Application of a Colorimetric Assay to Identify Putative Ribofuranosylaminobenzene 5'-Phosphate Synthase Genes Expressed with Activity in *Escherichia coli* [§]

Matthew E. Bechard[1], Sonya Chhatwal[1], Rosemarie E. Garcia[1], and Madeline E. Rasche[1*]

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[1]Microbiology and Cell Science Department, University of Florida, Gainesville, FL 32611-0700

*To whom correspondence should be addressed: Madeline E. Rasche, Microbiology and Cell Science Department, University of Florida, Gainesville, FL 32611-0700. Tel: 352-392-1192; Fax: 352-392-5922; Email: mrasche@ufl.edu

ABSTRACT

Tetrahydromethanopterin (H$_4$MPT) is a tetrahydrofolate analog originally discovered in methanogenic archaea, but later found in other archaea and bacteria. The extent to which H$_4$MPT occurs among living organisms is unknown. The key enzyme which distinguishes the biosynthetic pathways of H$_4$MPT and tetrahydrofolate is ribofuranosylaminobenzene 5'-phosphate synthase (RFAP synthase). Given the importance of RFAP synthase in H$_4$MPT biosynthesis, the identification of putative RFAP synthase genes and measurement of RFAP synthase activity would provide an indication of the presence of H$_4$MPT in untested microorganisms. Investigation of putative archaeal RFAP synthase genes has been hampered by the tendency of the resulting proteins to form inactive inclusion bodies in *Escherichia coli*. The current work describes a colorimetric assay for measuring RFAP synthase activity, and two modified procedures for expressing recombinant RFAP synthase genes to produce soluble, active enzyme. By lowering the incubation temperature during expression, RFAP synthase from *Archaeoglobus fulgidus* was produced in *E. coli* and purified to homogeneity. The production of active RFAP synthase from *Methanothermobacter thermautotrophicus* was achieved by coexpression of the gene *MTH0830* with a molecular chaperone. This is the first direct biochemical identification of a methanogen gene that codes for an active RFAP synthase.

INTRODUCTION

Tetrahydromethanopterin (H$_4$MPT), a structural analog of tetrahydrofolate (H$_4$F) (Fig. 1), was first discovered as a coenzyme required for the one-carbon metabolism of methanogenic archaea (1, 2). Since then, modified forms of H$_4$MPT have been identified in a variety of other hyperthermophilic and sulfur-dependent archaea that do not produce detectable amounts of H$_4$F (3-8). Curiously, H$_4$MPT has more recently been found in the (eu)bacterium *Methylobacterium extorquens*, a microorganism that also produces H$_4$F (9). With the discovery of H$_4$MPT in bacteria, it has become evident that H$_4$MPT analogs are more widely distributed among archaea and bacteria than previously thought (10). The full extent to which H$_4$MPT occurs among living organisms is currently unknown.
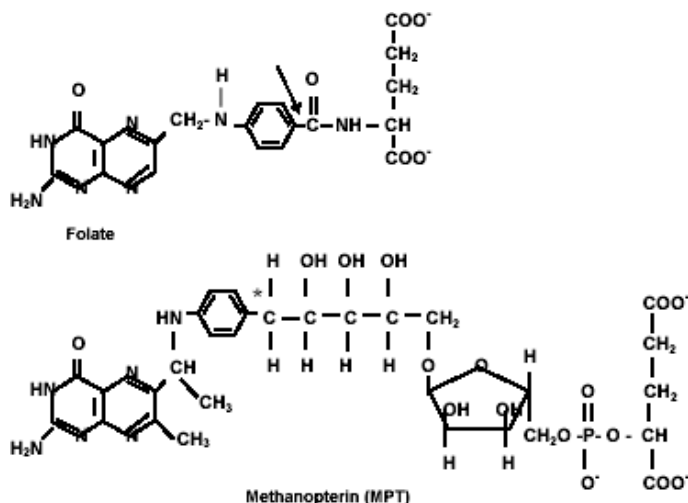

Fig. 1: The structures of folate and methanopterin. The arrow indicates the position of the carbonyl residue of folate. The absence of the carbonyl residue in methanopterin (*) is the defining structural feature of methanopterin and its derivatives.

