Purification, Overproduction, and Partial Characterization of β-RFAP Synthase, a Key Enzyme in the Methanopterin Biosynthesis Pathway†

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Methanopterin is a folate analog involved in the C₁ metabolism of methanogenic archaea, sulfate-reducing archaea, and methylotrophic bacteria. Although a pathway for methanopterin biosynthesis has been described in methanogens, little is known about the enzymes and genes involved in the biosynthetic pathway. The enzyme β-ribofuranosylaminobenzene 5′-phosphate synthase (β-RFAP synthase) catalyzes the first unique step to be identified in the pathway of methanopterin biosynthesis, namely, the condensation of p-aminobenzoic acid with phosphoribosylpyrophosphate to form β-RFAP, CO₂, and inorganic pyrophosphate. The enzyme catalyzing this reaction has not been purified to homogeneity, and the gene encoding β-RFAP synthase has not yet been identified. In the present work, we report on the purification to homogeneity of β-RFAP synthase. The enzyme was purified from the methane-producing archaeon Methanosarcina thermophila, and the N-terminal sequence of the protein was used to identify corresponding genes from several archaea, including the methanogen Methanococcus jannaschii and the sulfate-reducing archaeon Archaeoglobus fulgidus. The putative β-RFAP synthase gene from A. fulgidus was expressed in Escherichia coli, and the enzymatic activity of the recombinant gene product was verified. A BLAST search using the deduced amino acid sequence of the β-RFAP synthase gene identified homologs in additional archaea and in a gene cluster required for C₁ metabolism by the bacterium Methyllobacterium extorquens. The identification of a gene encoding a potential β-RFAP synthase in M. extorquens is the first report of a putative methanopterin biosynthetic gene found in the Bacteria and provides evidence that the pathways of methanopterin biosynthesis in Bacteria and Archaea are similar.

Methanopterin is a folate analog involved in the C₁ metabolism of methanogenic archaea, sulfate-reducing archaea, and methylotrophic bacteria (5, 7, 19, 31, 39). This coenzyme is used during the production of methane by methanogenic archaea and during the oxidation of growth substrates by sulfur-metabolizing archaea and certain methylotrophic bacteria. The recent discovery of methanopterin in the bacterium Methyllobacterium extorquens (5) was surprising because methanopterin had been thought to be exclusive to Archaea. This discovery has raised interesting questions about the evolutionary relationships between archaea and bacteria that use methanopterin. While M. extorquens is the only bacterium in which methanopterin itself has been detected, methanopterin-dependent enzyme activity has been observed in a number of other methylotrophic bacteria (34).

One approach to investigating the evolutionary relationships among organisms that use methanopterin is to compare the enzymes and genes involved in methanopterin biosynthesis. At present, the pathway of methanopterin biosynthesis in the Bacteria is unknown. However, a methanopterin biosynthetic pathway has been described for the methane-producing archaeon Methanosarcina thermophila (38, 39). To date, the genes encoding only 2 of the 18 proposed enzymes of the biosynthetic pathway have been identified. MIJ425 encodes an enzyme that converts alpha-ketoglutarate to hydroxylglutaric acid, a component of the side chain of methanopterin (10), and MIJ0301 codes for an enzyme that joins the pterin ring system to a unique p-aminobenzoic acid (pAB) derivative called β-ribofuranosylaminobenzene 5′-phosphate (β-RFAP) (41). Homologs of these two genes have not yet been identified in methylotrophic bacteria.

One of the key enzymes which distinguishes the pathway of methanopterin biosynthesis from that of folate biosynthesis is β-RFAP synthase (17, 38, 39). This enzyme catalyzes the condensation of pAB with 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) to produce β-RFAP, CO₂, and inorganic pyrophosphate (PP) (38), as shown in Fig. 1. β-RFAP synthase is unusual among phosphoribosyltransferases in that it is one of only two phosphoribosyltransferases known to catalyze the formation of a C-glycoside rather than an N-glycoside product with PRPP as the ribosyl donor. It is also one of only two phosphoribosyltransferases known to catalyze the decarboxylation of one of its substrates, pAB (4, 22). The decarboxylation of pAB by β-RFAP synthase leads to the defining chemical difference between folate and methanopterin, namely, the absence in methanopterin of the carboxyl group of pAB. This structural difference contributes significantly to the distinct functional differences observed between methanopterin and folate (17). Thus, it is anticipated that all organisms which synthesize methanopterin by the archaeal pathway will also contain a gene for β-RFAP synthase.

