Characterization of an Fe^{2+}-Dependent Archaeal-Specific GTP Cyclohydrolase, MptA, from *Methanocaldococcus jannaschii*†

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ABSTRACT: The first step in the biosynthesis of pterins in bacteria and plants is the conversion of GTP to 7,8-dihydro-D-neopterin triphosphate catalyzed by GTP cyclohydrolase I (GTPCHI). Although GTP has been shown to be a precursor of pterins in archaea, homologues of GTPCHI have not been identified in most archaeal genomes. Here we report the identification of a new GTP cyclohydrolase that converts GTP to 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate, the first intermediate in methanopterin biosynthesis in methanogenic archaea. The enzyme from *Methanocaldococcus jannaschii* is designated MptA to indicate that it catalyzes the first step in the biosynthesis of methanopterin. MptA is the archetype of a new class of GTP cyclohydrolases that catalyzes a series of reactions most similar to that seen with GTPCHI but unique in that the cyclic phosphate is the product. MptA was found to require Fe^{2+} for activity. Mutation of conserved histidine residues H200N, H293N, and H295N, expected to be involved in Fe^{2+} binding, resulted in reduced enzymatic activity but no reduction in the amount of bound iron.

Methanopterin, as its 5,6,7,8-tetrahydro derivative, is one of several methanogenic cofactors required for the biochemical reduction of carbon dioxide to methane, an important greenhouse gas. This coenzyme is also likely to be involved in anaerobic methane oxidation or reverse methanogenesis. Despite the central role of methanopterin in methane metabolism, the enzymes involved in the early steps of its biosynthesis are unknown.

The first canonical step in pterin biosynthesis by GTP cyclohydrolase I (GTPCHI) is the formation of 7,8-dihydro-D-neopterin 3′-triphosphate from GTP.[1] This reaction has been extensively studied and requires at least six discrete chemical steps, with the first being the hydrolytic opening of the imidazole ring of GTP (*1*). Enzymes known to catalyze this reaction are numerous (Figure 1). The first enzyme identified to carry out this reaction was GTPCHI, involved in folate biosynthesis (*2*). This was quickly followed by GTP cyclohydrolase II (GTPCHII), shown to be involved in riboflavin biosynthesis (*3*). Later came the enzymes involved in molybdopterin biosynthesis (*4*, *5*) and finally an archaeal signature GTP cyclohydrolase III (GTPCHIII), which adds only 1 mol of water to the imidazole ring to produce a formamide product. Currently, the function of archaeal GTP cyclohydrolase III is unknown, but it may catalyze the first step in the biosynthesis of riboflavin and coenzyme F_{420} (*6*). A GTP cyclohydrolase II orthologue in *Streptomyces coelicolor* was also recently found to have GTP cyclohydrolase III activity (*7*). Most archaea do not possess homologues of GTPCHI and GTPCHII, thus supporting the idea that GTP cyclohydrolase III is really involved in riboflavin biosynthesis. None of these enzymes are related in either sequence or structure (*8*).

The one common characteristic of these enzymes is that they all catalyze the hydrolytic addition of one water to the imidazole ring of GTP to form a formamide derivative of a triaminopyrimidine. With the exception of molybdopterin biosynthesis and GTPCHIII, a second common characteristic of each of these enzymes is the hydrolysis of the resulting formamide with the release of formate. GTPCHII and GTPCHIII also catalyze the release of the β- and γ-phosphates of GTP as pyrophosphate.

Early biosynthetic studies using labeled GTP's indicated that GTP is a precursor to pterins in the archaea (*9*, *10*). Further work implicated the involvement of 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate as an early intermediate in the pathway (*11*). Despite the demonstrated involvement of GTP in pterin biosynthesis, no homologues of either GTP cyclohydrolase I or GTP cyclohydrolase II have been identified in the *Methanocaldococcus jannaschii* genome (*12*). Recently, El Yacoubi et al. (*13*) identified a new family of prokaryotic GTP cyclohydrolase I enzymes, GTPCHIB. Many archaeal genomes possess distant homologues of the GTPCHIB protein family. El Yacoubi et al. proposed that the archaeal homologues of bacterial GTPCHIB may be involved in methanopterin biosynthesis (*13*).

In order to test for the involvement of the archaeal GTPCHIB homologue in methanopterin biosynthesis, we

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[1] Abbreviations: APCI, atmospheric pressure chemical ionization; ICP, inductively coupled plasma spectrometry; LC-MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; DTT, dithiothreitol; fapyGMP, 2-amino-5-formylamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-phosphate; GTP, guanosine 5′-triphosphate; α,β-methylene-GTP, α,β-methyleneguanosine 5′-triphosphate; β,γ-methylene-GTP, β,γ-methyleneguanosine 5′-triphosphate; GTP-γ-S, guanosine 5′-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; TES, *N*-[tris-(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid.

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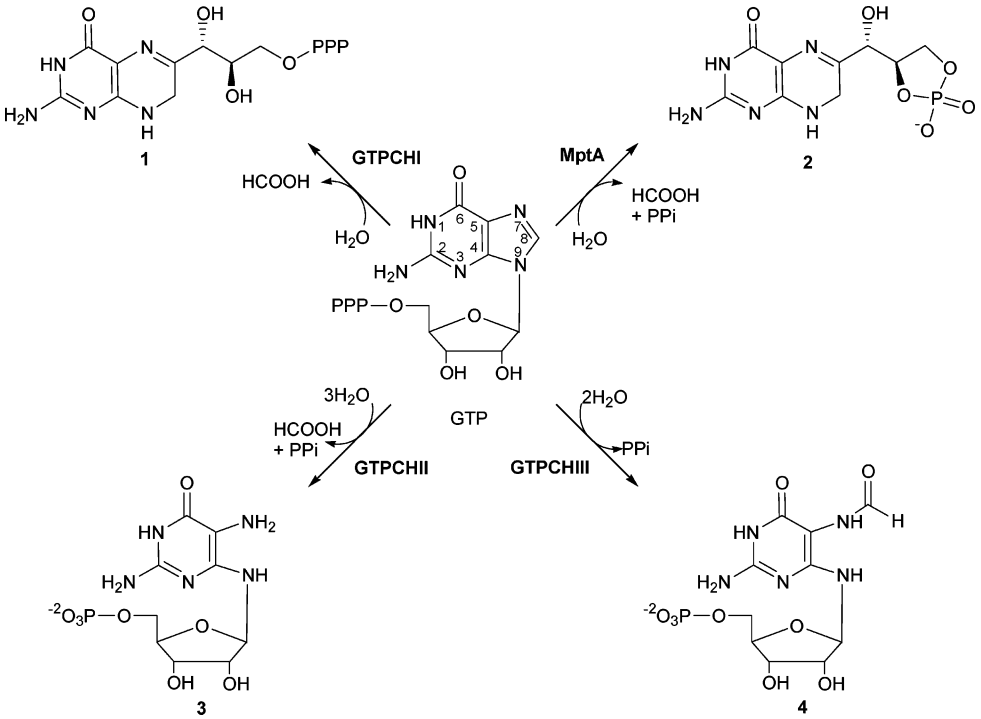

FIGURE 1: Reactions catalyzed by GTP cyclohydrolases I, II, and III and MptA. The reaction products are as follows: **1**, 7,8-dihydro-D-neopterin 3′-triphosphate; **2**, 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate; **3**, 2,5-diamino-6-ribosylamino-4-(3*H*)-pyrimidinone 5′-phosphate; **4**, 2-amino-5-formylamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-phosphate (fapyGMP).

have cloned, overexpressed, and characterized the recombinant enzyme from *M. jannaschii* (MJ0775). In this paper we describe the characterization of this new type of GTP cyclohydrolase, which we are designating MptA to indicate that it catalyzes the first step in methanopterin biosynthesis. We show that the enzyme catalyzes the conversion of GTP to 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate and pyrophosphate. On the basis of sequence, requirement for Fe^{2+}, and the unique reaction product, we conclude that this enzyme is distinct from any of the other GTPCH's. Considering the enzyme's dependency on Fe^{2+}, we propose that MptA may be the most primitive of the GTPCH's.