Direct measurement of H$_4$MPT in cells is challenging because the coenzyme is present in low concentrations and is labile to light and oxygen. An alternative approach to investigating H$_4$MPT distribution among organisms is to identify the genes

and enzymes involved in H_4MPT biosynthesis. One advantage of this approach is that homologs of known H_4MPT biosynthesis genes can be identified in the sequenced genomes of various organisms. Putative H_4MPT biosynthesis genes can be expressed heterologously, and the activity of the corresponding enzymes can be tested in vitro (11-13). The key enzyme which distinguishes the biosynthetic pathways of H_4MPT and H_4F is ribofuranosylaminobenzene 5'-phosphate synthase (RFAP synthase) (14). This enzyme catalyzes the reaction between phosphoribosylpyrophosphate and *p*-aminobenzoic acid (*p*AB) to form RFAP, carbon dioxide, and inorganic pyrophosphate (Fig. 2) (12, 14, 15). This decarboxylation reaction results in the defining structural difference between H_4MPT and H_4F, which is the absence in H_4MPT of the carbonyl group derived from *p*AB (Fig. 1) (3). Due to the uniqueness of RFAP synthase as a H_4MPT biosynthetic enzyme, the identification of an active RFAP synthase in different organisms could give strong evidence that they contain H_4MPT.
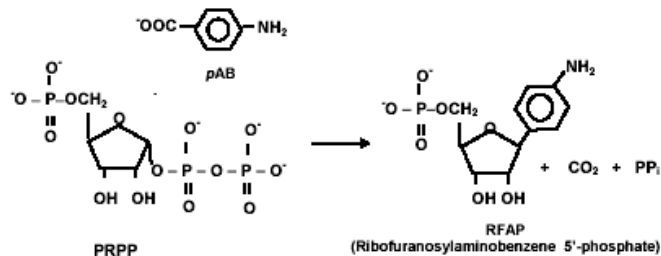

Fig. 2: The reaction of RFAP synthase. Abbreviations: *p*AB, *p*-aminobenzoic acid; PRPP, phosphoribosylpyrophosphate; PPi, inorganic pyrophosphate.

RFAP synthase has recently been purified to homogeneity from the methanogen *Methanosarcina thermophila*, and the N-terminal amino acid sequence used to identify homologs in the genomes of other organisms, including methanogens, non-methanogenic archaea, and the bacterium *M. extorquens* (12). By lowering the temperature of induction, the RFAP synthase homolog from the non-methanogenic archaeon *A. fulgidus* (AF2089) was expressed with activity, and the enzyme partially purified. However, the enzyme was not purified to homogeneity, and attempts to verify the function of methanogen genes failed because the proteins formed inactive inclusion bodies when produced in *Escherichia coli* (12; M. Bechard and M. Rasche, unpublished data).

Because of the central role of H_4MPT in methanogenesis, it would be valuable to verify the identity of RFAP synthase genes in methane-producing archaea. In the current work, this has been accomplished by coexpressing a methanogen gene in the presence of a molecular chaperone. Herein we describe detailed procedures for the RFAP synthase assay, the heterologous production and purification to homogeneity of RFAP synthase from *A. fulgidus*, and the successful production of active RFAP synthase from the methanogen *M. thermautotrophicus*. This is the first biochemical demonstration that the methanogen gene *MTH0830* encodes RFAP synthase.

MATERIALS AND METHODS

Colorimetric assay for RFAP synthase

The RFAP synthase assay (14) is based on the conversion of the arylamine product, RFAP, to its colored azo-dye derivative using nitrite and N-naphthylethylene diamine (Aldrich Chemical Co., Inc., Milwaukee, WI). Because these reagents also react with the arylamine substrate *p*-aminobenzoic acid (*p*AB) (Aldrich Chemical), RFAP must first be separated from *p*AB using a C18 column (14, 15). Detailed procedures for this assay are described in the protocols section of the current work. The optimum temperature for the enzymatic reaction varies for each organism and is typically similar to the optimal temperature for growth of the organism. For the studies presented here, the pH optimum was determined using 100 mM acetate (pH 5.0 to 5.5), 100 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (pH 6.0 to 6.5), or 100 mM piperazine-N,N'-bis(ethanesulfonic acid) PIPES (pH 7.0 to 9.0). The RFAP synthase inhibitor *p*-methylaminobenzoic acid (Aldrich Chemical) was used at a concentration of 5 mM.

Production and purification of RFAP synthase from *A. fulgidus* (AF2089)

RFAP synthase from *A. fulgidus* was produced in *E. coli* using the plasmid pJWS1 as described previously (12). Briefly, *E. coli* BL21 (DE3) cells (Stratagene, La Jolla, CA) containing pJWS1 were grown at 30°C on Luria-Bertani (LB) medium with kanamycin (50 µg/mL) to an optical density at 600 nm of 0.6 to 0.8. Expression of the *AF2089* gene was induced at 30°C with 1 mM isopropylthiogalactoside (IPTG; Inalco Pharmaceuticals, San Luis Obispo, CA) for 2 h. Cells were harvested by centrifugation and lysed using a French Press (12). After centrifugation at 31,000 x g for 45 min, the supernatant (cell-free extract) was heated to 65°C for 15 min. The mixture was centrifuged at 13,000 x g for 10 min. The proteins in the supernatant (heated cell-free extract) were separated on a 40-mL ceramic hydroxyapatite (Bio-Rad, Hercules, CA) column as described in the detailed protocol. Selected fractions were loaded onto a 1-mL MonoQ 5/5 anion exchange column (Amersham-Pharmacia Biotech, Piscataway, NJ), and RFAP synthase was purified using the gradient described in the detailed protocol.