In a previous study, the mechanism of β-RFAP synthase was investigated by using partially purified enzyme preparations (22). However, the enzyme had not yet been purified to homogeneity and the gene encoding β-RFAP synthase had not been identified. In this work, we purified to homogeneity the β-RFAP synthase from M. thermophila, overexpressed the corresponding gene from the archaeon Archaeoglobus fulgidus,
and identified β-RFAP synthase homologs in several additional archaea and in the bacterium *M. extorquens*. This is the first report of a putative methanopterin biosynthesis gene found outside the *Archaeaea* domain.

**MATERIALS AND METHODS**

**Growth of *M. thermophila* strain TM-1.** *M. thermophila* cells were grown anaerobically on acetate at 50°C in 10-liter batch cultures as described by Sowers et al. (28). A starting culture of *M. thermophila* was kindly provided by J. G. Ferry. A pH auxostat and acetic acid were used to maintain a pH of 6.8 and replenish the carbon and energy source. The medium contained the following per 10 liters of distilled water: 12.6 g of NH₄Cl, 11.3 g of K₂HPO₄, 9.0 g of KH₂PO₄, 5.0 g of yeast extract (Difco, Detroit, Mich.), 5.0 g of tryptone (Difco), 136 g of sodium acetate, 0.0137 g of Fe(II)(NH₄)₂(SO₄)₂, 0.048 g of NiCl₂, 377 ml of mineral II solution (described below), 20 ml of vitamin solution (described below), 100 ml of Wolfe's mineral solution (described below), 10 ml of 0.1% resazurin, and 50 μl of Antifoam 289 (Sigma Chemical Corp., St. Louis, Mo.). After autoclaving the medium and cooling to 50°C with nitrogen purging, 10 ml of 25% (wt/vol) cysteine-HCl in water and 10 ml of 29% (wt/vol) Na₂S in water were added. The mineral II solution contained the following per liter: 6.0 g of KH₂PO₄, 4.8 g of NH₄Cl, 12.0 g of NaCl, 2.4 g of MgSO₄·7H₂O, and 1.6 g of CaCl₂·2H₂O. The vitamin solution contained the following per liter: 0.0020 g of thiamine, 0.0020 g of folic acid, 0.0100 g of pyridoxine-HCl, 0.0050 g of riboflavin, 0.0050 g of nicotinic acid, 0.0050 g of pantothenic acid, 0.0010 g of vitamin B₁₂, 0.0050 g of p-aminobenzoic acid, and 0.0050 g of lipidic acid. Wolfe's mineral solution contained the following per liter: 1.5 g of nitrilotriacetic acid, 3 g of MgSO₄·7H₂O, 0.050 g of MnSO₄·H₂O, 1 g of NaCl, 0.10 g of FeSO₄·7H₂O, 0.10 g of CoSO₄ or CoCl₂, 0.10 g of CuCl₂, 0.08 g of ZnCl₂, 0.01 g of CuSO₄·5H₂O, 0.01 g of AlK(SO₄)₂, 0.01 g of H₃BO₃, 0.025 g of sodium molybdate, and 0.024 g of NiCl₂·6H₂O.

