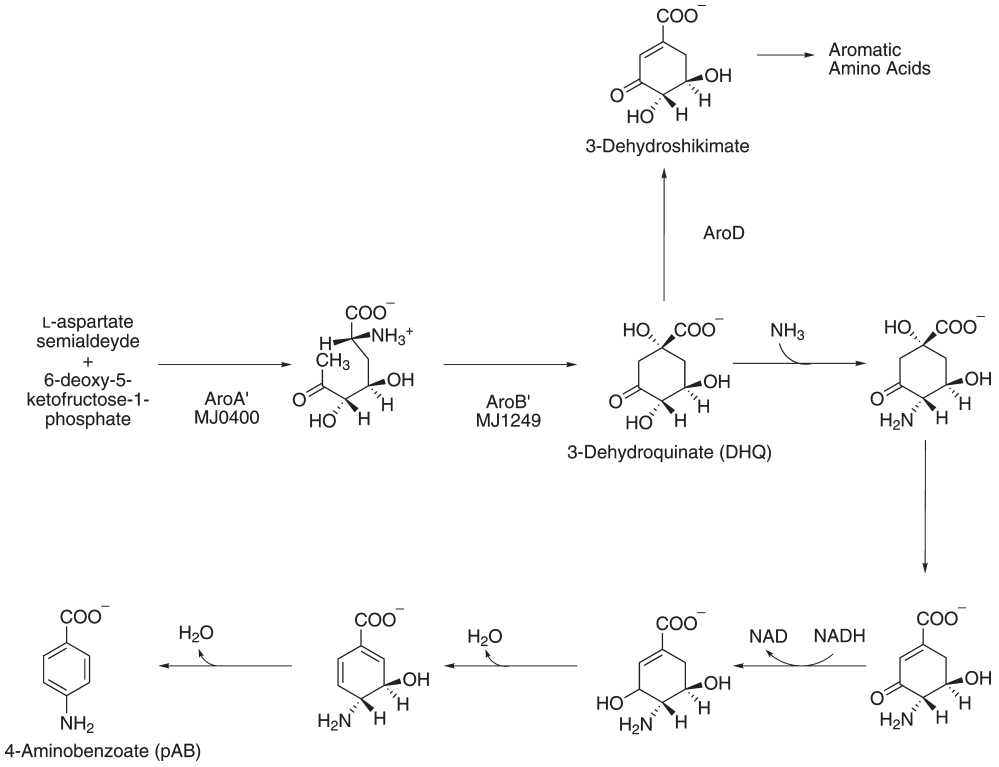

Figure 2. Hypothetical alternate route to AB.

unreacted AB, some of the azo dye derivative of the AB precipitated and was removed by centrifugation prior to separation on the BioGel P-4 (200−400 mesh) column (0.5 cm × 5.0 cm) as described below. This separation completely separated the β-RFA-P azo dye from the AB azo dye and allowed the measurement of the yield of the reaction and identification of the product by absorbance spectroscopy. LC−ESI-MS analysis was also used to confirm that the desired product was obtained.

Chemoenzymatic Synthesis of 4-(β-D-Ribofuranosyl)-*N*-succinylaminobenzene 5′-Phosphate (β-RFSA-P). Model reactions demonstrated that (±)-bromosuccinic acid readily alkylated aniline to form *N*-phenylaspartate when the two compounds were heated together in equal molar amounts at 100 °C for 20 min. The same product could also be obtained when these two compounds were heated together at 100 °C at neutral pH in water. In each case, the product was identified by DI-MS as the dimethyl trifluoroacetyl derivative ($M^+ = m/z$ 333). Heating solutions of 4-aminophenylalanine with (±)-bromosuccinate was used to determine the optimal condition for the alkylation. The progress of the reaction was followed by TLC with UV and ninhydrin detection of the products. Optimal formation of the *N*-succinyl adduct was achieved by heating 25 mM 4-aminophenylalanine and 150 mM (±)-bromosuccinate adjusted to pH 7−8 at 100 °C for 0.5 h. Analysis of the resulting product by TLC showed one major new UV and ninhydrin positive band. ESI-MS analysis of the sample showed a trace amount of the starting 4-aminophenylalanine $MH^+ = m/z$ 181.3 and $MNa^+ = m/z$ 203.2 ions, as well as some of the dialkylated product with $MH^+ = m/z$ 413.1 and $MNa^+ = m/z$ 435.1 ions. The major signal was from the *N*-monoalkylated product showing the following ions: $MH^+ = m/z$ 297.3, $MNa^+ = m/z$ 319.3, and $MK^+ = m/z$ 335.1. It is assumed, on the basis of the pH of the reaction, that only the aniline nitrogen was alkylated with the bromosuccinate.

A sample of 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate (β-RFA-P) was prepared as described above but on a 10-fold larger scale. Because the sample contained a large amount of unreacted AB, this was removed by passing the sample, made 0.1% in formic acid, through a 0.55 cm × 9.0 cm column containing preparative C18 125A resin (55−105 μm, Waters) equilibrated with 0.1% formic acid. The first material to elute (1−3 mL) was the β-RFA-P, with the AB being completely retained on the column. The β-RFA-P fractions were evaporated to dryness with a stream of nitrogen gas to remove the formic acid, and the residue was dissolved in 200 μL of water and adjusted to pH 7−8 with NaOH. To this sample was added 50 μL of 0.2 M (±)-bromosuccinate, and the sample was heated at pH 7−8 for 30 min at 100 °C. Analysis of the presence of an arylamine by the Bratton−Marshall assay showed a 95% reduction in the amount of β-RFA-P. The first incubation (Table 3) was conducted using a portion of this crude preparation of β-RFSA-P.

To ensure complete removal of the β-RFA-P from the sample as well as any fumarate that may have been produced in the reaction, the sample was purified by FPLC on a MonoQ HR 5/5 column using a 0 to 1 M NH_4HCO_3 gradient. In this gradient, the β-RFA-P eluted at 0.5 M NH_4HCO_3 and the β-RFSA-P eluted at 0.9 M NH_4HCO_3, approximately the same positions of a known sample of adenylosuccinate. This is expected because both compounds contained four negative charges. The production of the desired compound was identified by its 245 nm absorbance and DI-ESI-MS in 0.1% HCOOH or 0.1% NH_4OAc. DI-ESI-MS showed in +ESI the expected $MH^+ = m/z$ 422.0, $MNa^+ = m/z$ 444.0 and in −ESI the expected $(M - H)^- = m/z$ 420.1, $(M - 2H + Na)^- = m/z$ 442.3. Adenylosuccinate showed in +ESI the expected $MH^+ = m/z$ 464.2, $MNa^+ = 486.1$, $MK^+ = m/z$ 502.0 and in −ESI the expected $(M - H)^- = m/z$ 462.2, $(M - 2H +$