MATERIALS AND METHODS

Chemicals. GTP, dGTP, GMP, β,γ-methyleneguanosine 5′-triphosphate, α,β-methyleneguanosine 5′-diphosphate, and guanosine 5′-[γ-thio]triphosphate were obtained from Sigma. α,β-Methyleneguanosine 5′-triphosphate was prepared from α,β-methyleneguanosine 5′-diphosphate and ATP using nucleoside 5′-diphosphate kinase (EC 2.7.4.6) (*14*). Diammonium D-neopterin 3′-phosphate and other pterins were obtained from Schircks Laboratories, Jona, Switzerland.

Cloning and Overexpression of the M. jannaschii MJ0775 Gene in Escherichia coli. The MJ0775 gene (Swiss-Prot accession number Q58185) was amplified by PCR from genomic DNA using oligonucleotide primers pMJ0775Fwd and pMJ0775Rev (Supporting Information, Table 1). PCR amplification was performed as described previously (*15*) using a 55 °C annealing temperature. Purified PCR product was digested with *Nde*I and *Bam*HI restriction enzymes and ligated into compatible sites in plasmid pT7-7. Sequence of the resulting plasmid, pMJ0775, was verified by sequencing. pMJ0775 was transformed into *E. coli* strain BL21-Codon Plus (DE3)-PIL. Heterologous expression of the MJ0775 protein was performed by standard methods (*15*). Induction of the desired protein was confirmed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of total cellular proteins.

Construction of MptA Mutants. The Quik-Change site-directed mutagenesis kit (Stratagene) was used to construct MJ0775 mutants according to the manufacturer's instructions using template pMJ0775. Primers used for the mutants are named and shown in Supporting Information, Table 1. The correct sequences of plasmids carrying *mptA* and its mutations were confirmed by sequencing. A second, unintentional mutant site E50D was also identified in MJ0775-H200N.

Purification of Recombinant MptA. *E. coli* cells expressing recombinant protein (~0.4 g wet weight) were resuspended in 4 mL of extraction buffer [50 mM *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES), pH 7.0, 10 mM $MgCl_2$, 20 mM DTT] and lysed by sonication. After the majority of *E. coli* proteins were precipitated by heating the cell lysate to 80 °C for 10 min, the MJ0775-derived protein was purified by anion-exchange chromatography on a MonoQ HR column (1 × 8 cm; Amersham Bioscience) with a linear gradient from 0 to 1 M NaCl in 25 mM TES, pH 7.5, buffer over 55 mL at 1 mL/min. Protein concentration was determined by both Bradford analysis (*16*) and BCA protein analysis (Pierce).

Synthesis and Characterization of D-*Neopterin 2′,3′-Cyclic Phosphate*. The diammonium salt of D-neopterin 3′-phosphate (3.7 mg, 10 μmol) was dissolved in 100 μL of water, and 1.5 μL of triethylamine was added followed by 0.75 mL of methanol and 51 mg (250 μmol) of dicyclohexylcarbodiimide. The mixture was heated in a sealed vial for 30 min at 100 °C. The sample was then dissolved in 5 mL of water and extracted three times with 5 mL of diethyl ether. TLC analysis using acetonitrile−water−formic acid

(88%), 40:10:5 v/v/v as the developing solvent, showed that the water layer contained two fluorescent spots with the top $R_f = 0.2$ spot being about twice as fluorescent as the bottom $R_f = 0.15$ spot. No neopterin 3′-phosphate, which had an R_f of 0.08, was detected. ^{1}H NMR analysis (D_2O) also showed the presence of two pterins in the ratio of 2 to 1. Proton-decoupled ^{31}P NMR showed two singlets, the one at +17.8 ppm being twice as intense as the one at +1.9 ppm. On the basis of the expected phosphorus chemical shifts of the 1,2-cyclic phosphate diester (*17*), the most intense resonance corresponded to D-neopterin 2′,3′-cyclic phosphate and the less intense resonance to D-neopterin 1′,3′-cyclic phosphate. The D-neopterin 2′,3′-cyclic phosphate had δ at 8.846 (1H, s, H-7 of pterin), 4.874 (1H, d, $J_{1'\rightarrow2'} = 3.55$ Hz, H-1′), 4.84 (1H obscured by the DOH peak, H-2′), 4.204 (1H, ddd, $J_{3'\rightarrow2'} = 6.15$ Hz, $J_{3'\rightarrow3''} = 9.52$ Hz, $J_{3'\rightarrow P} = 10.8$ Hz, H-3′), and 4.122 (1H, ddd, $J_{3''\rightarrow2'} = 6.15$ Hz, $J_{3''\rightarrow3'} = 9.52$ Hz, $J_{3''\rightarrow P} = 10.9$ Hz, H-3″), and the neopterin 1′,3′-cyclic phosphate had δ at 8.825 (1H, s, H-7 of pterin), (1H, d, $J_{1'\rightarrow2'} = 7.61$ Hz, H-1′), the H-2′ and H-1′ were obscured by the water peak, 4.105 (1H, ddd, $J_{2'\rightarrow3'} = 3.9$ Hz, $J_{2'\rightarrow1'} = 7.8$ Hz, H-2′), 4.105 (1H, ddd, $J_{3'\rightarrow2'} = 2.98$ Hz, $J_{3\rightarrow P} = 5.66$ Hz, $J_{3'\rightarrow3''} = 11.11$ Hz, H-3′), and 4.057 (1H, ddd, $J_{3''\rightarrow2'} = 5.56$ Hz, $J_{3''\rightarrow P} = 5.7$ Hz, $J_{3''\rightarrow3'} = 11.11$ Hz, H-3″). Irradiation of the 17.8 ppm phosphorus resonance collapsed the 4.204 and 4.122 H-3′ and H-3″ hydrogen resonances of the D-neopterin 1′,2′-cyclic phosphate each to doublet of doublets patterns with no effect on the H-3′ and H-3″ hydrogens of the neopterin 1′,3′-cyclic phosphate. The converse was true for the radiation of the 1.9 ppm resonance of the phosphate. D-Neopterin 3′-phosphate (D_2O) showed δ at 8.84 (1H, s, H-7 of pterin), 5.002 (1H, d, $J_{1'\rightarrow2'} = 7.61$ Hz, H-1′), 4.186 (1H, dt, $J_{2'\rightarrow3'} = 3.9$ Hz, $J_{2'\rightarrow1'} = 7.8$ Hz, H-2′), and 4.078 (2H, dd, $J_{3'\rightarrow2'} = 4.0$ Hz, $J_{3'\rightarrow P} = 6.74$ Hz, H-3′).