For the first 10-liter fermentor, the medium was inoculated with a 500-ml batch culture of *M. thermophila* grown on acetate. Prior to harvesting the cells, 1 liter of the fully grown culture was used to inoculate 10 liters of fresh medium. The cells were harvested anaerobically with a Sharples model T-1R (Alfa Laval Grass Lake, Mich.) containing a gas phase of 2% hydrogen and 98% nitrogen. The cell suspension was loaded anaerobically into a French pressure cell (Scientific Instruments, Inc., Rochester, N.Y.). The cells were broken at 20,000 lb/in² and captured in a 160-ml vial that was sealed in the anaerobic chamber. The broken cells were poured into two 40-ml polycarbonate Oak Ridge tubes (Fisher Scientific, Swanson, Ga.) in the anaerobic chamber, and 10 ml of breakage buffer was added to each tube. The mixture was centrifuged at 27,000 × g for 25 min at 4°C. The supernatant was regarded as the cell extract.

**Purification of β-RFAP synthase from *M. thermophila*.** All purification steps were performed at ambient temperature. Only Q-Sepharose anion exchange chromatography (Amersham Pharmacia Biotech, Piscataway, N.J.) was performed in the anaerobic chamber. Cell extract (36 ml) was loaded onto a 200-ml Q-Sepharose column (3.2 by 25 cm) equilibrated with buffer A (50 mM TES [pH 6.8], 10 mM MgCl₂, 5% [vol/vol] glycerol, 2 mM DTT). The column was then washed with buffer A, and a 600-ml linear gradient from 0 to 600 mM KCl in buffer A was applied to the column. β-RFAP synthase activity in each fraction was assayed as described below. The highest specific activity was detected in fractions eluting between 400 and 450 mM KCl.

Active Q-Sepharose fractions were combined and loaded onto a ceramic hydroxyapatite column (1 by 16 cm; Bio-Rad, Hercules, Calif.) equilibrated with 50 mM TES (pH 6.8) and 2 mM DTT. The column was washed with equilibration buffer, and the proteins were eluted with 19 mM NaH₂PO₄, 27 mM NaHPO₄, and 300 mM Na₂HPO₄ in the buffer mentioned above. β-RFAP synthase activity was detected in fractions eluting at 27 mM NaH₂PO₄.

The most active hydroxyapatite fractions were combined, concentrated in a Centricon 20 Plus (Millipore, Bedford, Mass.) ultrafiltration device with a molecular mass cutoff of 30 kDa, and diluted to 2 ml with 850 mM (NH₄)₂SO₄, 50 mM TES (pH 6.8), 10 mM MgCl₂, and 2 mM DTT. The sample was then loaded onto a Phenyl Sepharose 26/10 column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The column was washed with 20 ml of buffer, followed by a 400-ml linear gradient from 500 to 0 mM (NH₄)₂SO₄. The most active fractions eluted between 400 and 300 mM (NH₄)₂SO₄.

Active fractions from the Phenyl Sepharose column were concentrated as above; diluted to 2 ml with 50 mM TES (pH 6.8), 10 mM MgCl₂, and 2 mM DTT; and loaded onto a Mono Q 5/5 anion exchange column (Amersham Pharmacia Biotech). The column was washed with 5 ml of 50 mM KCl in the above-mentioned buffer, followed by a 20-ml linear gradient from 50 to 300 mM KCl. Active fractions eluting between 150 and 175 mM KCl were concentrated by a Centricon 50 microconcentrator.

The concentrated sample was applied to a Superdex 75 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM TES (pH 6.8), 10 mM MgCl₂, and 2 mM DTT; and loaded onto a Mono Q 5/5 anion exchange column (Amersham Pharmacia Biotech). The column was washed with 5 ml of 50 mM KCl in the above-mentioned buffer, followed by a 20-ml linear gradient from 50 to 300 mM KCl. Active fractions eluting between 150 and 175 mM KCl were concentrated by a Centricon 50 microconcentrator.

Determination of the N-terminal sequence. The purified protein was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (9) and electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad) (18). Edman degradation was performed at the University of Florida Protein Chemistry Core Facility. The N-terminal sequence was used to identify analogs of *M. thermophila* genes MJ1427 from *M. extorquens*. A BLAST search revealed that an analog of MJ1427 was added to each tube. The mixture was centrifuged at 27,000 × g for 25 min at 4°C. The supernatant was regarded as the cell extract.