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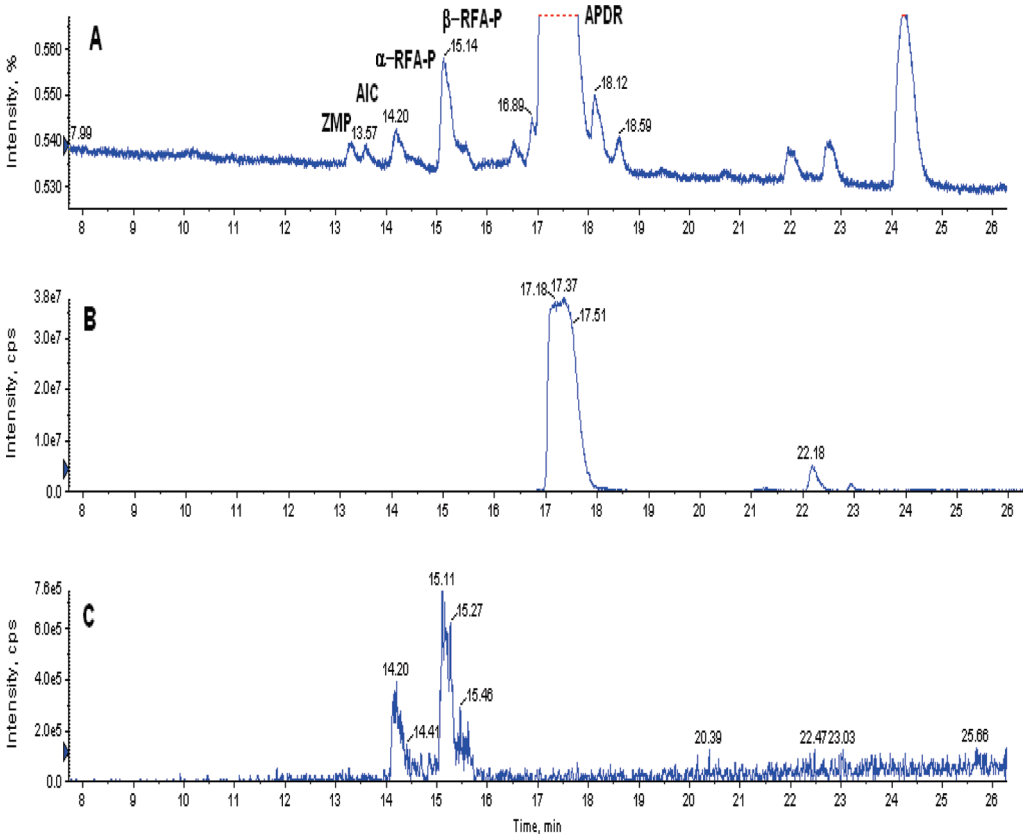

Figure 3. LC−ESI-MS data and UV analysis of sample 8 in Table 2: (A) UV trace of the separated sample at 545 nm, (B) m/z 425 ion trace for APDR, and (C) m/z 503 ion trace for α-RFA-P and β-RFA-P.

$Na)^- = m/z$ 444.3, $(M - 2H + K)^- = m/z$ 500.0. The total yield (0.2 μmol) was 5% on the basis of the amount of PRPP used. Because this product was produced with (±)-bromosuccinate, it is expected to consist of two stereoisomers at the position of the addition of the succinate. Although the yield was low, sufficient product was purified to determine if it served as a precursor to β-RFA-P.

Measurement of β-RAH-P Synthesis in Cell Extracts. To 70 μL of cell extract were added 10 μL of 0.1 M PRPP and 10 μL of 0.1 M HB, and the mixture was incubated at 70 °C for 15 min. After the sample had cooled to room temperature, 10 μL of 2 M trichloroacetic acid was added and the sample centrifuged (16000*g* for 10 min) to remove the protein. The pellet was washed by suspension in 44 μL of 0.2 M TCA and centrifuged (16000*g* for 10 min), and the combined supernatants were adjusted to pH 7.0 by the addition of 6 M NaOH prior to HPLC analysis. A Varian PursuitXRs 250 mm × 4.6 mm, 5 μm particle size column was used for the separation using a solvent system that consisted of 95% sodium acetate buffer [25 mM (pH 6.0) and 0.02% NaN_3] and 5% methanol for 5 min followed by a linear gradient to 20% sodium acetate buffer and 80% methanol over 40 min at a rate of 0.5 mL/min.

Incubation of Cell Extracts with Precursors and Quantitation of Products. The procedure consisted of incubating 70 μL of *M. jannaschii* cell extract (~30 mg/mL protein) in a total volume of 100 μL containing ~10 mM HB, PRPP, L-aspartate, L-glutamine, ammonium chloride, and/or the stable isotopically labeled compounds. When ammonia was tested, the ammonium concentration was 100 mM. Samples were incubated for 15 min at 70 °C and cooled to room temperature, and 10 μL of 6 M HCl was added and the insoluble material separated by centrifugation (16000*g* for 15 min). The recovered soluble fraction was assayed directly for β-RAH-P content by HPLC as described above. For analysis of arylamines, the liquid was separated and diluted with 150 μL of water and the arylamines were converted to their azo dye derivatives as previously described.[19,20] In most cases, the azo dyes were purified on a small BioGel P-4 (200−400 mesh) column (0.5 cm × 5.0 cm) eluted in 0.1 M HCl and the desired factions assayed directly by LC−ESI-MS and/or HPLC. Products were quantified from both the intensities of the specific MH^+ ion from the LC−ESI-MS data with UV data recorded at 545 nm as well as by HPLC recorded with a diode array detector. The peak areas of the intensities of the $MH^+ = m/z$ 503 ion for α-RFA-P, the $MH^+ = m/z$ 503 ion for β-RFA-P, the $MH^+ = m/z$ 505 ion for ADPR-P, and the $MH^+ = m/z$ 425 ion for APDR were used. A typical example of a separation and analytical data obtained is shown in Figure 3. The calculated values assume that all these ions are produced from their respective molecules in proportion to the molar concentration of each compound. This was confirmed because the measured areas of the 545 nm absorbance trace corresponded with that obtained from the m/z 425 and 503 ion traces. The amount of APDR resulting from oxidative cleavage of MPT in the cell extracts was used as an internal standard. Measurement of the amount of APDR in the cell extract was accomplished using the Bratton−Marshall assay of the cell extract with a sample of 4-aminophenethyl alcohol serving as a standard. This analysis showed that the concentration of APDR was 3.0 mM in the cell extracts used. This level is very similar to the level of methanopterin (~100 mmol/mg of