Identification of the MptA Reaction Product. Chromatographic separation of pterins was performed on a Shimadzu HPLC System with a C18 reverse-phase column (Varian PursuitXRs, 250 × 4.6 mm, 5 μm partical size). The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% MeOH followed by a linear gradient to 20% sodium acetate buffer/80% MeOH over 40 min at 0.5 mL/min. Pterins were detected by fluorescence using an excitation wavelength of 356 nm and an emission wavelength of 450 nm. Under these conditions, pterins were eluted in the following order (minutes): neopterin 3′-phosphate (6.0), neopterin 2′-phosphate (6.5), D-neopterin 2′,3′-cyclic phosphate (8.4), neopterin (11.0), pterin (17.5), 6-hydroxymethylpterin (17.5), and 6,7-dimethylpterin (27.0).

Acid hydrolysis of the MptA reaction product was conducted by addition of 1 M HCl to an incubation mixture to a final concentration of 0.3 M. After the acidified sample was incubated for 30 min at room temperature, the sample was neutralized with 1 M NaOH. A control sample included the addition of an equivalent amount of 1 M NaCl. The neutralized and control samples were subjected to HPLC analysis as described above. Alternately, the neutralized samples were evaporated to dryness under a stream of nitrogen gas and resuspended in 0.2 mL of 0.1 M glycine buffer, pH 10.0, containing 1 mM Zn^{2+}, and 0.2 unit of *E. coli* alkaline phosphatase was added. Samples were incubated at 37 °C for 2 h and then analyzed by HPLC as described above.

In addition, HPLC−mass spectral analysis of the reaction mixture was used to confirm the identity of the MptA reaction product. HPLC-MS/MS was performed on a Thermo Finnigan Quantum Triple Quad LC-MS with atmospheric pressure chemical ionization following capillary C18 chromatography using a 150 × 2.0 mm, 3.5 μm column.

Measurement of the Enzymatic Activities of MptA. The standard assay for MptA was performed in a total volume of 50 μL containing 35 mM TES buffer, pH 7.2, 1.9 μg of MptA, 2 mM $MnCl_2$, and 2 mM GTP. Incubations containing GTP analogues were identical, except for the substitution of 2 mM analogue in place of GTP; inhibition assays included both 2 mM GTP and 2 mM potential inhibitor. Assays were incubated for 30 min at 70 °C and quenched by the addition of 60 μL of methanol. 7,8-Dihydropterin reaction products were oxidized to the fluorescent pterins by addition of 5 μL of iodine in MeOH (50 mg/mL), and the samples were incubated at room temperature for 30 min. Excess iodine was reduced by addition of 5 μL of 1 M $NaHSO_3$. Following centrifugation (14000*g*, 10 min), the supernatant was separated and combined with 600 μL of H_2O for HPLC analysis. Air oxidation of the MptA reaction product was accomplished by adding 600 μL of H_2O to the reaction mixture and shaking vigorously for about 5 min and then allowing the sample to stand at room temperature for at least 30 min prior to analysis. Assays used to determine MptA kinetic parameters were carried out in a total volume of 50 μL containing 35 mM TES buffer, pH 7.2, 1.9 μg of MptA, 2 mM $MnCl_2$, and 10−500 μM GTP. Reaction mixtures were incubated for 15 min at 70 °C and processed as described above. Reaction velocity was determined by measuring the neopterin 2′,3′-cyclic phosphate peak area and subsequent conversion to nanomoles of product based on a standard curve generated for D-neopterin. Kinetic parameters were estimated from the slope and intercepts of the Lineweaver−Burk plot using Microsoft Excel software.

Identification of a Reaction Intermediate. Imidazole ring-opened intermediates of the MptA reaction were converted into 6,7-dimethylpterin for HPLC analysis. Following incubation, the MptA reaction mixtures were subjected to acid hydrolysis for 5 min at 95 °C in 0.5 M HCl to form triaminopyrimidine. Samples were then neutralized with an equivalent amount of NaOH. 2,3-Butanedione (34 mM in 0.5 M Tris-HCl, pH 8.5) was then added to a final concentration of 12 mM, and the samples were heated at 95 °C for 45 min. The resulting 6,7-dimethylpterin was analyzed by HPLC as described above.

Alkylation of MptA and Analysis of the Alkylated Protein by LC-MS/MS. Alkylation of cysteine residues in MptA was accomplished by preincubation of 4 μg of MptA with 11 mM iodoacetamide or *N*-ethylmaleimide (NEM) in 25 mM TES buffer, pH 7.0, at room temperature for 30 min. An identical sample without the addition of iodoacetamide or NEM served as the control. Following incubation 20% of the preincubation mixtures were combined with 3 mM GTP, 3 mM $MnCl_2$, 39 mM TES/K^+ buffer, pH 7.2, 7.8 mM $MgCl_2$, and 16 mM DTT in a final volume of 32 μL and assayed for activity as described above. The remaining portion of the control or NEM-modified MptA was separated on a SDS−polyacrylamide gel. Four 1 mm^3 samples were

excised from each protein band. Samples were reduced with dithiothreitol and alkylated with iodoacetamide. An in-gel tryptic digest was performed overnight at 37 °C with gel pieces overlaid with a 25 mM NH_4HCO_3 solution. The digest was adjusted to pH ~4 with the addition of acetic acid (5% aqueous solution), and peptides were gently vortexed out of the gel for 15 min. Native and NEM-modified MptA peptides were analyzed utilizing an Ultimate capillary HPLC system (LC Packings) interfaced with a Thermo Finnigan LCQ DecaXP ion trap mass spectrometer in NSI mode. Peptides were eluted from a pulled-tip 75 i.d. capillary column packed with 4 cm of Synergi 4u Hydro-RP with a linear gradient from 95% water to 95% acetonitrile (both solvents contained 0.5% acetic acid) over 30 min at a flow rate of 100 nL/min. The most abundant peptides were collected twice, and collision-induced MS/MS spectra were obtained. MASCOT software was employed to analyze the tandem mass spectrometry peak lists. The *E. coli* database was searched with the sequence for MJ0775 from *M. jannaschii* added. Peptide identification was considered significant and correct when a minimum of three peptides with an individual ion score ≥23, each of which had a $p < 0.05$ for random matches, was observed.