**Heterologous expression of gene AF2089 in *Escherichia coli*.** The BLAST search revealed that an analog of β-RFAP synthase was likely to be encoded by genes MJ1427 from *Methanococcus jannaschii* and AF2089 from the sulfate-reducing archaeon *A. fulgidus*. To test the hypothesis that these genes coded for β-RFAP synthase, PCR was used to amplify the genes for cloning and expression in *E. coli* (24). Attempts to clone the MJ1427 gene into an expression vector were
unsuccessful. However, the AF2089 gene was successfully cloned and expressed in E. coli. The 939-bp gene encodes a protein of 313 amino acids with a predicted molecular mass of 34 kDa. The template for the PCR was a plasmid containing the AF2089 gene. The plasmid was purified from E. coli clone GAFHD40 (American Type Culture Collection, Manassas, Va.). The primers were synthesized commercially (Sigma Genosys, St. Louis, Mo.) and designed to introduce an Ndel site at the 5’ end of the gene and a BamHI site at the 3’ end. These primers were 5’-CTATGATCCATATGCTAGATATAGGAAGACCCC-3’ and 5’-CTATCTATGATCCATATGCTAGATATAGGAAGACCCC-3’. Pfu polymerase (Stratagene, La Jolla, Calif.) was used. The PCR product was digested overnight with Ndel and BamHI (New England Biolabs, Beverly, Mass.) at 37°C. The DNA was separated by electrophoresis on a 0.8% agarose gel, and the appropriate band was extracted using a gel extraction kit (Qiagen, Valencia, Calif.). The cassette was then cloned into a pET41a (+) vector (Novagen, Inc., Madison, Wis.) that had been digested with the same restriction endonucleases. The new construct was called pWS1. The plasmid was transformed into chemically competent E. coli DH-1 cells (23), and the gene sequence was verified using dideoxy sequencing by Jack Shelton at the University of Florida.

For expression of the AF2089 gene, pWS1 was transformed into E. coli BL21(DE3) cells (Stratagene). The cells were grown in 1 liter of Luria-Bertani broth at 30°C to an optical density at 600 nm of 0.6 to 0.8. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Inalco Pharmaceutica, San Luis Obispo, Calif.) to a specific protein concentration of 75 nmol of β-RFAP produced per min (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

Partial purification of the AF2089 protein. The heat-stable A. fulgidus enzyme was partially purified by heat treatment and column chromatography. Cells (2.5 g) were suspended in 5 ml of breakage buffer containing DNase I, disrupted with a French press, and centrifuged at 27,000 × g for 20 min. One milliliter of the supernatant was heated for 20 min at 90°C to precipitate heat-sensitive E. coli proteins, and the sample was centrifuged at 13,000 × g for 20 min. The supernatant was applied to a 2-ml hydroxyapatite column equilibrated with 50 mM TES (pH 8.0), 10 mM MgCl2, and 2 mM DTT. A step gradient of 27, 50, 75, and 400 mM sodium phosphate in buffer was used. Activity was found in fractions eluting at 75 mM sodium phosphate.

The active fractions were dialyzed against 4 liters of 1 M (NH4)2SO4 in buffer (50 mM TES [pH 6.8], 10 mM MgCl2, 2 mM DTT). The sample was then loaded onto a Phenyl Sepharose column (2 ml) equilibrated with the same buffer. The step gradient consisted of 1.0, 0.5, 0.4, 0.3, and 0 M (NH4)2SO4 in buffer. The active protein eluted at 0.4 M (NH4)2SO4.

Determination of enzyme activity. The β-RFAP synthase activity of M. thermophila TM-1 protein fractions was measured by converting the product to the azo-dye derivative essentially as described previously (22). For M. thermophila, the samples were incubated at 50°C for 3 h. For A. fulgidus, the reactions were incubated at 70°C for 1 h in a buffer containing 50 mM sodium acetate (pH 5.3). To determine the pH optimum for the A. fulgidus enzyme, the activity was measured in a mixed buffer containing 50 mM sodium acetate and 50 mM bis-trispropane at a pH ranging from 4.0 to 9.0.