Heterologous expression of *MTH830* in *E. coli*

The polymerase chain reaction (PCR) was used to amplify the RFAP synthase homolog *MTH0830* from the *M. thermautotrophicus* genome using established protocols (16). The primers were synthesized commercially (Sigma Genosys, St. Louis, MO) and designed to engineer in an NdeI site 5' and a BamHI site 3' of the gene. The PCR product and the vector pET41a(+) (Novagen, Inc., Madison, WI) were digested with restriction enzymes overnight at 37°C, and the two pieces were ligated yielding the plasmid designated pED2. pED2 was

transformed into electrocompetent *E. coli* DH5-alpha cells, and the gene sequence was verified by dideoxy sequencing (Sambrook and Russell, 2001). *Pfu* DNA polymerase was purchased from Stratagene. Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA).

When *MTH0830* was expressed in *E. coli* at 37°C, most of the protein aggregated as inactive inclusion bodies (data not shown). A previous study (12) using RFAP synthase from *A. fulgidus* showed that lowering the induction temperature to 30°C resulted in the production of soluble enzyme. Therefore, to increase the chances of producing soluble, active *M. thermautotrophicus* RFAP synthase, different induction temperatures and times were tested. In addition, the *MTH0830* gene was coexpressed with the genes for the chaperone GroEL/ES and trigger factor provided on plasmid pG-Tf2 (17). For overproduction of RFAP synthase (MTH0830), pED2 was transformed into *E. coli* BL21(DE3) cells containing pG-Tf2 (kindly provided by Dr. Tsunetaka Ohta). The expression cell line with pED2 and pG-Tf2 was called KB1. To test the effect of variable induction times and temperatures in the absence of chaperone, KB1 was grown in 1 liter of LB medium at 37°C to an optical density at 600 nm of 0.6 to 0.8. *MTH0830* expression was induced with IPTG at 1 mM, and the cultures were incubated at 37°C for 2 h, 30°C for 6 h, or 20°C for 16 h. After the appropriate incubation times, cells were harvested by centrifugation. For expression in the presence of the chaperone, KB1 cells were grown to an optical density of 0.4, and tetracycline was added (final concentration, 50 ng per mL) to induce the chaperone genes (17). After a 30-min incubation, expression of *MTH0830* was induced with IPTG. The cultures were then transferred to a 20°C incubator, and cells were grown for 16 hours before centrifugation.

Proteins were separated by reducing SDS-PAGE (18) using 12% acrylamide gels (Bio-Rad) stained with Coomassie Blue. Protein concentrations were measured using the method of Bradford (19) (Bio-Rad) with bovine serum albumin as the standard. N-naphthylethylene diamine, *p*-aminobenzoic acid, and *p*-methylaminobenzoic acid were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). PRPP was from Sigma Chemical Corp. (St Louis, MO), and gases were obtained from Strate Welding (Gainesville, FL). Except where noted above, all other chemicals were purchased from Fisher Scientific (Suwanee, GA).

RESULTS AND DISCUSSION

Because RFAP synthase plays a key role in the biosynthesis of H_4MPT (14), characterization of RFAP synthase activity can be a useful initial step for investigating the range of microorganisms that produce H_4MPT. Previous work has indicated that archaeal RFAP synthases produced in *E. coli* tend to form inactive inclusion bodies (12). The work presented here describes modified expression techniques that allow for the production of soluble, active RFAP synthases in

E. coli along with the procedure for measuring RFAP synthase activity using a colorimetric assay.

The RFAP synthase gene homolog from the hyperthermophilic archaeon *A. fulgidus* was previously designated as *AF2089* (12). This gene was previously expressed in *E. coli* BL21(DE3) when the temperature for growth and induction was lowered from 37°C to 30°C (12). The lower temperature presumably slowed the rate of protein synthesis, enabling proteins to fold into an active conformation. For RFAP synthase from *A. fulgidus*, the use of an expression cell line containing rare codon tRNA synthetases did not facilitate the synthesis of active enzyme, but rather exacerbated the production of inclusion bodies (data not shown). Previous attempts to purify the enzyme using an 80°C heat step followed by hydroxyapatite and Phenyl Sepharose column chromatography resulted in 95% pure protein with a specific activity of 290 nmol per min per mg protein at the temperature optimum of 70°C. However, attempts to further purify the enzyme resulted in complete loss of activity (12). In the current work, we have purified AF2089 to homogeneity using a gentler 65°C heat step followed by hydroxyapatite and anion exchange chromatography with linear gradients rather than step gradients (Fig. 3). The highest specific activity obtained for the purified fraction (240 nmol per min per mg protein) was comparable to the specific activity reported previously (12).
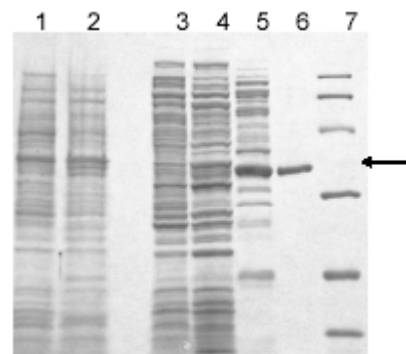