Synthesis of 2-Amino-5-formylamino-6-ribofuranosylamino-4(3H)-pyrimidinone 5′-Phosphate (fapyGMP). GTP cyclohydrolase III, product of the MJ0145 gene, was used to generate fapyGMP to test as a substrate and an inhibitor of MptA (*6*). For the preparation of fapyGMP, 9.5 μg of GTPCHIII was combined with 2.3 mM GTP in a final volume of 43 μL of buffer containing 43 mM TES, pH 7.3, 8.6 mM $MgCl_2$, and 17 mM DTT. The sample was incubated at 70 °C for 30 min, and then the GTPCHIII was removed by passing the sample through a Microcon YM3 centrifugal concentrator. During this procedure most of the GTP was converted into fapyGMP. In order to test if fapyGMP was a substrate or an inhibitor for MptA, 2.4 μg of enzyme or 2.4 μg of enzyme plus 1 μL of 0.1 M GTP was added directly to the concentrator flow-through (43 μL) supplemented with 2 mM $MnCl_2$ and incubated for an additional 30 min at 70 °C. Samples were analyzed for pterins by HPLC as described above.

pH Optimum of the Recombinant Enzyme. The activity of MptA was determined at 0.5 pH increments between pH 5 and pH 10 using a three-component buffer system consisting of 75 mM BisTris, 38 mM HEPPS, and 38 mM CHES (*18*) in place of TES buffer used in the standard assay.

Temperature Stability of MptA. The temperature stability of MptA was determined by incubating 1.9 μg of enzyme in assay buffer containing 2 mM $MnCl_2$ at 70, 80, 90, or 100 °C for 10 min in sealed tubes. Following heating, the enzyme mixture was cooled on ice and centrifuged, and GTP was added. The final composition and volume of the reaction mixture were as described for the standard assay above. The samples were incubated at 70 °C for 30 min and analyzed as described above.

Metal Ion Analysis of MptA. Metal analysis of MptA was performed at the Virginia Tech Soil Testing Laboratory using inductively coupled plasma emission spectrophotometry. Instrumentation included a Spectro CirOS VISION made by Spectro Analytical Instruments equipped with a Crossflow nebulizer with a Modified Scott spray chamber; the nebulizer rate was 0.75 L/min. A 50 mg/L yttrium internal standard was introduced by a peristaltic pump. Samples were analyzed for iron, manganese, and zinc.

Measurement of the Native Molecular Weight of MptA. The native molecular weight of MptA was determined by size exclusion chromatography on a Superose 12HR column (10 mm × 300 mm) with anaerobic buffer containing 50 mM HEPES, pH 7.2, 50 mM NaCl, and 10 mM DTT at 0.5 mL/min with detection at 280 nm. Protein standards used to calibrate the column included alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

RESULTS

Purification of Recombinant MptA. The MJ0775 gene (coding for MptA) was cloned from *M. jannaschii* genomic DNA and overexpressed in *E. coli*. The resulting protein was purified by heating to 80 °C to denature the *E. coli* proteins and subsequent anion-exchange chromatography. Purified MptA was judged to be >90% pure by SDS−PAGE analysis with Coomassie staining. MptA ran in the gel at approximately 37 kDa, consistent with the predicted monomeric molecular mass of 36.3 kDa. The multimeric structure of MptA was determined by size exclusion chromatography under anaerobic conditions and was found to be 84 kDa, corresponding to a dimer.

Identification of the MptA Reaction Product. The pterin-containing product of the MptA reaction with GTP was identified as D-neopterin 2′,3′-cyclic phosphate (compound **2**, Figure 1). HPLC analysis of the oxidized MptA reaction mixture with fluorescence detection at pterin excitation and emission wavelengths identified the major peak with an elution time that corresponded to authentic D-neopterin 2′,3′-cyclic phosphate.

The two synthetic isomers of neopterin cyclic phosphate were separated by preparative TLC using the TLC solvent acetonitrile−water−formic acid (88%), 40:10:5 v/v/v. The R_f of the MptA reaction product was identical to that of the D-neopterin 2′,3′-cyclic phosphate. LC-ES-MS of the MptA reaction mixture as well as the preparative TLC-separated synthetic neopterin cyclic phosphate isomers each showed $M - H^- = m/z$ 314 and $MH^+ = m/z$ 316. LC-APCI-MS/MS of the M − phosphoric acid fragment ion at m/z 218 was used to further confirm the identity of these compounds.

The presence of a 2′,3′-cyclic phosphate in the MptA reaction product was further confirmed by its resistance to alkaline phosphatase until after mild acid hydrolysis, which is consistent with the presence of 5-membered cyclic phosphate diester (*19*). It was possible to hydrolyze the 2′,3′-cyclic phosphate by treatment of the reaction mixture with 0.3 M HCl at room temperature for 30 min. Such hydrolysis resulted in the formation of two new peaks in the HPLC trace at 6.0 and 6.5 min in a ratio of 1:4. The first of these peaks was identified as D-neopterin 3′-phosphate based on retention time and fluorescence as compared to a known sample, and the second peak is presumably D-neopterin 2′-phosphate. Mild acid hydrolysis of the reaction mixture followed by neutralization and incubation with alkaline phosphatase resulted in the removal of the phosphate and formation of D-neopterin, identified by HPLC analysis.

It has been demonstrated that neopterin 3′-triphosphate can be chemically converted into neopterin 2′,3′-cyclic phosphate

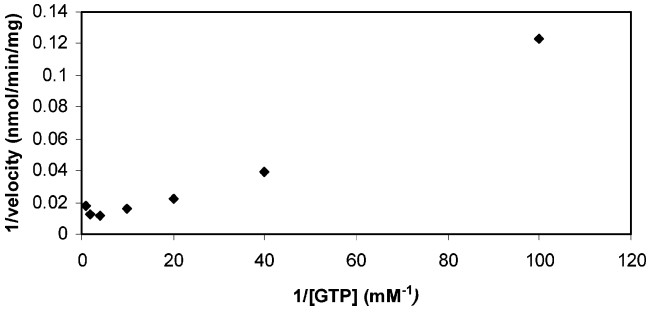

FIGURE 2: Lineweaver−Burk plot of MptA activity.

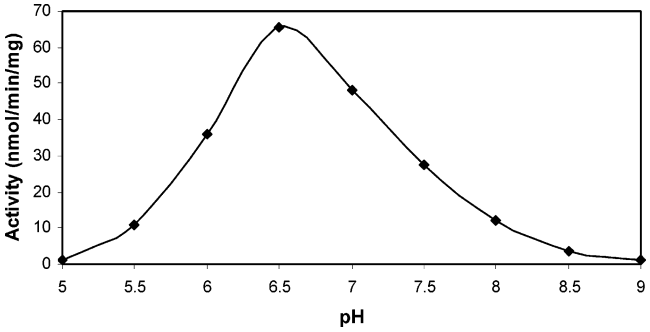

FIGURE 3: pH profile of MptA activity.

when exposed to basic work-up conditions or certain chromatographic conditions (*20*, *21*). Such a transformation could explain our detection of neopterin 2′,3′-cyclic phosphate. However, because all of our manipulations were performed at or near neutral pH, that is not conducive to such cyclization, such a side reaction appears very unlikely. Additionally, the neopterin 2′,3′-cyclic phosphate was the only product observed following air oxidation of the dihydropterin product that involved no changes in the pH of the solution or exposure to other reagents, indicating that it is the true product of the MptA-catalyzed reaction.