Protein quantitation and gel electrophoresis. Proteins were separated by SDS-PAGE (9) and stained with either Coomassie blue or the Silver Stain Plus kit (Bio-Rad). Protein concentrations were determined by the Bradford assay (Bio-Rad) (2) with BSA as the standard. Chemicals. Gases were purchased from Strate Welding (Gainesville, Fl.). Gases were purchased from Strate Welding (Gainesville, Fl.).

RESULTS

Purification β-RFAP synthase from M. thermophila. β-RFAP synthase was purified to homogeneity from M. thermophila cell extract by Q-Sepharose, hydroxyapatite, Phenyl Sepharose, and Mono Q column chromatography. The native molecular mass was estimated by gel filtration on a Superdex 75 column to be 63.5 kDa. Silver staining of an SDS-PAGE gel showed that the purified protein ran as a single band of 32.6 kDa (Fig. 2, lanes 5 and 6), consistent with a homodimeric structure for the enzyme. The enzyme was purified 2,800-fold to a specific activity of 75 nmol of β-RFAP produced per min per mg of protein (Table 1). In different preparations of the enzyme, the total activity of the Q-Sepharose fraction was consistently higher than the total activity of the cell extract. It is likely that this increase in total activity resulted from the removal of an inhibitor of β-RFAP synthase during Q-Sepharose chromatography. The yield of β-RFAP synthase activity recovered in different preparations varied between 1% and 26%. By using this purification procedure, a sufficient amount of highly purified protein was isolated to determine the N-terminal amino acid sequence.

Identification of putative β-RFAP synthase genes. The N-terminal sequence of the protein purified from M. thermophila was MFLVVTSPRLHILTLDLNAE. A search of the non-redundant database with the program BLAST did not identify a protein with this N-terminal sequence, indicating that the amino acid sequence of the corresponding protein from M. thermophila was not available in the public databases. Therefore, the N-terminal sequence was used to search for an analogous protein in the genome of M. jannaschii (3), a methanogen in which two other methanoterpin biosynthetic genes have been identified (10, 41). Ten out of 20 amino acids of the N-terminal sequence matched the N terminus of the protein encoded by gene MJ1427 of M. jannaschii. Using the BLAST program, we identified homologs of the MJ1427 gene with Expect values of less than 4e-19 in 13 prokaryotes, including the sulfate-reducing archaeon A. fulgidus (AF2089) (15); the methanogens Methanobacterium thermautotrophicum ΔH (MTH0830) (27), Methanopyrus kandleri (26), Methanosarcina

| TABLE 1. Purification of β-RFAP synthase from M. thermophila |
|------------------|------------------|------------------|------------------|------------------|
| Purification step | Total activity (nmol of β-RFAP/min) | Specific activity (nmol of β-RFAP/min/mg of protein) | Yield (%) | Increase in purification (n-fold) |
| Cell extract | 31 | 0.027 | 100 | 1 |
| Q-Sepharose | 49 | 0.42 | 160 | 16 |
| Hydroxyapatite | 25 | 8.0 | 81 | 300 |
| Phenyl Sepharose | 7.0 | 57 | 22 | 2,100 |
| Mono Q | 0.3 | 75 | 1 | 2,800 |

* β-RFAP synthase assays were performed at pH 5.0 for 3 h at 50°C as described in Materials and Methods.
methanococcus maripaludis (orf322) (11), and Methanosarcina acetivorans (8); and the methylo trophic bacterium M. extorquens (orf4) (5). No specific functions had previously been assigned to any of the homologous sequences.