Fig. 3: SDS-PAGE of steps in the purification of RFAP synthase from *A. fulgidus*. *E. coli* BL21(DE3) with pJWS1 was induced for the production of RFAP synthase encoded by the *AF2089* gene. Samples were boiled for 10 min with reducing SDS-PAGE sample buffer prior to loading on a 12% polyacrylamide gel, and the gel was stained with Coomassie blue. Lane 1: uninduced cells (20 µg protein); lane 2: cells induced at 30°C with 1 mM IPTG (20 µg protein); lane 3: cell-free extract (26 µg); lane 4: heated cell-free extract (27 µg); lane 5: hydroxyapatite fraction (17 µg); lane 6: MonoQ fraction (2 µg). The molecular mass markers (97.4, 66.2, 45, 31, 21.5, and 14 kilodaltons) are shown in lane 7. The protein encoded by *AF2089* has a predicted molecular mass of 34 kilodaltons and is indicated by the arrow.

The time course for the reaction of purified AF2089 (Fig. 4) shows that at the optimal temperature of 70°C, the rate of RFAP production was constant for 1 h. Thus, all subsequent activity measurements for AF2089 were completed at 70°C within a 1-h time period. By comparison, the reaction rate of RFAP synthase from the moderate (50°C) thermophile *Methanosarcina thermophila* is constant for 3 h at the optimal temperature of 50°C (15). The above results illustrate that,

when measuring the activities of RFAP synthases from different sources, it is important to determine the optimal temperature for the reaction (generally similar to the optimal growth temperature for the organism) and the time period over which the rate is constant. Previously, RFAP synthase from *A. fulgidus* was shown to be active over a wide range of pH values from 5 to 7, with a pH optimum at 5.3 (12). In this work, the enzyme was also found to be active over a broad pH range, but with an optimum around pH 7.0. We suspect that this difference is due to the replacement in this study of TES buffer (tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) (12) with PIPES, which is more stable than TES as a buffer at high temperatures (20).
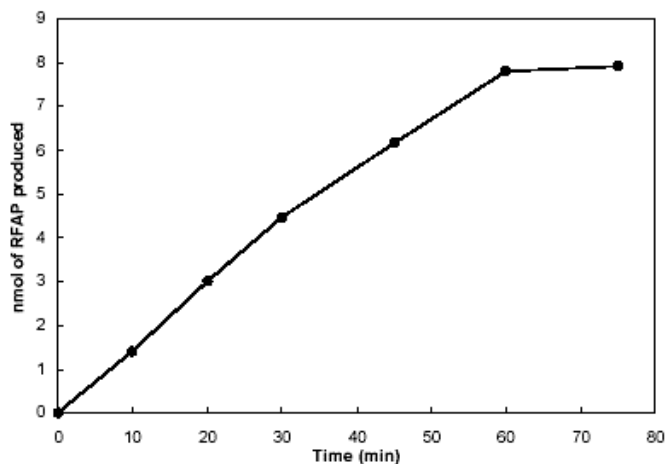

Fig. 4: Time course for the reaction of purified RFAP synthase (AF2089) from *A. fulgidus*. The reaction mixtures (125 µL) contained 100 mM PIPES, pH 7.0, 8 mM MgCl$_2$, 2 mM dithiothreitol, 12 mM *p*AB, 8.3 mM PRPP, and 1.8 µg of protein. The assays were incubated at 70˚C for the indicated times and terminated by placing the reaction vials on ice and adjusting the pH to 3.6. Samples were processed as described in the detailed protocol.