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Table 1. MS/MS Data for the Azo Dye Derivatives α-RFA-P, [^{15}N]-α-RFA-P, β-RFA-P, [^{15}N]-β-RFA-P, and [$^{2}H_4$]-β-RFA-P

	observed MS/MS ions originating from MH^+ ions							
compound							$(MH^+ - HPO_3)$	$(MH^+ - H_2O)$
α-RFA-P, $MH^+ = m/z$ 503.3		170.3		187.2		333.5	423.5	
[^{15}N]-α-RFA-P, $MH^+ = m/z$ 504.1		170.3	171.2	187.2	188.2	334.5	424.2	486.5
β-RFA-P, $MH^+ = m/z$ 503.3	169.2				198.2	333.4	423.3	485.3
[^{15}N]-β-RFA-P, $MH^+ = m/z$ 504.1	169.2				198.2	337.3	424.3	486.2
[2,3,5,6-$^{2}H_4$]-β-RFA-P, $MH^+ = m/z$ 507.2	169.1				198.2	337.3	427.3	489.1

protein) known to be present in the methanogens.[21,22] Because this value is close to the measured amount of methanopterin, we must conclude that most of the methanopterin was cleaved to APDR during sample preparation. Because this cleavage is known to occur by air oxidation of 6-substituted tetrahydropterins,[23] it is likely the nitrous acid used in formation of the azo dye is responsible for the cleavage.[20]

Measurement of Incorporation into Precursors. The method consisted of forming and purifying the azo dye adducts of the arylamines present in the cell extracts as described above. The compounds eluted as a broad purple band that allowed the separation of the azo dyes from most of the other cellular constituents. Two additional compounds, 5-aminoimidazole-4-carboxamide and 5-aminoimidazole-4-carboxamide ribonucleotide, known to be present in the methanogens,[24] were consistently identified in all samples analyzed that contained cell extracts.

Three different HPLC methods were used to analyze and quantitate the amounts of these azo dyes. Chromatographic separation and analysis were performed on a Shimadzu HPLC system with a diode array and equipped with a C18 reverse phase column (Varian PursuitXRs 250 mm × 4.6 mm, 5 μm particle size, or Agilent Zorbax Eclipse XDB-C-18, 50 mm × 4.6 mm, 1.8 μm particle size). The elution profile consisted of 5 min at 96.5% buffer A and 3.5% buffer B followed by a linear gradient to 45% buffer B over 50 min at a rate of 1 mL/min.

In solvent system 1, buffer A consisted of 0.1% TFA in water and buffer B consisted of 0.1% TFA in methanol. In solvent system 2, buffer A consisted of 0.1% formic acid in water and buffer B consisted of 1% formic acid in acetonitrile. The advantage of the TFA solvent is that the azo dyes are completely protonated and purple with absorbance around 560 nm, whereas in the formic acid solvent, mixed states of ionization are observed, resulting in more complex UV−visible spectra. For LC−MS, the formic acid system was used and the samples were separated on a Zorbax column with mass spectra being recorded using a AB SCIEX Triple Quad 3200 Qtrap LC/MS/MS instrument. The following compounds of interest eluted at the indicated times from the Zorbax column: ZMP, 13.29 min; AIC, 13.66 min; α-RFA-P, 14.24 min; β-RFA-P, 15.32 min; APDR-P, 17.03 min; APDR, 17.4 min. A similar elution order was observed in solvent system 2. Identification of each of these compounds was confirmed by its retention time and absorbance spectrum compared to knowns in each of these solvent systems. Solvent system 3 was used for the separation of α-RAH and β-RAH, and the elution consisted of 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% methanol for 5 min followed by a linear gradient to 20% sodium acetate buffer and 80% methanol over 40 min at a rate of 0.5 mL/min.

Chemical Interconversion of Azo Dye Derivatives of α-RFA-P and β-RFA-P. A mixture of the azo dye derivatives of α-RFA-P and β-RFA-P was prepared as described above, and each was isolated by preparative HPLC separation using a TFA-containing solvent system. The compounds were then subjected to the different acidic conditions used for the production of the azo dyes, and each was analyzed by HPLC and LC−ESI-MS to establish the extent of interconversion and the final position of equilibrium.

Analysis and Identification of Known Azo Dyes. In trifluoroacetic acid solvent system 1, 4-aminobenzyl-containing compounds such as α-RFA-P, β-RFA-P, and 4-aminobenzyl alcohol have absorbance maxima between 560 and 561 nm. Reducing the incipient benzyl alcohol to a methylene as occurs in APDR, APDR-P, or methaniline, the oxidative cleavage product of MPT,[23] increased the absorbance maximum to 569 nm. Azo dye derivatives of 5-amino-4-imidazolecarboxamide-1-β-D-ribofuranosyl 5′-monophosphate (ZMP and AICAR) and 5-amino-4-imidazolecarboxamide (AIC), 2-aminobenzoic acid, 3-aminobenzoic acid, AB, and aniline had absorbance maxima at 552, 543, 554, 543, 546, and 554 nm, respectively. Each compound with a unique λ_{max} can be readily distinguished by its visible color. These λ_{max} absorbance values are helpful in determining the chemical nature of the compound being detected. All the azo dyes that contained polar side chains such as α-RAF-P, β-RAF-P, APDR, APDR-P, and methaniline were isolated together as a group after BioGel P-4 chromatographic purification. Eluting after these compounds were ZMP and AIC followed by the azo dyes derivatives of AB and aniline.[20]

Identification of Azo Dye Derivatives of α-RFA-P, β-RFA-P, APDR-P, and APDR. Each of these compounds was converted to its azo dye derivative and purified by Bio-Gel P-4 chromatography. The resulting samples were used as knowns to establish their chromatographic retention times, absorbance spectra, and LC−ESI-MS and ESI-MS/MS data. Each compound has its own HPLC retention time. α-RFA-P and β-RFA-P, with the same MH^+, could be distinguished on the basis of their different retention times and MS/MS data with the ions at m/z 170 and 187 being present in α-RFA-P and m/z 169 and 198 being present in β-RFA-P (Table 1). As expected, many of the observed MS/MS ions are shifted in the labeled compounds. MS/MS of APDR-P showed the following product ions for $MH^+ = m/z$ 505 at 169, 198, 407, 425 $(MH^+ - HPO_3)$, and 487 $(MH^+ - H_2O)$ and for $MH^+ = m/z$ 506 for the ^{15}N-labeled material at 146, 169, 198, 408, 426 $(MH^+ - HPO_3)$, and 488 $(MH^+ - H_2O)$. Even with the observed labeling, it has not yet been possible to assign structures to any of the observed MS/MS ions.