Detection of Reaction Intermediates. The MptA reaction is expected to proceed through triaminopyrimidine intermediates. In order to test for the presence of such intermediates, a reaction mixture was subjected to acid hydrolysis in 0.5 M HCl followed by derivitization of the released 2,4,5-triamino-4(1*H*)-pyrimidinone with 2,3-butanedione to form 6,7-dimethylpterin. The levels of the triaminopyrimidines detected were observed to be at 10−15% of the corresponding neopterin 2′,3′-cyclic phosphate levels when either GTP or β,γ-methylene-GTP was used as substrate. Incubation of MptA with α,β-methylene-GTP did not result in the formation of any triaminopyrimidines. Due to the use of an acid hydrolysis step that would also hydrolyze any formamide, it was not possible to determine if the observed intermediate contained a formamide moiety.

Catalytic Properties of MptA. Steady-state kinetic analysis of the MptA reaction showed that substrate inhibition was occurring at GTP concentrations greater than 0.3 mM (Figure 2). For this reason, data for substrate concentrations less than 0.25 mM were used for the calculation of apparent K_m and V_{max}. The kinetic parameters for MptA are as follows: $K_M^{app} = 96\ \mu M$, $V_{max} = 0.12\ \mu mol\ min^{-1}\ mg^{-1}$, and $k_{cat}/K_M^{app} = 750\ M^{-1}\ s^{-1}$. The standard assay for MptA included GTP concentrations sufficient for substrate inhibition and is reflected in the lower specific activities reported in other

Table 1: Specific Activity of MptA with GTP and GTP Analogues

substrate	specific activity (nmol min^{-1} mg^{-1})	substrate	specific activity (nmol min^{-1} mg^{-1})
GTP	30	GMP	<0.001
GDP	13	ATP	<0.001
GTP-γ-S	39	ITP	<0.001
β,γ-methylene-GTP	3	fapyGMP	<0.001
dGTP	<0.001		

Table 2: Effect of Divalent Metal Ions on the Specific Activity (nmol min^{-1} mg^{-1}) of MptA

	concn of metal[a]		
	20 μM	200 μM	2000 μM
Fe^{2+}	7	25	49
Mn^{2+}	4	6	74
Zn^{2+}	ND[b]	ND[b]	4

[a] Incubations were conducted as described in the text. [b] ND is not detected.

experiments (Tables 1−4). The specific activity of MptA was about 10 times higher than that reported for bacterial GTPCHIB homologues (*13*). MptA was found to have a sharp pH activity profile with maximum activity at pH 6.5 (Figure 3). Relative to a sample of the enzyme heated at 70 °C for 10 min, MptA lost 27% activity when it was incubated for 10 min at 80 °C, 60% activity at 90 °C, and 89% activity at 100 °C.

Specificity of the MptA Reaction. MptA was able to utilize a variety of GTP analogues as substrates, including GDP, β,γ-methyleneguanosine 5′-triphosphate, and guanosine 5′-[γ-thio]triphosphate, for the generation of dihydroneopterin 2′,3′-cyclic phosphate (Table 1). Other purine nucleotides including ATP, ITP, dGTP, GMP, and 7-methyl-GTP did not serve as substrates for MptA as no fluorescent products were observed by HPLC following incubation with the enzyme. Partial inhibition of MptA activity was observed when 2 mM GMP, dGTP, or 7-methyl-GTP was included along with 2 mM GTP in the incubation mixture (43%, 28%, and 36% inhibition, respectively). Similarly, fapyGMP was tested as both a substrate and an inhibitor of MptA. It was found that although fapyGMP does not serve as a substrate, it did act as an inhibitor, and coincubation of MptA with fapyGMP (∼1 mM) and GTP (2 mM) resulted in a 5-fold reduction in activity.

Metal Ion Dependency of the MptA Reaction. MptA was found to readily lose activity upon exposure to air. Some activity could be recovered after aerobic anion-exchange purification as described in Materials and Methods; however, in the absence of DTT, MptA lost all activity within 4 days at −20 °C. This activity could be restored by adding specific divalent metal ions to the oxygen-inactivated enzyme under anaerobic conditions in the presence of DTT. Only Fe^{2+} was capable of restoring activity at the physiologically relevant concentrations of 20 μM; however, higher concentrations of Mn^{2+} (200 μM) were able to restore activity to 24% of that seen with the ferrous ion (Table 2). Interestingly, at very high concentrations (2 mM) Mn^{2+} was able to restore MptA activity to a higher level than Fe^{2+} (Table 2). As the Mn^{2+} enzyme is not inactivated by exposure to oxygen, most characterization experiments were done with Mn^{2+}-activated enzyme because it did not require the maintenance of

Table 3: Restoration of Air-Inactivated MptA Activity with Different Reductants

	specific activity (nmol min^{-1} mg^{-1})
no reductant	<0.01
2 mM Fe^{2+}	47
20 mM dithionite	17
20 mM ascorbic acid	8
12 mM DTT	8

Table 4: Specific Activity and Iron Content of MptA Mutants

	specific activity (nmol min^{-1} mg^{-1})	Fe per protomer
MptA wild type	36	1.0
MptA H101N	36	1.3
MptA H200N	2	1.2
MptA H293N	3	0.9
MptA H295N	1	*a*

[a] The iron content of MptA H295N was not determined.

anaerobic conditions. Although zinc was found to restore some activity at high concentrations (2 mM, Table 2), the level of activity was such that the involvement of trace amounts of Fe^{2+} or Mn^{2+} cannot be ruled out.

It was found that the addition of reducing agents to the assay mixture was able to partially restore activity in oxygen-inactivated MptA (Table 3). Dithionite appeared to restore MptA activity to 37% that observed with the addition of 2 mM Fe^{2+}; however, addition of dithionite resulted in the production of a complicated HPLC trace, indicating that nonenzymatic reactions were occurring. The addition of ascorbic acid or DTT to the MptA reaction mixture also restored some of the activity, 17% relative to Fe^{2+} addition, and did not result in the complicated mixture of reaction products seen with dithionite.

Metals Present in MptA. ICP was used to determine the identity and quantity of metal ions present in the purified enzyme prior to activation. MptA was found to contain iron in a 1:1 molar ratio per protomer using both the Bradford and BCA protein assays to measure the protein concentration. MptA was also analyzed for manganese and zinc, and although no manganese was found, a 0.5:1 molar ratio of zinc to MptA monomer was measured. The metal content of three histidine mutants of MptA (H101N, H200N, H293N) was also determined and found to be similar to that observed for the wild type (Table 4). The zinc content of the mutants was also similar to that of the wild type, with the exception of H101N which had an increased amount of zinc (1:1 molar ratio of zinc to monomer).

Identification of Catalytically Important Residues. Alignment of MptA homologues from several archaea allowed for the identification of four conserved histidine residues, H101, H200, H293, and H295. Of these four histidines, the last three were universally conserved among members of COG1469 including both bacterial and archaeal members. The first residue, H101, was found to be conserved in a majority of the archaeal members of COG1469 but not the bacterial members. Among the archaeal MptA homologues analyzed, a few members including members of genus *Pyrococcus* and *Thermococcus* possessed a histidine residue that was shifted C-terminal by one residue from *M. jannaschii* H101 while a few archaea, including members of genus *Thermoplasma*, do not have a histidine in this region.