Native RFAP synthase from the methanogen *M. thermophila* is inhibited by *p*-methylaminobenzoic acid, an analog of the substrate *p*AB. At the optimal pH of 5, the methanogen enzyme was completely inhibited by 5 mM methylaminobenzoic acid (15). Because methylaminobenzoic acid is the most potent characterized inhibitor of the methanogen RFAP synthase, we were interested in using the *A. fulgidus* enzyme to study the effect of methylaminobenzoic acid on RFAP synthases. To compare the properties of the *M. thermophila* and *A. fulgidus* enzymes, both proteins were treated with methylaminobenzoic acid under conditions known to inhibit the methanogen RFAP synthase. In the presence of 5 mM methylaminobenzoic acid and 100 µM *p*AB at pH 6.0, RFAP synthase from *M. thermophila* retained only 15% of its specific activity compared to control enzyme without inhibitor added. Unexpectedly, when methylaminobenzoic acid was added to the enzyme from *A. fulgidus* at pH 6.0, 79% of the activity was retained. The activity of AF2089 at pH 5.0 was too low to be measured reliably; however, at the optimal pH of 7.0 for AF2089, 74% of the activity was retained in the presence of 5 mM methylaminobenzoic acid. Higher concentrations of methylaminobenzoic acid led to nonspecific

inhibition due to the precipitation of assay components. The results above indicate that the inhibitor profiles of the two enzymes differ substantially, possibly due to subtle structural differences in the active sites of the two enzymes. Because the *A. fulgidus* enzyme is not a good model system to study methylaminobenzoic acid inhibition of RFAP synthase, the results led us to further develop techniques to identify an active methanogen RFAP synthase that is more similar to the *M. thermophila* enzyme.

BLAST searches indicate that the gene *MTH0830* from the methanogen *M. thermautotrophicus* is an RFAP synthase homolog (12). Initially, we attempted to express the gene by transforming *E. coli* BL21(DE3) cells with a plasmid (pED2) containing *MTH0830*. However, this procedure resulted in a complete lack of RFAP synthase activity even when the induction temperature was lowered to 30ºC (data not shown). This led us to create an expression strain (KB1) containing both pED2 and the plasmid pG-Tf2, which carries the genes for the GroEL/ES chaperone under the control of a tetracycline promoter (17). Fig. 5 illustrates the effects of induction temperature and the presence of chaperone on the production of soluble versus insoluble *M. thermautotrophicus* RFAP synthase.
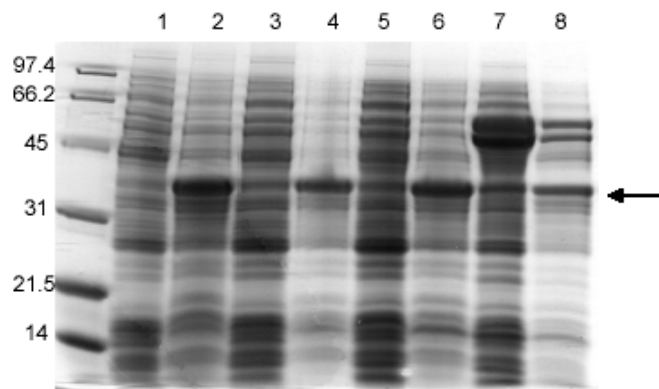

Fig. 5: SDS-PAGE of soluble versus insoluble fractions of KB1 cells induced for the production of RFAP synthase from *M. thermautotrophicus*. KB1 cells were grown at 37˚C and then induced with IPTG at the indicated temperatures. Cells were broken with a French press and centrifuged, producing a soluble fraction (supernatant) and insoluble fraction (pellet). Induction at 37˚C for 2 h, soluble (lane 1) and insoluble (lane 2); induction at 30˚C for 6 h, soluble (lane 3) and insoluble (lane 4); induction at 20˚C for 16 h, soluble (lane 5) and insoluble (lane 6). Induction of chaperone, followed by induction of *MTH0830* and incubation at 20˚C for 16 h, soluble (lane 7) and insoluble (lane 8). Each lane contained between 15 and 30 µg of protein. The arrow indicates the position of RFAP synthase from *M. thermautotrophicus*.

In these experiments, KB1 cells were grown at 37ºC, and then *MTH0830* expression was induced with IPTG at the indicated temperatures and times. In the absence of chaperone induction, when *MTH0830* was induced and incubated at 37ºC for 2 h, SDS-PAGE analysis showed that a large quantity of the overproduced protein was present in the insoluble fraction (Fig. 5, lanes 1 and 2). The resulting soluble fraction had only a trace of RFAP synthase activity (0.0056 nmol of RFAP produced per min per mg of protein, at pH 7.0 and 70°C),

possibly due to leaky expression of the chaperone from the pG-Tf2 plasmid. When the induction temperature was lowered to 30ºC and the induction time increased to 6 hours, the specific activity (0.033 nmol of product per min per mg of protein) increased 6-fold when compared to the enzyme produced at 37°C, and a corresponding increase in soluble protein was observed (Fig. 5, lanes 3 and 4). The specific activity was further increased when the induction temperature was lowered to 20ºC for 16 hours (0.074 nmol of product per min per mg of protein), accompanied by a corresponding increase in soluble enzyme (Fig. 5, lanes 5 and 6).