**Heterologous production of β-RFAP synthase from A. fulgidus.** Because the genome sequences of M. jannaschii (3) and A. fulgidus (15) were readily available and DNA from these organisms could be purchased commercially, we attempted to amplify and express the putative β-RFAP synthase genes from M. jannaschii (MJ1427) and A. fulgidus (AF2089). Numerous attempts to clone the MJ1427 gene into an expression vector were unsuccessful, possibly indicating a harmful effect of the DNA construct on E. coli cells. However, the AF2089 gene was successfully expressed in E. coli. The overproduced protein had an estimated molecular mass of 35.5 kDa by SDS-PAGE and an estimated molecular mass of 35.5 kDa by SDS-PAGE and E. coli successfully expressed in E. coli cells. However, the AF2089 gene was successfully expressed in E. coli. The overproduced protein had an estimated molecular mass of 35.5 kDa by SDS-PAGE.

**Heat treatment and column chromatography (Table 2).** Heat treatment was achieved by incubating the enzyme with pyridoxal phosphate (6). Attempts to restore β-RFAP synthase activity by incubating the enzyme with pyridoxal phosphate did not increase the specific activity of the enzyme (data not shown), and the inclusion of pyridoxal phosphate in buffers during Phenyl Sepharose chromatography did not prevent the loss of enzyme activity.

**Phylogenetic relationships among putative β-RFAP synthases.** The amino acid sequence of β-RFAP synthase from A. fulgidus shared 47% identity and 66% similarity with the sequence from M. jannaschii. The A. fulgidus sequence also shared 31% identity and 45% similarity with the putative homologs from the bacteria M. extorquens. These values are consistent with a role for these proteins as β-RFAP synthases. By using the BLAST program, homologs of β-RFAP synthase were found in 13 species of prokaryotes. Three organisms contained two putative β-RFAP synthase sequences (paralogs) each. A model of the phylogenetic relationships of the homologs (Fig. 3) shows that the putative β-RFAP synthases can be separated into three main groups. The first group consists of the proteins from the methanogens, A. fulgidus, and two Sulfolobus species. Within this clade, the proteins from the two Methanosarcina species (M. mazei and M. acetivorans) are closely related and the sequences from the two Sulfolobus species (S. solfataricus and S. tokodaii) are clustered together. The homolog from A. fulgidus appears most closely related to the homolog from M. jannaschii. The second major group of β-RFAP synthases includes one of the paralogs from Pyrococcus abyssi (Pab-1), Pyrococcus horikoshii (Pho-1), and Aeropyrum pernix (Ape-1), along with the homolog from the sole bacterial representative M. extorquens. The M. extorquens homolog appears most closely related to that of the strictly aerobic archaean A. pernix (14). The third clade consists of the second paralog from P. abyssi (Pab-2), P. horikoshii (Pho-2), and A. pernix (Ape-2) and the homolog from Pyrococcus furiosus.

**DISCUSSION**

To gain insight into the pathways of methanopterin biosynthesis in archaea and bacteria, we have purified the enzyme β-RFAP synthase to homogeneity, identified the corresponding gene, and investigated the distribution of putative β-RFAP synthase genes among different microorganisms. β-RFAP synthase was purified 2,800-fold from cell extracts of M. ther mophila. The low abundance of this enzyme (less than 0.04% of the total soluble protein) is consistent with the role of β-RFAP synthase in the biosynthesis of a coenzyme. The level of β-RFAP synthase in M. thermophila is on the same order of magnitude as the amount of the folate biosynthetic enzyme dihydropteroate synthase found in extracts of E. coli (0.014%)
The native molecular mass of the purified β-RFAP synthase was 63.5 kDa, similar to the value of 65 kDa previously obtained for the partially purified protein (22). SDS-PAGE analysis of the purified protein revealed a single band at 32.6 kDa (Fig. 2), indicating that the enzyme is a dimer of identical subunits.