Measurement of Incorporation of ^{15}N into Soluble Amino Acids. After incubation of cell extracts with [^{15}N]aspartate, the proteins were precipitated with TCA and the soluble amino acids recovered and analyzed by GC−MS as their trifluoroacetyl methyl ester derivatives.[25]

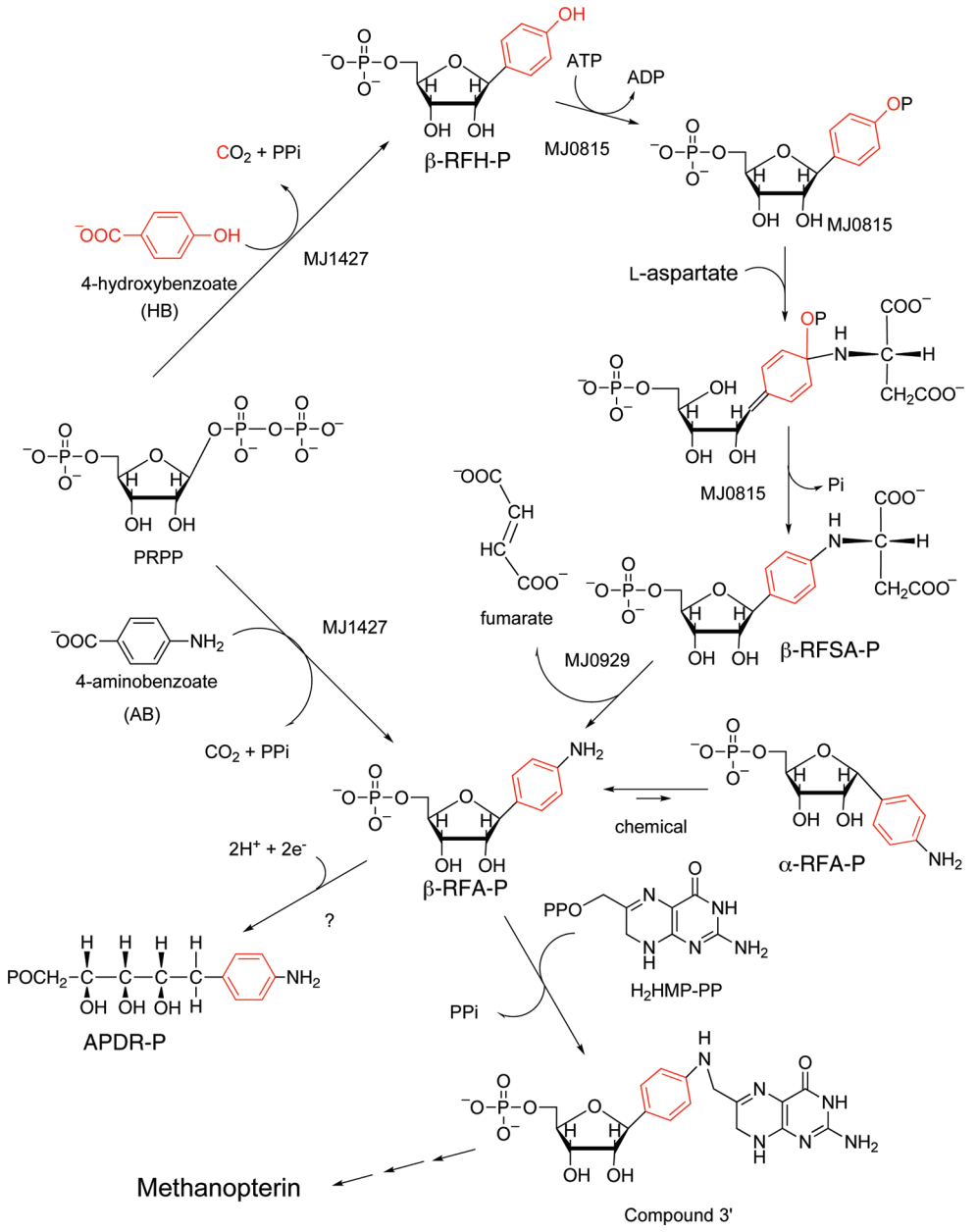

Figure 4. Pathways to β-RFA-P and Compound 3′ from either 4-aminobenzoic acid (AB) or 4-hydroxybenzoic acid (HB).

RESULTS

Defining the Pathway from HB to β-RFA-P. The first step in this three-step transformation occurs by the reaction of 4-hydroxybenzoic acid with PRPP to form β-RAH-P. The β-RAH-P then condenses in the presence of ATP with L-aspartate to form β-RFSA-P. Elimination of fumarate from β-RFSA-P produces β-RFA-P, a known precursor to MPT. The proposed steps in this reaction are shown in Figure 4. The details of how this was delineated are presented below.

Testing the Chemical Conversion of 4-Hydroxybenzyl Alcohol to an Aniline. The chemical conversion of a phenol to an aniline is not a very facile organic reaction. If ammonia is the nucleophile, this is an example of aromatic substitution by the $S_{RN}1$ mechanism[26] and requires reaction temperatures of >450 °C. Conversion of the phenol to a phosphate ester, a much better leaving group, can greatly facilitate the reaction.[27] To test if an aniline could be produced from a phenol or an aryl bromide, I heated both HB and bromobenzoic acid with concentrated ammonia at 110 °C for 24 h and analyzed the production of AB by GC−MS of their methyl esters. No detectable AB was produced in either reaction.

Two additional experiments were conducted to establish if it was chemically possible to convert a phenol to an aniline and to give mechanistic insight into how this may occur. In these experiments, the phenol used was 4-hydroxybenzyl alcohol because it was a closer structural analogue to the phenol in β-RAH-P than HB. The first experiment addressed the exchange of the oxygens of 4-hydroxybenzyl alcohol with ^{18}O-labeled water, and the second tested for the replacement of the hydroxyl groups with aqueous ammonia. Analysis of the product from the first experiment by GC−MS showed a single peak for the

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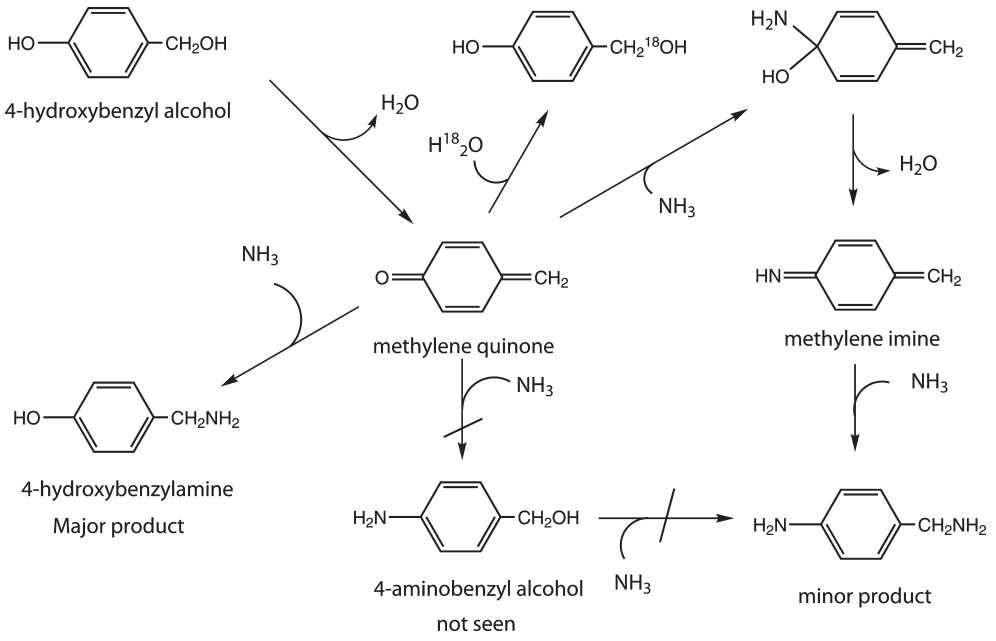