In order to investigate the potential involvement of each of these histidine residues in catalysis or metal binding, each was mutated to an asparagine. Each of the four single mutations were heterologously expressed, purified, and analyzed for metal composition and MptA activity. We found that one mutation, H295N, resulted in a majority of the protein being expressed in an insoluble form; however, an adequate amount of protein was in the soluble form for enzymatic but not metal analysis. Mutation of each of the three universally conserved histidines (H200, H293, H295) resulted in a decrease in the specific activity of the enzyme (Table 4). Mutation of the fourth residue (H101) that was partially conserved in the archaea did not affect the ability of the enzyme to form dihydro-D-neopterin 2′,3′-cyclic phosphate nor did it have a reduced amount of iron. Mutant H200N was found to also contain an additional mutation, E50D; however, this mutation is not believed to be responsible for the decreased activity because a MptA E50D single mutation did not affect the specific activity of the enzyme (data not shown).

In addition to the conserved histidine residues, members of COG1469, including MptA, contain a conserved cysteine residue, C163 in *M. jannaschii* MptA. We proposed that C163 may be catalytically important and, as such, alkylation of this residue would be expected to inhibit the enzyme. When incubated with NEM, MptA lost 67% activity, suggesting the presence of a catalytically important cysteine residue. Under the same conditions iodoacetamide reduced activity by 33%. LC-MS/MS analysis of tryptic digests of NEM-modified and unmodified MptA indicated that the conserved C163 residue was not modified by NEM, nor was C165 that is conserved in a subgroup of COG1469 that includes the methanogens. The one cysteine residue that was found to be modified by NEM was C8, which is not conserved across members of COG1469. MptA contains two other cysteines, C90 and C261, that are conserved in MptA homologues from methanogens. Unfortunately, fragments containing these residues were not detected by LC-MS/MS, and as a result it could not be established if these residues were alkylated by the NEM.

DISCUSSION

Relationship of MptA to Other GTPCH's. MptA is unique among the known GTP cyclohydrolases in several regards. The most obvious difference between MptA and the other GTP cyclohydrolases is its primary sequence. Members of COG1469, including MptA, have no recognizable sequence similarity to either GTPCHI (FolE), GTPCHII (RibA), or GTPCHIII. A recent bioinformatic analysis of pterin biosynthetic clusters in bacteria identified COG1469 as a potential GTPCHI (*13*). Several bacterial representatives of COG1469 were subsequently shown to catalyze the formation of 7,8-dihydroneopterin 3′-triphosphate and designated GTPCHIB (*13*). Although MptA is a member of this COG, a phylogenetic analysis of this protein family using the Neighbor−Joining method shows that the archaeal members containing MptA are clearly distinct from the bacterial members containing GTPCHIB (Figure 4). Phylogenetic analysis utilizing Parsimony (PAUP program) produced a