The N-terminal sequence of the purified protein from *M. thermophilus* was instrumental in identifying homologs of the putative β-RFAP synthase gene. Because the gene from *M. thermophilus* was not available in the public database, the homolog from *A. fulgidus* was cloned and overexpressed to verify the enzymatic activity of the gene product. Cell extracts of *E. coli* containing the AF2089 gene showed high levels of β-RFAP synthase activity (Table 2). The recombinant *A. fulgidus* enzyme, like the *M. thermophilus* enzyme, was a dimer of identical subunits. Both enzymes had a pH optimum near 5, and it has been proposed that this relatively low pH optimum indicates the need for an ionized form of pAB during catalysis (22). In contrast, the temperature optimum for the *A. fulgidus* enzyme (70°C) was higher than that for the *M. thermophilus* enzyme (50°C) (22). This is consistent with the hyperthermophilic nature of *A. fulgidus*, which grows at a temperature optimum of 83°C (29), compared to *M. thermophilus*, which grows at a temperature optimum of 50°C (43).

During the purification of both enzymes, a large decrease in specific activity occurred during the Phenyl Sepharose step (Tables 1 and 2). One explanation for the observed loss of activity could be the separation of a required coenzyme from the protein during this step. Pyridoxal phosphate catalyzes the nonenzymatic decarboxylation of pAB and has been implicated in the activity of β-RFAP synthase (22). Previous experiments have shown that sodium borohydride inactivates partially purified β-RFAP synthase from *M. thermophilus* and also produces a fluorescence spectrum characteristic of reduced pyridoxal phosphate, which is consistent with a role for pyridoxal phosphate in β-RFAP synthase activity. In addition, when radioactively labeled sodium borohydride (NaB³H₄) is added to a solution containing partially purified β-RFAP synthase, a 64-kDa protein is labeled with radioactivity. Furthermore, β-RFAP synthase is inhibited by pyridoxol phosphate, an analog of pyridoxal phosphate. Unexpectedly, however, β-RFAP synthase is not inhibited by cysteine or by several carbonyl reactive reagents known to inactivate pyridoxal phosphate-dependent enzymes (22). These observations led to the proposal that pyridoxal phosphate might be involved in β-RFAP synthase activity through a mechanism that does not require the carboxyl group of pyridoxal phosphate.

In the present work, to test whether pyridoxal phosphate could stabilize the activity of β-RFAP synthase, we included pyridoxal phosphate in the purification buffers and added pyridoxal phosphate to the partially purified enzyme. Neither of these treatments increased the specific activity of the enzyme.

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In the present work, to test whether pyridoxal phosphate could stabilize the activity of β-RFAP synthase, we included pyridoxal phosphate in the purification buffers and added pyridoxal phosphate to the partially purified enzyme. Neither of these treatments increased the specific activity of the enzyme.
In addition, partially purified β-RFAP synthase from *A. fulgidus* showed no UV-visible absorbance at wavelengths characteristic of pyridoxal phosphate and a consensus pyridoxal phosphate binding motif was not found in the amino acid sequence of β-RFAP synthase. Therefore, further biochemical analysis will be necessary to determine if pyridoxal phosphate is involved in the mechanism of β-RFAP synthase. Although the low abundance of β-RFAP synthase in methanogen cells has precluded detailed enzymatic studies in the past, the ability to produce large quantities of enzyme through the overexpression system described will enable us to address fundamental questions about the structure and mechanism of β-RFAP synthase.

The amino acid sequence of the *A. fulgidus* enzyme was used to identify homologs of β-RFAP synthase in 12 archaea and 1 bacterium. A phylogenetic comparison of the proteins revealed three major groups of β-RFAP synthase homologs (Fig. 3). In one group, the *A. fulgidus* homolog clustered with the homologs from five methanogens and two aerobic sulfur-oxidizing archaea (*S. tokodaii* and *S. solfataricus*) (13, 25). In the second group, the bacterial protein from *M. extorquens* clustered with the first of two paralogs from the archaea *P. abyssi* (Pab-1), *P. horikoshii* (Pho-1), and *A. pernix* (Ape-1). In the third clade, the second paralogs from *P. abyssi* (Pab-2), *P. horikoshii* (Pho-2), and *A. pernix* (Ape-2) were related to the homolog from *P. furiosus*.