Figure 5. Proposed chemical mechanism for the replacement of the hydroxyl groups of 4-hydroxylbenzyl alcohol with an amine.

ditrifluoroacetyl derivative of 4-hydroxybenzyl alcohol showing a molecular ion at m/z 316 containing 36% ^{18}O. The base peak in the spectrum at m/z 203 represented the benzyl cation radical resulting from the loss of $CF_3COO^{\bullet}$ from the molecular ion. This benzyl ion contained no ^{18}O enrichment, indicating that only the oxygen of the benzyl alcohol had exchanged with the water. When the 4-hydroxybenzyl alcohol was heated with 12 M aqueous ammonia, the generated amine-containing compounds included 4-hydroxybenzylamine and 4-aminobenzylamine, both being identified by GC−MS as their ditrifluoroacetyl derivatives. The identification of these compounds confirmed that both the phenol hydroxyl group and the benzyl alcohol can be chemically replaced with ammonia to generate an amino group. The expected reactions leading to these compounds are summarized in Figure 5. Key to the proposed mechanism of these reactions is the formation of the methylene quinone by the elimination of water from 4-hydroxybenzyl alcohol. Addition of ammonia produces 4-hydroxybenzylamine as the observed major product. Conversion of the methylene quinone to a methylene imine followed by the addition of ammonia would produce 4-aminobenzylamine.

Enzymatic Formation and Characterization of *β*-RAH-P. Incubation of HB and PRPP with recombinant MJ1427 protein (70 °C for 0.5 h) readily produced β-RAH-P. HPLC analysis showed two fluorescent peaks, a minor peak at 7.5 min for α-RAH-P and a major peak at 8.60 min for β-RAH-P. The ratio of the minor peak to the major peak, based on the fluorescence intensities of the peak areas, was 0.032. Removal of the phosphate with alkaline phosphatase changed the retention times of the two peaks to 15.17 and 16.29 min, respectively. The ratio of the minor to the major peak representing α-RAH and β-RAH, based on the fluorescence intensities of the peak areas, was 0.031. LC−ESI-MS analysis of the α-RFH-P and β-RFH-P peaks showed $(M - H)^-$ ions at m/z 305.3, and both of the α-RFH and β-RFH peaks showed an MH^+ ion at m/z 227.0 consistent with the proposed structures. The major peak also showed an $(M_2 - H)^-$ ion at m/z 611.2 and an $(M_2 - H + Na)^-$ ion at m/z 633.2. Using [2,3,5,6-$^2H_4$]HB as a substrate increased the observed mass of all of these products by m/z 4, confirming their origin from HB. The yield of β-RFH-P in the incubation was 40% based of the amount of fluorescence observed by HPLC analysis of the sample and the amount of HB in the incubation. No synthesis of α-RFH-P and β-RFH-P was observed in the absence of enzyme.

Measurement of *β*-RAH-P Synthesis in Cell Extracts. Cell extracts of *M. jannaschii* incubated with HB and PRPP and analyzed using HPLC showed α-RFH-P and β-RFH-P with the same retention times and ratios of α- and β-isomers that were observed for the sample generated with the recombinant enzyme. The β-isomer is assumed to be the more stable and major isomer due to steric hindrance between the C-2 hydroxyl and the phenyl ring in the α-isomer. LC−ESI-MS confirmed the identity of the peaks as described above. The yield of β-RAH-P was 10% based of the amount of fluorescence observed and the amount of HB used in the incubation. These peaks were detected in the incubation mixture only after incubation with these substrates.

Chemical Interconversion of Azo Dye Derivatives of α-RFA-P and *β*-RFA-P. The ratio of the α-RFA-P and β-RFA-P azo dye derivatives prepared from an enzymatically generated sample of β-RFA-P was 0.11. In the preparation of these azo dye derivatives, the final acid hydrolysis step was eliminated because the side chain of the methanopterin did not need to be removed. This ratio remained stable for samples held at −20 °C for periods of up to 4 months, the longest tested. The low ratio indicates that β-RFA-P is the enzymatic product and that the azo dye derivative of this compound can equilibrate to the azo dye derivative of α-RFA-P under the acidic conditions used to prepare the samples. To test this idea, a chromatographically pure sample of the azo dye derivative of β-RFA-P was heated with different concentrations of hydrochloric acid and the ratios of α-RFA-P and β-RFA-P measured by HPLC. Heating a pure sample of the β-RFA-P derivative (ratio of α-RFA-P to β-RFA-P of 0.024) in 0.6 M HCl for 5 min at 100 °C produced a sample with a ratio of α-RFA-P to β-RFA-P of 0.13. Repeating the experiment with

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Table 2. Incorporation of Labeled Precursors into α-RFA-P, β-RFA-P, and APDR Measured from LC−ESI-MS Data[a]

experiment[b]	α-RFA-P, $MH^+ = m/z$ 503	β-RFA-P, $MH^+ = m/z$ 503	APDR-P, $MH^+ = m/z$ 505
(1) control	0.0%	0.0%	nd[d]
	∼2 μM	∼6 μM	
(2) [2,3,5,6-$^2H_4$]HB and PRPP	83.2%	79.6%	nd[d]
	12 μM	30 μM	–
(3) [^{15}N]Asp, HB, and PRPP	39.6%	36.5%	0
	19 μM	60 μM	0.3 μM
(4) [^{15}N]Asp, HB, PRPP, and ATP	33%	32%	29%
	8.7 μM	75 μM	6.4 μM
(5) [^{15}N]Asp, HB, PRPP, and GTP	26%	29%	14%
	4.8 μM	18 μM	6.4 μM
(6) [^{15}N]Asp, HB, PRPP, and GTP	28%	31%	10%
	4.0 μM	19 μM	7 μM
(7) H_2, [^{15}N]Asp, [2,3,5,6-$^2H_4$]HB, PRPP, and ATP	same as β-RFA-P	34.4% $^2H_4$, 26.3% $^2H_4$ and ^{15}N	10%
	6.6 μM	54 μM	2 μM
(8) [2,3,5,6-$^2H_4$]-α-RFH-P and [2,3,5,6-$^2H_4$]-β-RFH-P at 220 μM,[c] ATP, and L-aspartate	91%	91%	∼66%
	35.1 μM	173 μM	12 μM

[a] The percentages are the percentages of the total molecules that were derived from the incubated labeled precursor. The concentrations are the concentrations of the indicated products measured in the incubation mixtures after incubation. The concentrations were calculated from measured MH^+ ion intensities and confirmed from the UV absorbance data. The MH^+ for the APDR was used as the internal standard. [b] Each component was present at a concentration of 10 mM in the cell extract in experiments 2 and 3 and 9.2 mM in experiments 4−7. Incubations were conducted for 15 min at 70 °C. [c] The concentration of these compounds in the incubation mixture was based on its fluorescence using phenol as the standard. [d] Not detected.