To investigate whether the induction of the chaperone would further aid in producing soluble, active enzyme, KB1 cells were grown at 37ºC, and chaperone gene expression was induced with tetracycline, prior to the induction of *MTH0830*. Induction of the chaperone, in conjunction with *MTH0830* expression at 20ºC for 16 hours, resulted in the highest production of soluble, active enzyme. The specific activity (0.100 nmol of product per min per mg of protein) was approximately 20-fold higher than the activity of enzyme produced at 37ºC, with a significant increase in soluble protein and a marked decrease in protein found in the insoluble fraction (Fig. 5, lanes 7 and 8). The results presented here show that lower induction temperatures, longer induction times, and the use of a chaperone all aided in the successful production of active RFAP synthase from *M. thermautotrophicus*. This is the first direct biochemical demonstration of a methanogen gene (*MTH0830*) that codes for an active RFAP synthase. This enzyme is currently being purified for detailed biochemical characterization and analysis.

The expression techniques and colorimetric RFAP synthase assay described here may have broad applications for the overproduction and identification of active RFAP synthase homologs in other organisms. Twenty-one homologs of RFAP synthase have been identified in the sequenced genomes of archaea and bacteria (12). Some of these organisms, such as the non-methanogenic hyperthermophilic archaeon *Aeropyrum pernix* (21) are not known to contain H_4MPT. Indeed, the genomes of *A. pernix*, *Pyrococcus horikoshii*, and *Pyrococcus abyssi* contain two distantly related homologs of the RFAP synthase gene, and it is unknown if either or both of these homologs function as RFAP synthase genes (12). The RFAP synthase assay and the modified expression techniques provide simple procedures to begin to answer these questions. Due to the uniqueness of RFAP synthase as a H_4MPT biosynthetic enzyme, the identification of an active RFAP synthase in these organisms could give strong initial evidence that they contain H_4MPT and enable us to address questions about the distribution and evolutionary relationships of organisms that produce H_4MPT.

ACKNOWLEDGEMENTS

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PROTOCOLS

Protocol I: Colorimetric RFAP synthase assay

Overview

In the RFAP synthase assay (Fig. 2), the substrates *p*-aminobenzoic acid (*p*AB) and phosphoribosylpyrophosphate (PRPP) are mixed with buffer, $MgCl_2$, dithiothreitol, and a solution containing RFAP synthase. The components are combined in 2-mL glass vials sealed with silicone or rubber septa under a gas phase of N_2 to avoid oxygen inactivation of the product RFAP. The final assay volume can vary between 100 and 250 µL, but should be consistent when making kinetic comparisons. Vials are incubated at the appropriate temperature for the enzyme of interest, usually similar to optimal temperature for growth of the organism. The reaction is terminated by placing the vial on ice and adjusting the pH to 3.6. After terminating the reaction, the product RFAP is separated from *p*AB using a C18 column (Fig. 6). Nitrite and N-naphthylethylene diamine are added to convert RFAP to its pink azo-dye derivative, which absorbs strongly at 562 nm.
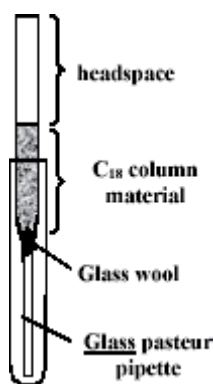

Fig. 6: Diagram of the C18 column used to separate RFAP from the substrate *p*AB.

N-naphthylethylene diamine hydrochloride and *p*-aminobenzoic acid can be purchased from Aldrich Chemical Co. (Milwaukee, WI). PRPP can be obtained from Sigma Chemical (St. Louis MO), and the preparative C18 125 Å bulk packing material from Waters Corp. (Milford, MA). All other chemicals for this reaction are available from Fisher Scientific (Suwanee, GA).

A. Pouring the C18 column.

1. To prepare the C18 columns as illustrated in Fig. 6, loosely plug a 5.75-inch glass Pasteur pipette by inserting glass wool through the top and into the narrow stem. Use the minimum amount of glass wool needed to retain the packing material in the column. Place the pipette in a glass test tube.
2. Weigh out 0.375 g of Preparative C18 125 Å bulk packing material. Mix the material in a test tube with about 2 mL of methanol to form a slurry. To avoid trapping air in the bottom of the column, pipette methanol into the plugged 5.75-inch pipette (above) and while methanol is still in the column, transfer the C18 slurry to the column using a 9-inch glass Pasteur pipette.
3. When the packing material settles, cover the top of the bed with a very thin layer of glass wool to prevent disturbing the packing material during subsequent addition of liquid samples.
4. Store the column at room temperature in a test tube containing 1 to 2 mL of liquid to prevent the column from drying. If the packing material dries between uses, the column must be poured again.