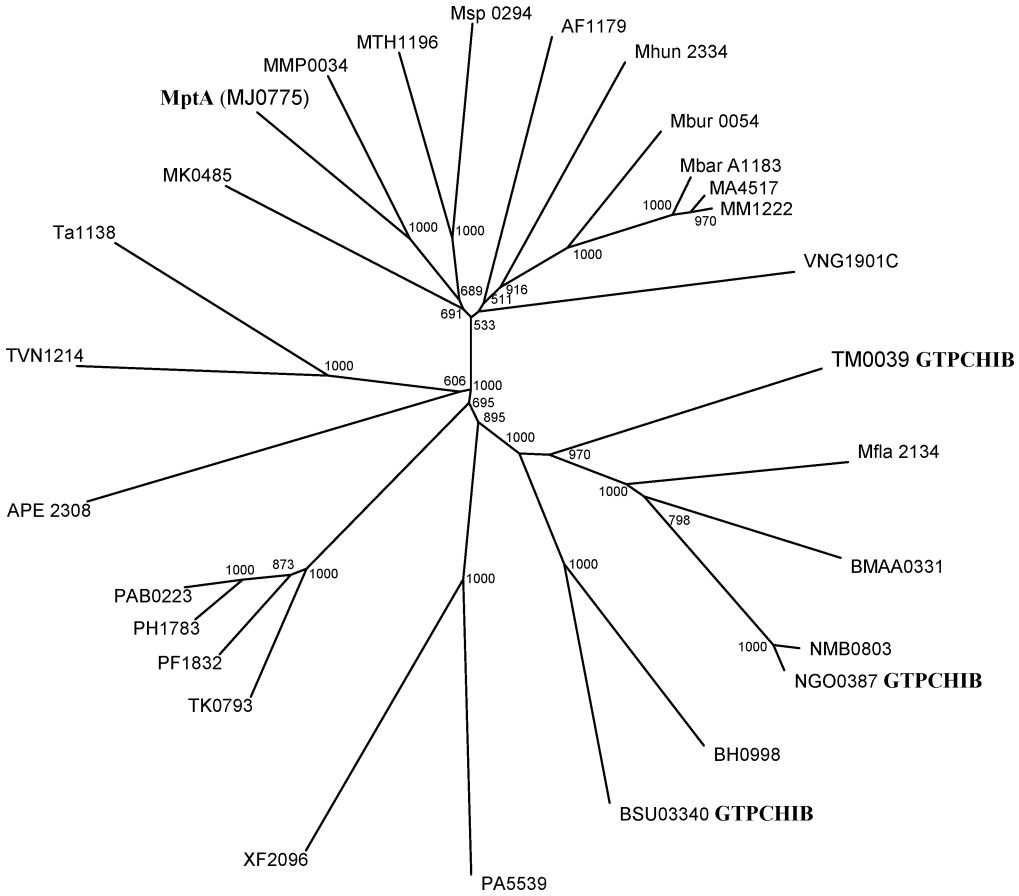

FIGURE 4: Phylogenetic tree of COG1469 GTP cyclohydrolases. The phylogenetic tree was generated using the Neighbor−Joining method with ClustalX and drawn with TreeView. Bootstrap analysis was performed using the ClustalX program and is based on 1000 iterations. Proteins previously determined to have GTPCHIB activity are indicated. Protein sequences used to generate the tree are as follows: MJ0775, *Methanocaldococcus jannaschii* DSM 2661, gi:15668956; MMP0034, *Methanococcus maripaludis*, gi:115305917; MTH1196, *Methanothermobacter thermautotrophicus* str. Delta H, gi:88953299; Msp_0294, *Methanosphaera stadtmanae* DSM 3091, gi:84489115; MK0485, *Methanopyrus kandleri*, gi:114158577; NGO0387, *Neisseria gonorrhoeae* FA 1090, gi:66773830; NMB0803, *Neisseria meningitidis* MC58, gi:15676701; BSU03340, *Bacillus subtilis*, gi:59800463; BH0998, *Bacillus halodurans* C-125, gi:15613561; TM0039, *Thermotoga maritima* MSB8, gi:15642814; PA5539, *Pseudomonas aeruginosa* PAO1, gi:15600732; XF2096, *Xylella fastidiosa* 9a5c, gi:15838687; PF1832, *Pyrococcus furiosus*, gi:108884804; PH1783, *Pyrococcus horikoshii*, gi:62291742; PAB0223, *Pyrococcus abyssi*, gi:68828933; TK0793, *Thermococcus kodakarensis* KOD1, gi:62511150; TVN1214, *Thermoplasma volcanium* GSS1, gi:13542045; Ta1138, *Thermoplasma acidophilum*, gi:110288498; APE2308, *Aeropyrum pernix*, gi:62292366; VNG1901C, *Halobacterium* sp. NRC-1, gi:15790790; Mbur_0054, *Methanococcoides burtonii* DSM 6242, gi:91772134; Mbar_A1183, *Methanosarcina barkeri* str. Fusaro, gi:73668716; MA4517, *Methanosarcina acetivorans*, gi:92081595; MM1222, *Methanosarcina mazei*, gi:85718589; Mhun_2334, *Methanospirillum hungatei* JF-1, gi:88603579; AF1179, *Archaeoglobus fulgidus*, gi:11498779; Mfla 2134, *Methylobacillus flagellatus*, gi:91776486; BMAA0331, *Burkholderia mallei* ATCC 23344, gi:53716442.

phylogenetic tree with the same monophyletic topology as the tree generated by the Neighbor-Joining method. Our characterization here of an archaeal member of COG1469 demonstrates that, despite the sequence similarities, MptA produces a unique reaction product, 7,8-dihydroneopterin 2′,3′-cyclic phosphate. This confirms the phylogenetic distinction between the bacterial and archaeal members of this protein family and supports a different designation for this enzyme.

Bacterial GTPCHI's have been observed to exist as both homooctamers and homodecamers (*22*−*25*). However, MptA was found to exist as a dimer, the same as with GTP cyclohydrolase II and the plant GTP cyclohydrolase I (*21*). GTP cyclohydrolase III is reported to be a trimer (*6*).

Proposed Mechanisms for the Reactions Catalyzed by MptA. Each of the four known GTP cyclohydrolases produces a different reaction product (Figure 1). Despite their different final products, similar reaction mechanisms are expected to be utilized by the different GTP cyclohydrolases. *M. jannaschii* MptA catalyzes the formation of 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate (compound **2**, Figure 1), a product that has been shown to be an intermediate in methanopterin biosynthesis (*11*) and is metabolized to 7,8-dihydro-D-neopterin and 7,8-dihydro-6-hydroxymethylpterin in *M. jannaschii* (unpublished results). Of each of the GTP cyclohydrolase reaction products, the MptA reaction product most closely resembles that produced by GTPCHI, indicating that it may be produced by MptA using a similar series of reactions.

Studies of *E. coli* GTP cyclohydrolase I (FolE; EC 3.5.4.16) suggest that the enzyme uses a zinc-bound water to produce a zinc-bound hydroxyl ion that initiates the reversible opening of the imidazole ring of GTP by nucleophilic attack at C8 (*26*). The intermediate produced by this first hydrolysis reaction, 2-amino-5-formylamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone triphosphate, is rapidly hydrolyzed by FolE through a repeat of the initial reaction to release formate and produce 2,5-diamino-6-ribofuranosyl-

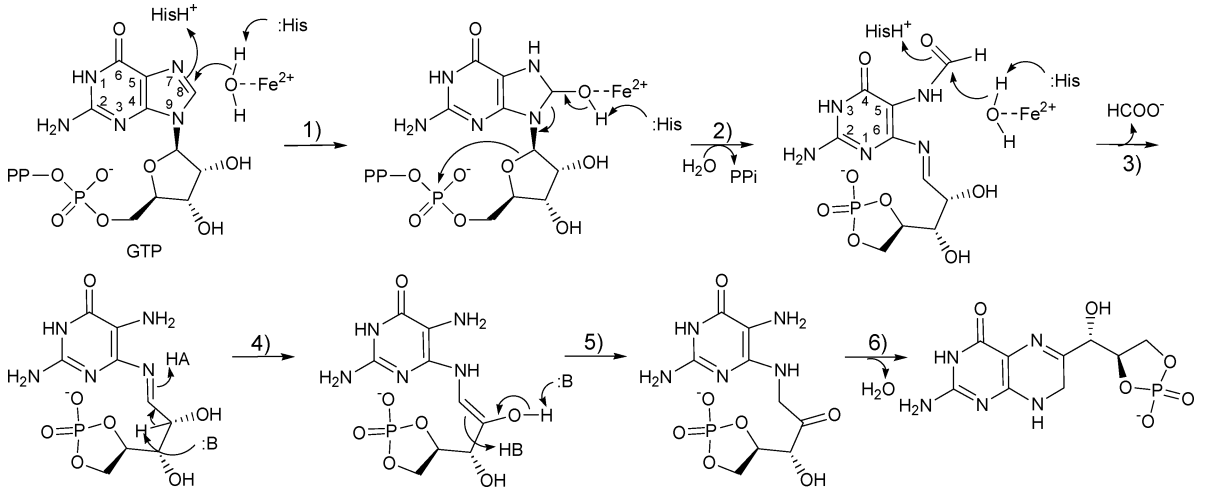

FIGURE 5: Proposed chemical steps in the MptA reaction. The second step is a concerted mechanism involving the hydrolysis of the imidazole ring and formation of the cyclic phosphate. A third step is then required for hydrolysis of the formate.

amino-4(*3H*)-pyrimidinone 5′-triphosphate (*1*, *27*, *28*). The FolE protein subsequently produces 7,8-dihydro-D-neopterin 3′-triphosphate through a slow, multistep series of intramolecular rearrangements (*1*, *29*). A H179 mutant of the *E. coli* GTPCHI, that is unable to form 2,5-diamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-triphosphate, readily catalyzed the reversible conversion of GTP to 2-amino-5-formylamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-triphosphate with a K_{eq} of 0.1 (*30*). Although the complete biosynthesis of 7,8-dihydro-D-neopterin 3′-triphosphate catalyzed by FolE is a slow reaction (with a turnover number of 0.05 s^{-1}), rates for the partial reactions of ring opening and formate release are relatively fast (with respective rate constants of 0.9 and 2.0 s^{-1}) (*29*).

GTPCHII releases pyrophosphate from GTP and hydrolytically opens the imidazole ring to form 2,5-diamino-6-ribosylamino-4-(3*H*)-pyrimidinone 5′-phosphate by way of a formylaminopyrimidine-containing intermediate (*31*). Although the total number of reactions leading to this final product is less than that of GTPCHI, it also proceeds slowly with an apparent rate constant of 0.06 s^{-1} (*32*). The release of pyrophosphate by GTPCHII has been proposed to be the rate-limiting step (*33*). This would also appear to be the case for MptA as it has a similarly low turnover number (0.07 s^{-1}), and use of the substrate analogue with a γ-thiophosphate resulted in an increase in the specific activity of the enzyme (Table 1). This may be due to the decrease in the apparent pK_a of the pyrophosphate sulfur analogue. The thiol would contribute to a decrease in the apparent pK_a for the pyrophosphate (*34*). Similarly, use of β,γ-methylene-GTP resulted in a lower specific activity (Table 1).