6 M HCl increased this ratio to 0.29. Thus, the different values for the ratios of α-RFA-P to β-RFA-P reported in Table 2, ranging from 0.12 to 0.32, indicate different extents of chemical conversion of β-RFA-P to α-RFA-P during the preparation of the samples. Together, these data indicate that β-RFA-P is the biologically important isomer. Data for both compounds are listed in Table 2 because they produce two independently derived sets of incorporation data for each incubation.

Incubation of *M. jannaschii* Cell Extracts with Precursors and LC−ESI-MS Measurement of Concentrations and Incorporation of Isotopes into Products. Incubation of cell extracts with [2,3,5,6-$^2H_4$]HB and PRPP resulted in the incorporation of the four deuterium atoms of HB into both α-RFA-P and β-RFA-P to an extent of ∼80% (Table 2, experiment 2). This experiment also showed an increase in the total amount of α-RFA-P and β-RFA-P over a control incubated without the addition of [2,3,5,6-$^2H_4$]HB and PRPP (Table 2, experiment 1). This result demonstrated not only that the cell extracts readily incorporate HB into β-RFA-P but also that cell extracts were able to catalyze the replacement of the phenolic hydroxyl group of HB with an amine. To test for the source of the nitrogen used for the formation of the amine, incubations were conducted with 10 mM [^{15}N]-L-aspartic acid, 10 mM [*amide*-^{15}N]glutamine, and 100 mM [^{15}N]ammonium chloride each incubated along with HB and PRPP. ^{15}N was incorporated from only [^{15}N]-L-aspartic acid, where ∼40% of the α-RFA-P and β-RFA-P molecules were found to contain ^{15}N (Table 2, experiment 3). Also, a further increase in the total amount of α-RFA-P and β-RFA-P produced in the extract was observed. Incubation of extracts with PRPP and ammonium chloride or glutamine followed by HPLC analysis using solvent system 1 showed no increase in the amount of α-RFA-P or β-RFA-P, indicating that these compounds were not produced from RFH-P using ammonia or glutamine as the nitrogen source. Because the extent of labeling was much lower that expected on the basis of the 98% enrichment in the [^{15}N]-L-aspartic acid used in the experiment, a reason for this reduction in the amount of label incorporated was sought. This reduction could occur either by dilution with unlabeled aspartate present in the cell extract or through transamination reactions with α-keto acids and/or amino acids present in the cell extracts. Extracts of other methanogens generally contain ∼0.2 mM α-ketoglutarate,[28] and *M. jannaschii* contains ∼0.4 M α-glutamate and ∼0.5 M β-glutamate that serve as osomolites in this as well as other methanogens.[29] Thus, the soluble free amino acids were recovered from an incubation mixture, and the ^{15}N abundances in the individual amino acids were measured by GC−MS analysis of their trifluoroacetyl methyl ester derivatives. The major amino acids found were aspartate, α-glutamate, and β-glutamate. Aspartate was found to contain 44% ^{15}N and α-glutamate 38% ^{15}N. β-Glutamate, alanine, valine, proline, Leu/Ilu, and proline contained no ^{15}N enrichment (<2%). These results showed that the aspartate nitrogen had undergone extensive mixing with the glutamate via transamination with α-ketoglutarate, thus explaining the observed reduction of ^{15}N in the aspartate. Thus, the L-aspartate nitrogen was likely the sole source of the amino group of β-RFA-P.

To test if a nucleoside triphosphate was involved in the reaction, cell extracts were incubated with [^{15}N]-L-aspartic acid, PRPP, HB, and either ATP or GTP. In both experiments, ∼30% ^{15}N was incorporated into both α-RFA-P and β-RFA-P (Table 2, experiments 4 and 5). The amount of both of the isomers, however, increased in the ATP over the GTP incubation, with the amount in the GTP incubation being approximately 2−3 times that seen in the control. Although the levels of APDR-P remained approximately the same in both experiments, a 29% incorporation of ^{15}N into APDR-P was observed in the ATP incubation and 14% in the GTP incubation. Experiment 6 was a repeat of experiment 5.

A cell extract was incubated with [^{15}N]-L-aspartic acid, [2,3,5,6-$^2H_4$]HB, ATP, and PRPP under hydrogen (Table 2,