B. Preparation of RFAP synthase reaction mixtures.

1. The reaction mixtures can vary in volume between 100 and 250 µL. The following is a procedure for a 125-µL reaction containing 100 mM PIPES, pH 7.0, 8 mM $MgCl_2$, 2 mM dithiothreitol, 24 mM *p*AB, and 8.3 mM PRPP.
2. To a 2-mL glass vial, add the following:
   - 25 µL of 0.5 M PIPES, pH 7.0 (pH adjusted at the appropriate temperature)
   - 10 µL of 0.1 M $MgCl_2$
   - 7.5 µL of 0.4 M *p*AB, adjusted to pH 7.0
   - up to 70 µL of cell-free extract or a solution containing RFAP synthase

• distilled deionized water to adjust the volume to 112.5 µL

(Note: if the cell-free extract or solution containing RFAP synthase is already well buffered at the appropriate pH, the concentrated buffer (25 µL of 0.5 M PIPES, pH 7.0) can be omitted and the sample volume can be increased up to 105 µL).

3. Seal the vial with a rubber stopper or with a teflon seal (Supelco, Bellefonte, PA) inserted into a screw cap with a small drilled hole. Purge the gas phase of the vial with N$_2$ for 1 min.
4. Use a syringe to add 2.5 µL of 0.1 M dithiothreitol.
5. Initiate the reaction by adding 10 µL of a PRPP stock solution (1 mg PRPP per mL of 50 mM TES, pH 7.0, 10 mM MgCl$_2$). Transfer the vial to a water bath or incubator at the appropriate temperature.
6. Terminate the reaction by placing the vial on ice and adjusting the pH to 3.6 with 1 M citric acid. Careful adjustment of the pH is critical for successful separation of RFAP from *p*AB in the next step (Note: If the sample contains a large quantity of protein, precipitated proteins might interfere with adjustment of the pH. In this case, centrifuge the sample for 1 to 2 min in a microcentrifuge, and continue to adjust the pH of the supernatant to 3.6.).

C. Use of the C18 column to separate RFAP from *p*AB.

1. Equilibrate the C18 column from step A with two "headspace" volumes of 0.1 M citrate, pH 3.6 buffer (see Fig. 6). To do this, fill the space above the C18 column material with the buffer. Allow the liquid to drain through the column, and discard the liquid that flows into the test tube. Repeat once.
2. Transfer the column to a fresh test tube. Using a 9-inch glass Pasteur pipette, carefully transfer the pH-adjusted sample from step B6 to the equilibrated column. The best results are obtained when the sample is placed gently and directly onto the glass wool on top of the column bed.
3. After the sample drains onto the column, add 100 µL of 0.1 M citrate, pH 3.6. (Note: if the RFAP synthase reaction in step B was 250 µL, do NOT add 100 µL of 0.1 M citrate, pH 3.6 at this point.)
4. Add an additional 300 µL of 0.1 M citrate, pH 3.6 to the column. The total 525 µL of liquid that flows into this test tube is called fraction 1.
5. Transfer the column to a new test tube. To the tube containing fraction 1, add 275 µL of 0.1 M citrate, pH 3.6 to raise the volume to 800 µL.
6. With the column in a new test tube, add 800 µL of 0.1 M citrate, pH 3.6 to the column. The liquid that flows through contains RFAP and is designated as fraction 2.
7. Transfer the column to a new test tube. Add 800 µL of 0.1 M citrate, pH 3.6 to the column and collect fraction 3. *p*AB remains on the column.
8. To remove *p*AB, transfer the column to a new tube, and wash with two headspace volumes of 50% methanol, discarding the flow-through between washes. Transfer the column to a clean test tube containing 1 to 2 mL of distilled water to avoid drying during storage.

D. Conversion of RFAP to the azo-dye derivative.

1. To each of the three fractions from step C, add 100 µL of 6 N HCl.
2. Add 100 µL of 1.5% NaNO$_2$ (w/v in water) to each tube and mix gently but completely.
3. After 2 min, add 100 µL of 7.5% (w/v) ammonium sulfamate and mix vigorously to inactivate the NaNO$_2$. (Note: Any remaining NaNO$_2$ will interfere with the final color reaction by producing a yellow product.)
4. After 2 min, add 300 µL of 1% (w/v) N-naphthylethylene diamine hydrochloride and mix vigorously.
5. After 60 min, read the absorbance at 562 nm. Convert the absorbance to nmol using the extinction coefficient of 48,000 per cm per M (7). (Note: The pink RFAP derivative should appear in fraction 2, but a small amount might appear in fraction 3. If fraction 3 is more intensely colored than fraction 2 (or if fraction 1 is has color), it is likely that the pH of the sample was not adjusted to 3.6, causing *p*AB to elute in an early fraction).