One important consideration of the MptA reaction is the order in which the different steps in the overall sequence of reactions occur. The MptA reaction mechanism would have to consist of at least five distinct named chemical reactions, two separate hydrolytic reactions releasing the C8 of the imidazole ring as formate, an Amadori rearrangement of the ribose, cyclization of the Amadori product to a dihydroneopterin, and formation of the cyclic phosphate. All but the last of these steps must also occur with GTPCHI. Because of chemical constraints, hydrolysis of the N9−C8 bond must occur either prior to or simultaneous with the opening of the furanoside ring. Likewise, the opening of the furanoside ring must occur before or during the formation of the cyclic phosphate. Only in this way will the C4 hydroxyl group of the ribose be made available for the formation of the cyclic phosphate. The hydrolysis of the N9−C8 bond, the opening of the furanoside ring, and the formation of the cyclic phosphate diester can all occur in a concerted mechanism after the first addition of the iron-bound water to the imidazole ring as shown in reaction 2, Figure 5. In this manner the free energy of hydrolysis of the α,β-phosphate anhydride can be used to drive the reaction.

An alternate route for the sequence of reactions leading to product would begin by the initial hydrolysis of the N7−C8 bond of the GTP. A concerted hydrolytic removal of formate from the 5-formamide group of the pyrimidine would proceed to produce the same product generated as reaction 3, Figure 5.

The sequence of reactions shown in Figure 5 was supported by the detection of a small amount of a ring-opened triaminopyrimidine intermediate when either GTP or β,γ-methylene-GTP was used as substrate and its absence when α,β-methylene-GTP, which cannot form the cyclic phosphate, was used as substrate. As dihydroneopterin formation cannot occur until the C8 carbon is removed as formate, removal of the formate must be the third step in the reaction, followed by the isomerization of the imine, the first step in the final cyclization reaction leading to the formation of the dihydropterin. On the basis of this information and analogy to the GTPCHI reaction sequence, we proposed the reaction sequence shown in Figure 5 to explain the mechanism of MptA.

Metal Ion Dependency of the MptA Reaction. A third unique feature of *M. jannaschii* MptA is its requirement for Fe^{2+}. Although GTPCHI and GTPCHII are also metal-dependent enzymes, they have only been studied with Zn^{2+} as the catalytic metal (*33*). Recombinant GTP cyclohydrolase III was originally reported to only require Mg^{2+} to function (*6*). We have now analyzed the metal content of the recombinant GTPCHIII (MJ0145) and determined that it is also likely a Fe^{2+}-dependent enzyme (unpublished data). Thus MptA is not the only Fe^{2+}-dependent GTPCH.

The first indication of a Fe^{2+} requirement for MptA was the loss of enzymatic activity observed after exposure of the enzyme to aerobic conditions during purification. Subsequent

studies found that at high concentrations either Mn^{2+} or Fe^{2+} was able to restore enzyme activity. At physiologically relevant concentrations, however, only Fe^{2+} was able to restore the activity of MptA. The ability of the enzyme to use Fe^{2+} and Mn^{2+} but not Fe^{3+} is consistent with the acid dissociation constants of the aquo complex of metallic cations where Fe^{2+} has a pK_a of 10.1 and Mn^{2+} a pK_a of 10.7 while Fe^{3+} has a pK_a of 2.2 (*35*).

ICP analysis of purified MptA indicated the presence of 1 mol of iron per protomer. This analysis was confirmed by the measurement of Fe^{2+} with *o*-phenanthroline (data not shown) (*36*). Although ICP analysis was able to detect the presence of iron, the location and ligands of the iron are still not known. Because the enzyme became inactivated in the presence of oxygen, and addition of Fe^{2+} was required for reactivation, we can conclude that the iron needs to be in the reduced state. This was also supported by the observed partial restoration of activity by the reduction of the Fe^{3+} with either DTT or dithionite (Table 3).

Only a few amide hydrolytic enzymes are known to function with a non-heme iron. Among these is *E. coli* cytosine deaminase (*37*), an enzyme that was only shown to require Fe^{2+} after its initial characterization (*36*). *E. coli* peptide deformylase was also initially considered to be a Zn enzyme, but later Fe^{2+} was found to be the physiologically relevant metal (*38*). To be added to this list are thioesterases containing Fe^{2+} (*39*), methionyl aminopeptidase (*40*), and atrazine chlorohydrolases (*41*).

Many non-heme iron enzymes have been shown to contain a 2-His-1-carboxylate facial triad (*42*) containing two histidines and one carboxylate in an array encompassing one face of an octahedrally bound iron. Analysis of the aligned sequences of both archaeal and bacterial members of COG1469 identified three histidine residues, three glutamate residues, and one aspartate residue that are conserved and are candidates for serving as the iron ligands in such a triad. Although mutation of each of the three conserved histidine residues in MptA resulted in a 10-fold reduction in specific activity, the amount of bound iron was not affected, and the role of these residues in iron binding and/or catalysis is not clear. Consequently, the organization of the metal in the active site has not yet been determined, and additional crystallographic and spectroscopic studies are underway to determine the nature of the iron center in MptA.

The effect of other non-transition-state metal ions also differed between MptA and the other GTPCHI's. Other known GTPCHI enzymes have exhibited varying responses to the presence of potassium and magnesium in the reaction mixtures (*22*, *23*, *43*). The addition of KCl to the MptA reaction mixture did not affect the enzyme's activity. MptA differed from other GTP cyclohydrolases in its requirement for Mg^{2+} ions for activity. *E. coli* GTP cyclohydrolase I activity was found to be dependent on the concentration of metal-free GTP, and divalent metal ions, including Mg^{2+}, inhibited the reaction (*43*). *Nocardia* sp. GTP cyclohydrolase I exhibited decreased activity with the addition of high concentrations (5 mM) of metal ions (*22*). Conversely, addition of Mg^{2+} to the MptA reaction mixture did not affect enzyme activity.

It has been proposed that prebiotic and the earliest biochemical reactions were able to utilize the abundant and soluble ferrous ion (*44*). This may have been initially true for all of the GTPCH's. The reason some of the present day aerobic GTPCH's use Zn^{2+} instead of Fe^{2+} may have been the emergence of an oxygen atmosphere resulting in the oxidation of Fe^{2+} to insoluble Fe^{3+}. Instead of evolving a system to reduce the resulting inactive ferric ion back to ferrous ion, many organisms may have simply elected to use a non-redox-active metal such as Zn^{2+}. *M. jannaschii* as well as the other methanogens is an obligate anaerobe and, as such, lives in an environment rich in soluble ferrous ions and thus did not experience the evolutionary pressure against the use of ferrous ion. Consequently, MptA may represent a very ancient form of the GTP cyclohydrolases.

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SUPPORTING INFORMATION AVAILABLE

Sequences of primers used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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