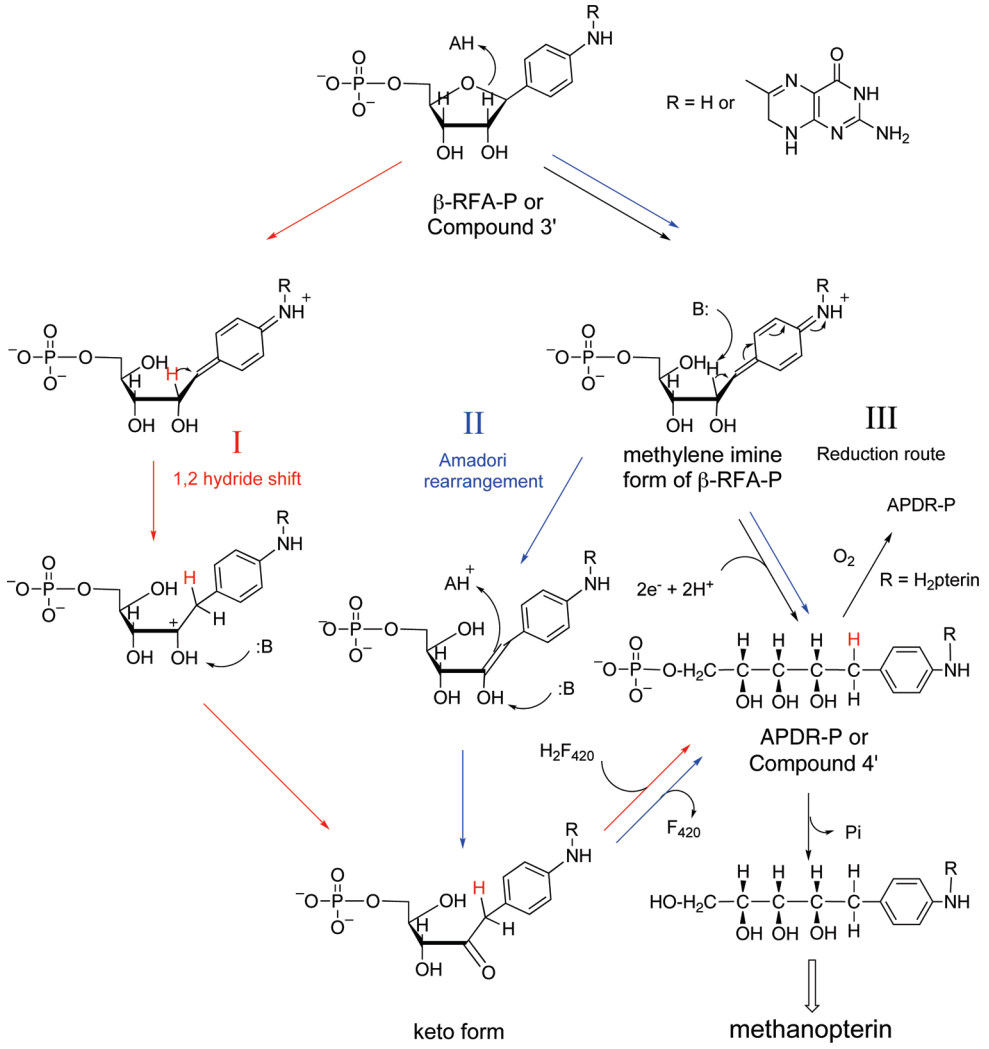

Figure 6. Three possible routes to APDR-P from β-RFA-P (R = H) or Compound 3′ (R = pterin). (I) Isomerization to the methylene imine derived from β-RFA-P or Compound 3′ (R = H2pterin), followed by a 1,2 hydride shift and reduction of the resulting ketone. (II) Amadori rearrangement of methylene imine from β-RFA-P or Compound 3′. (III) Direct reduction of methylene imine from β-RFA-P. The keto form is reduced to Compound 4′ that could undergo oxidative cleavage to APDR-P. The conversion of APDR-P to methanopterin has not been demonstrated.

experiment 7). Here, 34.4% of the total RFA-P molecules were derived from the labeled HB, and 26.3% of them had incorporated a single ^{15}N from the L-aspartate. From the amounts of the molecules that had increased by m/z 4−5, the nitrogen incorporated would have had an ^{15}N enrichment of 43.3%.

Finally, a mixture of [aromatic-$^{2}H_4$]-α-RFH-P and β-RFH-P was prepared and incubated along with ATP and aspartate at a total concentration of 220 μM (Table 2, experiment 8). After incubation, the recovered azo dyes of α-RFA-P and β-RFA-P were each found to be labeled to an extent of 91% with four deuterium atoms. The sum of the α-RFA-P and β-RFA-P produced was 210 μM, indicating that 95% of the α-RFH-P and β-RFH-P mixture had been converted into product. Surprisingly, the APDR-P level increased to 12 μM, and it contained 66% $^{2}H_4$.

APDR-P was detected in many of these experiments on the basis of the presence of its MH^+ = m/z 505 ion at the same retention time as a known sample of this material. Significant enrichment of ∼10−30% ^{15}N was seen in the ADPR-P in experiments 5−7 (Table 2). The level of label was less than that of the α-RFA-P or β-RFA-P, indicating that APDR-P was not derived solely from the β-RFA-P produced enzymatically in the cell extract. The dilution of label occurs from APDR-P present in or generated in the extract. The unlabeled material can arise from oxidative cleavage of the phosphorylated intermediate as shown in Figure 6.[23]

To establish that β-RFSA-P was an intermediate in biosynthesis, we prepared this molecule, incubated it with a cell extract, and measured the increase in the amount of α-RFA-P and β-RFA-P in the cell extract by LC−ESI-MS of their azo dye derivatives. The first incubation was conducted with a crude preparation of β-RFSA-P, and a large increase in the amount of α-RFA-P and β-RFA-P (Table 3, experiments 2 and 3) was observed. Incubation in a second experiment with a chromatographically pure sample of β-RFSA-P (82 μM) caused the generation of ∼45 μM α-RFA-P and β-RFA-P, consistent with half of the sample being converted into product. This is consistent with only one of the stereoisomers serving as the precursor. The other product of the reaction was confirmed to be fumarate by TLC analysis.

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Table 3. Conversion of DL-β-RFSA-P to β-RFA-P by Cell Extracts[a]

experiment	[α-RFA-P] (μM)	[β-RFA-P] (μM)	[APDR-P] (μM)
(1) control	2.7	6	0.1
(2) β-RFSA-P incubated with ~300 μM crude material	21	81	0.1
(3) 82 μM β-RFSA-P	11	42	0.1

[a] The concentrations reported were calculated from the intensities of the $MH^+ = m/z$ 503 ion for α-RFA-P, the $MH^+ = m/z$ 503 ion for β-RFA-P, the $MH^+ = m/z$ 505 ion for APDR-P, and the $MH^+ = m/z$ 425 ion for APDR. The values reported assume that all these ions are produced from their respective molecules in proportion to the number of moles of each compound and that the measured concentration of APDR in the cell extract is 3.0 mM.

DISCUSSION

The results presented here are consistent with the pathways shown in Figure 4 as being involved in the biosynthesis of β-RFA-P. Earlier in our work on the biosynthesis of methanopterin, we demonstrated that externally fed AB could serve as a precursor to the aminophenyl moiety of APDR,[6] and from this work, it was expected that this would also hold true for its in vivo biosynthesis. This was supported by the observation that cell extracts readily incorporated AB into β-RFA-P, a precursor of APDR.[15,30] The gene encoding the enzyme catalyzing this incorporation was identified as MJ1427 in *M. jannaschii*,[31] and the recombinant enzyme was shown to catalyze the proposed reaction.[31] The mechanism of the enzyme was studied both before[30] and after the gene had been identified.[32] One troubling aspect of the involvement of AB in ADPR biosynthesis was that no genes annotated for the biosynthesis of AB[9,10] are present in the genomes of the methanogens. From detailed biochemical and genetic studies of the archaeal pathway to aromatic amino acid and AB biosynthesis in the archaeon *M. maripaludis*, it was concluded that AB was derived from an early intermediate in aromatic biosynthesis and not from chorismate.[13] An alternate pathway based on this idea (Figure 3) was proposed and has now been shown not to be functional (unpublished data). One idea not considered in this earlier work was the possibility that chorismate-derived 4-hydroxybenzoic acid (HB) normally serves as a precursor to APDR but that HB could also be derived from DHQ. The conversion of HB to β-RFA-P resolves this problem because AB is not required to produce β-RFA-P. AB can, however, be converted into β-RFA-P by growing methanogens when it is present in the growth medium. Interestingly, this incorporation occurs with the same enzyme that incorporates HB and an enzyme that is inhibited by HB.[30]

Reaction Mechanism for β-RFSA-P Formation. The available data indicate the incorporation of the aspartate nitrogen into the β-RFA-P proceeds as shown in Figure 4. The mechanism of this reaction is similar to that recently observed by Balskus and Walsh in the biosynthesis of the mycosporine amino acids that use a ATP-grasp enzyme[33] to couple 4-deoxygadusol with different amino acids.[34] In our scheme, the first step in a series of reactions is the phosphorylation of the phenol of β-RFH-P by ATP. Subsequent nucleophilic attack by the aspartate nitrogen with expulsion of the protonated furan oxygen of the ribose produces a tetrahedral intermediate similar to that proposed for ATP-grasp reactions. In our reaction, however, this tetrahedral intermediate has delocalized the negative change not on the oxygen of a carboxyl group but to the C-4 hydroxyl group leaving from C-1 of the ribose of β-RFH-P. In other words, the opening of the ribose ring to form the carbocation provides an electron sink to drive the addition of the aspartate nitrogen to the phosphorylated phenol. Addition of the C-4 hydroxyl group back to the ribose C-1 atom with expulsion of phosphate leads to the formation of β-RFSA-P. The fact that the nitrogen comes from L-aspartate by this route strongly suggests the stereochemistry of the aspartate in the β-RFSA-P is (*S*), the same as that of the aspartate in adenylosuccinate.