Protocol II: Production and purification of active *A. fulgidus* RFAP synthase (AF2089) in *E. coli.*

1. Inoculate 50 mL of LB-kanamycin medium (50 µg kanamycin per mL) with *E. coli* BL21(DE3) cells containing pJWS1. Grow overnight at 37ºC with shaking (200 rpm).
2. Inoculate 1 L of LB-kanamycin medium with 10 to 20 mL of the culture above. Grow at 30ºC with shaking at 200 rpm until the optical density at 600 nm reaches 0.6 to 0.8.
3. To induce expression of *AF2089*, add 1 mL of 1 M IPTG. Grow for an additional 2 h.

4.   Harvest the cells by centrifugation at 5000 x g for 5 min. Wash the cells with 30 mL of 50 mM PIPES, pH 6.8, and centrifuge at 5000 x g for 15 min. Freeze cells at -20ºC until use.
5.   For each g of cells, add 2 mL of 50 mM PIPES, pH 6.8, 2 mM dithiothreitol. Break the cells at 20,000 psi using a French Press (Spectronic Instruments, Inc., Rochester, NY). Centrifuge at 31,000 x g for 45 min. Save the supernatant (cell-free extract).
6.   Aliquot the cell-free extract into 2-mL microcentrifuge tubes. Heat the tubes at 65ºC for 15 min, inverting the tubes several times during the heating. Centrifuge at 13,000 rpm for 10 min. Save the supernatant (heated cell-free extract).
7.   Filter the heated cell-free extract through a 0.45 µm filter. Load onto a 40-mL (2.5 x 8 cm) ceramic hydroxyapatite (Bio-Rad, Hercules, CA) column equilibrated with 50 mM PIPES, pH 6.8, 2 mM dithiothreitol. Wash the column with 40 mL of 50 mM PIPES, pH 6.8, 2 mM dithiothreitol. Elute the protein with a 400-mL linear gradient of 0 to 200 mM potassium phosphate in 50 mM PIPES, pH 6.8, 2 mM dithiothreitol. Collect 5-mL fractions. (Note: For purification to homogeneity, only the three most active, least contaminated fractions were chosen for further purification.)
8.   To reduce the salt concentration prior to anion exchange chromatography, concentrate the selected protein fractions in Centricon microconcentrators (10,000 molecular weight cut-off) (Millipore, Bedford, MA). Dilute the concentrated sample 20-fold with 25 mM PIPES, pH 6.8, 2 mM dithiothreitol. Load the protein onto a MonoQ 5/5 column (Amersham-Pharmacia Biotech) equilibrated with 25 mM PIPES, pH 6.8, 2 mM dithiothreitol. Wash the column with 5 mL of the same buffer. Elute the proteins with a 20-mL gradient of 0 to 130 mM sodium chloride in 25 mM PIPES, pH 6.8, 2 mM dithiothreitol, followed by 5 mL of buffer with 130 mM sodium chloride and 5 mL of buffer with 1 M sodium chloride. Collect 1-mL fractions.

Protocol III: Production of active *M. thermautotrophicus* RFAP synthase (MTH0830) in *E. coli.*

1.   Inoculate 50 mL of LB medium containing kanamycin (50 µg per mL) and chloramphenicol (17 µg per mL) with KB1 cells. (Note: Chloramphenicol is a suspect carcinogen. Handle with care.) Grow the cells overnight at 37ºC with shaking (200 rpm).
2.   For MTH0830 expression in the absence of chaperone, inoculate 1 L of LB-kanamycin-chloramphenicol medium with 7 mL of the culture above. Grow at 37ºC with shaking at 200 rpm until the optical density at 600 nm reaches 0.6 to 0.8. Add 1 mL of 1 M IPTG to induce expression of MTH0830. Then incubate the cells under one of the following conditions: (i) 37ºC for 2 h, (ii) 30ºC for 6 h, or (iii) 37ºC for 16 h. Harvest the cells by centrifugation at 5000 x g for 5 min. Wash the cells with 30 mL of 50 mM TES, pH 7.0, and centrifuge at 5000 x g for 15 min. Freeze the cell pellet at -20ºC until use.
3.   For MTH0830 expression in the presence of chaperone induction, inoculate 1 L of LB-kanamycin medium with 7 mL of the culture in step III-1 above. Grow at 37ºC with shaking at 200 rpm to an optical density at 600 nm of 0.4. To induce the chaperone genes, add tetracycline to a final concentration of 50 ng of tetracycline per mL, and continue shaking for 30 min. Add 1 mL of 1 M IPTG, and then transfer the flasks to a 20°C incubator for 16 hours. Harvest the cells as described above.