One intriguing aspect of the phylogenetic tree is that Pab-1, Pho-1, and Ape-1 are more closely related to each other than to the corresponding paralogs found within the same species (Pab-2, Pho-2, and Ape-2, respectively). This observation is consistent with a model in which duplication of the β-RFAP synthase gene appears to precede speciation. In this scenario, the common ancestor of all organisms that use β-RFAP synthase may have contained two β-RFAP synthase paralogs which diverged from one another over evolutionary time prior to the evolution of different archaeal species. The existence of only one β-RFAP synthase homolog in the clade containing the methanogens, *Archaeoglobus*, and *Sulfolobus* may have resulted from the loss of one paralog of β-RFAP synthase during the evolution of microorganisms of this clade. Although it is uncertain as to what extent horizontal gene transfer is involved in the distribution of methanopterin biosynthesis genes among the *Bacteria* and *Archaea*, it is apparent that the putative bacterial β-RFAP synthase is most closely related to that of *A. pernix*, an aerobic, hyperthermophilic archaeon positioned on a deep branch of the phylogenetic tree based on 16S rRNA sequencing (14, 23, 40).

A role for methanopterin or a structurally similar analog has been proposed for all of the microorganisms represented in Fig. 3 except for *A. pernix* (5, 17, 19, 31, 33, 35, 36, 42). The original molecule defined as methanopterin carries one-carbon units through different oxidation states during the reduction of carbon dioxide to methane by hydrogenotrophic methanogens (31) and during the oxidation of lactate by the sulfate-reducing archaeon *A. fulgidus* (19). Structurally similar compounds have been isolated from *Methanosarcina* (sarcipintherin) (33), *Pyrococcus* (36), *S. solfataricus* (solfaptherin) (16, 42), and *M. extorquens* (diphosphomethanopterin) (5). The structures of these compounds have been summarized in a review by Maden (17). All of the analogs contain the decarboxylated aminobenzene group that defines methanopterin and its derivatives. Because the decarboxylation of pAB is catalyzed by β-RFAP synthase, it is anticipated that all organisms which synthesize a methanopterin analog will contain a homolog of β-RFAP synthase. Therefore, although methanopterin has not yet been isolated from *A. pernix*, we would predict that this organism uses a metabolic pathway that is dependent upon methanopterin. In contrast, methanopterin has not been detected in extremely halophilic archaea, which contain folate at levels 10 times higher than those in typical bacteria (37). A BLAST search of the genome of the halophilic archaeon *Halobacterium sp. NRC-1* did not identify a protein with significant sequence similarity to β-RFAP synthase.

The discovery of a potential β-RFAP synthase gene (orf4) in *M. extorquens* represents the first example of a putative methanopterin biosynthesis gene found in the *Bacteria*. In *M. extorquens*, methanopterin plays a critical role in methanol oxidation by carrying C1 units through various oxidation states. Methanopterin is also required for the detoxification of formaldehyde produced as a metabolic intermediate during growth on C1 compounds (35). Chistoserdova et al. (5) previously showed that a mutation in the orf4 gene produces a mutant unable to grow on C1 compounds. The present work provides a rationale for the observed phenotype. An orf4 mutant would be unable to produce β-RFAP synthase and would therefore be deficient in the methanopterin required to catabolize methanol and prevent formaldehyde accumulation.

Finally, it is interesting to note that the putative β-RFAP synthase gene of *M. extorquens* is found within a cluster of twelve archaeal-like genes required for growth on C1 compounds (5). This cluster includes three genes which encode analogs of methanopterin-dependent enzymes as well as several open reading frames of unknown function. The identification of a potential β-RFAP synthase gene in *M. extorquens* provides the first indication that the pathways of methanopterin biosynthesis in the *Archaea* and *Bacteria* could be similar. We speculate that some of the remaining unidentified genes in this cluster may encode additional methanopterin biosynthetic enzymes. Importantly, it is anticipated that the available genetic system of *M. extorquens* will facilitate the identification of the remaining methanopterin biosynthesis genes. This information will contribute to an increased understanding of the evolutionary relationships among organisms that use methanopterin and the fundamental biochemical differences between pathways involving folate and methanopterin.

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