Enzymes in *M. jannaschii* annotated to be ATP-grasp enzymes that presently have no confirmed function include proteins derived from the MJ0815 and MJ0776 genes. MJ0815 is linked to many of the genes involved in MPT biosynthesis and likely encodes the enzyme catalyzing this reaction. We thus propose that the ATP phosphorylates β-RFH-P and the resulting phosphorylated phenol reacts with aspartate to form β-RFSA-P catalyzed by one of these enzymes. β-RFSA-P, after elimination of fumarate, then forms β-RFA-P. The enzyme that catalyzes the loss of fumarate is the adenylosuccinate lyase, the product of the MJ0929 gene (R. H. White, unpublished observations), an enzyme that is already known to function not only with adenylosuccinate but also with 5-aminoimidazole-*N*-succinylocarboxamide ribotide (SAICAR), another intermediate in purine biosynthesis.[35]

Formation of APDR-P. Early work demonstrated that β-RFA-P condensed with 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to form 7,8-dihydropterin-6-yl-4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (Compound 3′) that was then converted into 7,8-H_2pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5″-phosphate (Compound 4′) in a reaction stimulated by the addition of FMN and F_{420}.[15] After dephosphorylation, Compound 4′ is then converted into methanopterin (Figure 6). The discovery here that APDR-P was present in the incubation mixtures and was labeled by the different precursors was surprising. This molecule could be produced by the oxidative cleavage of Compound 4′ (Figure 6) and would be expected to be generated to a small extent in a cell extract. Labeled Compound 4′ should not have been generated in the incubation mixtures because no 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (H_2HMP-PP) (Figure 4) was added to the incubation mixtures. However, because the extent of labeling was always less than that seen in the β-RFA-P and the amounts were small, it is possible that the enzyme(s) responsible for the conversion of Compound 3′ to Compound 4′ in Figure 6 could also function to convert β-RFA-P to APDR-P by one of the pathways shown in Figure 6.

Three possible routes for this conversion can be envisaged. The first of these routes would be the direct reversible two-electron reduction of the methylene imine form of β-RFA-P as shown in reaction III in Figure 6. The formation of this methylene imine would be readily generated by an intramolecular elimination of an alcohol from the 4-aminobenzyl ether moiety present within β-RFA-P. Evidence of the formation of such an intermediate is supported by the facile acid-catalyzed equilibration of azo dye derivatives of α-RFA-P and β-RFA-P and the observed exchange of the oxygen of the 4-aminobenzyl alcohol with $H_2{}^{18}O$. This equilibration explains why we always see the mixture of α-RFA-P and β-RFA-P in the experiments reported here because the samples are exposed to acid during the formation and purification of the azo dyes derivatives of these molecules. As seen from the data in Table 2, the ratios of the isomers are different

from sample to sample because not all the samples have come to equilibrium. This process proceeding through a methylene quinone (Figure 5) can also explain why a mixture of α-RFH-P and β-RFH-P is observed. This process is expected to be enhanced by the addition of electron-donating groups on the aromatic ring as seen in formation of the carbocation in substituted triphenylmethane dyes.[36,37] Others have prepared molecules either identical or similar to β-RFA-P, but their anomerization has not been previously reported.[38−40] This may have been missed because the samples were likely never subjected to an acid treatment.

In route II, intramolecular loss of water would proceed, which occurs in the *N*-(5′-phospho-D-ribosylformimino)-5-amino-1-(5″-phosphoribosyl)-4-imidazole carboxamide isomerase (HisA)-catalyzed Amadori rearrangement of the phosphoribosylformimino-5-aminoimidazole carboxamide ribotide,[41] an intermediate in histidine biosynthesis.[42] A very likely candidate for the enzyme to catalyze the Amadori rearrangement (Figure 6, route II) is the product of the MJ0703 gene. This protein is homologous to HisA (MJ1532) and is linked to genes known to be involved in methanopterin biosynthesis. The sequence of reactions catalyzed with HisA is the same as that required for the opening and rearrangement of the ribose ring of β-RFA-P as shown in Figure 6. Route III would be via a pinacol rearrangement involving a 1,2 hydride shift. In route II or III, the resulting keto compound would then be reduced to the alcohol by an F_{420}-dependent dehydrogenase.[15]

M. jannaschii has three enzymes homologous to MJ0703 (HisA2). These include HisA (MJ1532), HisF (MJ0411), and TrpF (MJ0451), each of which conducts the same type of isomerase reactions but uses different substrates. The *hisA* homologous to these enzymes from *Mycobacterium tuberculosis* and *Streptomyces coelicolor* complemented both the *hisA* and *trpF* mutants in *E. coli*,[43] indicating that the HisA protein can catalyze the same type of isomerase reaction with different substrates. The wide assortment of reactions catalyzed by these enzymes indicated that widely different groups can be attached to a ribose 5-phosphate moiety and still undergo the same type of reaction reported here. We propose that one of these isomerases has also evolved into HisA2 that is involved in the biosynthesis of the APDR.

AUTHOR INFORMATION

Corresponding Author

*Phone: (540) 231-6605. Fax: (540) 231-9070. E-mail: rhwhite@vt.edu.

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ABBREVIATIONS

HB, 4-hydroxybenzoic acid; AB, 4-aminobenzoic acid; PRPP, phosphoribosyl pyrophosphate; β-RFH-P, 4-(β-D-ribofuranosyl)-hydroxybenzene 5′-phosphate; β-RFSA-P, 4-(β-D-ribofuranosyl)-*N*-succinylaminobenzene 5′-phosphate; β-RFA-P, 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; APDR, 1-(4-aminophenyl)-1-deoxy-D-ribitol; Compound 3′, 7,8-dihydropterin-6-methyl-4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; Compound 4′, 7,8-dihydropterin-6-methyl-1′-(4-aminophenyl)-1-deoxy-D-ribitol 5-phosphate; ZMP or AICAR, 5-amino-4-imidazolecarboxamide-1-β-D-ribofuranosyl 5′-monophosphate; AIC, 5-amino-4-imidazolecarboxamide; DTT, dithiothreitol; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid; PP_i, pyrophosphate; LC−ESI-MS, liquid chromatography with electrospray-ionization mass spectroscopy; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; TLC, thin layer chromatography.

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