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An Fe^{2+}-Dependent Cyclic Phosphodiesterase Catalyzes the Hydrolysis of 7,8-Dihydro-D-neopterin 2′,3′-Cyclic Phosphate in Methanopterin Biosynthesis[†]

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ABSTRACT: 7,8-Dihydro-D-neopterin 2′,3′-cyclic phosphate (H_2N-cP) is the first intermediate in biosynthesis of the pterin portion of tetrahydromethanopterin (H_4MPT), a C_1 carrier coenzyme first identified in the methanogenic archaea. This intermediate is produced from GTP by MptA (MJ0775 gene product), a new class of GTP cyclohydrolase I [Grochowski, L. L., Xu, H., Leung, K., and White, R. H. (2007) *Biochemistry 46*, 6658−6667]. Here we report the identification of a cyclic phosphodiesterase that hydrolyzes the cyclic phosphate of H_2N-cP and converts it to a mixture of 7,8-dihydro-D-neopterin 2′-monophosphate and 7,8-dihydro-D-neopterin 3′-monophosphate. The enzyme from *Methanocaldococcus jannachii* is designated MptB (MJ0837 gene product) to indicate that it catalyzes the second step of the biosynthesis of methanopterin. MptB is a member of the HD domain superfamily of enzymes, which require divalent metals for activity. Direct metal analysis of the recombinant enzyme demonstrated that MptB contained 1.0 mol of zinc and 0.8 mol of iron per protomer. MptB requires Fe^{2+} for activity, the same as observed for MptA. Thus the first two enzymes involved in H_4MPT biosynthesis in the archaea are Fe^{2+} dependent.

Methanopterin is one of a series of structurally related cofactors that serve as C_1 carrier coenzymes in methanogenesis: the conversion of CO_2 and acetate to methane (*1*). In addition to its functioning in the methanogens in methane production, it also serves as a substitute cofactor for many of the enzymes where the canonical C_1 carrier coenzyme folate would function (*2*). This cofactor substitution is required because folate is not found in most methanogenic archaea (*3*). The one known exception is the recently confirmed presence of tetrahydrofolate-dependent enzymes in *Methanosarcia barkeri* (*4*). GTP[1] is known to be the precursor for the synthesis of the pterin portions of both of these coenzymes.

A review of fully sequenced and annotated genomes of methanogenic archaea revealed that homologues of the genes of folate biosynthetic enzymes were absent in the methanogens despite the fact that cell extracts of methanogens readily converted GTP to 6-hydroxymethyl-7,8-dihydropterin with 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate (H_2N-cP) and 7,8-dihydro-D-neopterin (H_2N) serving as intermediates (*5*, *6*). A bioinformatics analysis of the different archaea genomes identified several genes that may be involved in archaeal pterin biosynthesis (*7*). Using this information it was established that the first enzyme in the archaeal pterin pathway is a new type of GTP cyclohydrolase, MptA, coded by the MJ0775 gene in *Methanocaldococcus jannaschii* (*8*). Unlike the analogous GTP cyclohydrolase I enzymes found in bacteria where the product is 7,8-dihydroneopterin 3′-triphosphate, the product of the *M. jannaschii* enzyme is H_2N-cP. Other known GTP cyclohydrolases have been characterized as Zn^{2+}-dependent enzymes (*9*), but MptA was found to be unique in its Fe^{2+} requirement for activity. To process the H_2N-cP product of MptA to 7,8-dihydro-D-neopterin, the cyclic phosphodiester must be hydrolyzed. Here we report the identification of a cyclic phosphodiesterase in *M. jannaschii*, MptB, coded by the MJ0837 gene that hydrolyzes this five-membered cyclic phosphodiester to produce both 7,8-dihydro-D-neopterin 2′-phosphate (H_2N-2′-P) and 7,8-dihydro-D-neopterin 3′-phosphate (H_2N-3′-P) (Figure 1). The MJ0837 gene product is named MptB because it catalyzes the second step in the methanopterin biosynthetic pathway.

MATERIALS AND METHODS

Chemicals. Diammonium D-neopterin 3′-phosphate, 7,8-dihydro-D-neopterin 3′-phosphate, and other pterins were obtained from Schircks Laboratories, Jona, Switzerland. GTP, ATP, 2′,3′-cAMP, 3′,5′-cAMP, 3′,5′-cGMP, bis(4-nitrophenyl) phosphate, *O*-(4-nitrophenylphosphoryl)choline, and all other chemicals were obtained from Sigma. 4′,5′-cFMN was prepared as previously described (*10*).

Cloning and Recombinant Expression of MptB and Its Mutants. The MJ0837 gene which encodes the protein identified by Swiss-Prot accession number Q58247 (*11*) was amplified by PCR from genomic DNA (*12*) using oligonucleotide primers pMJ0837Fwd, 5′-GGTCATATGGAGAGGTTAATAAAATTG-3′, and pMJ0837Rev, 5′-GCTGGATCCTTAATTGGATTCTTTATTTTC-3′. Purified PCR product was digested with *Nde*I and *Bam*HI restriction enzymes. DNA fragments were ligated into compatible sites

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[1]Abbreviations: H_2N-cP, 7,8-dihydro-D-neopterin 2′,3′-cyclic phosphate; H_2N-2′-P, 7,8-dihydro-D-neopterin 2′-phosphate; H_2N-3′-P, 7,8-dihydro-D-neopterin 3′-phosphate; GTP, guanosine 5′-triphosphate; 2′,3′-cAMP, adenosine 2′,3′-cyclic phosphate; PDE, phosphodiesterase; bis-pNPP, bis(4-nitrophenyl) phosphate; pNPPC, 4-nitrophenylphosphorylcholine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; ICPES, inductively coupled plasma emission spectrophotometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAP, purple acid phosphatase.

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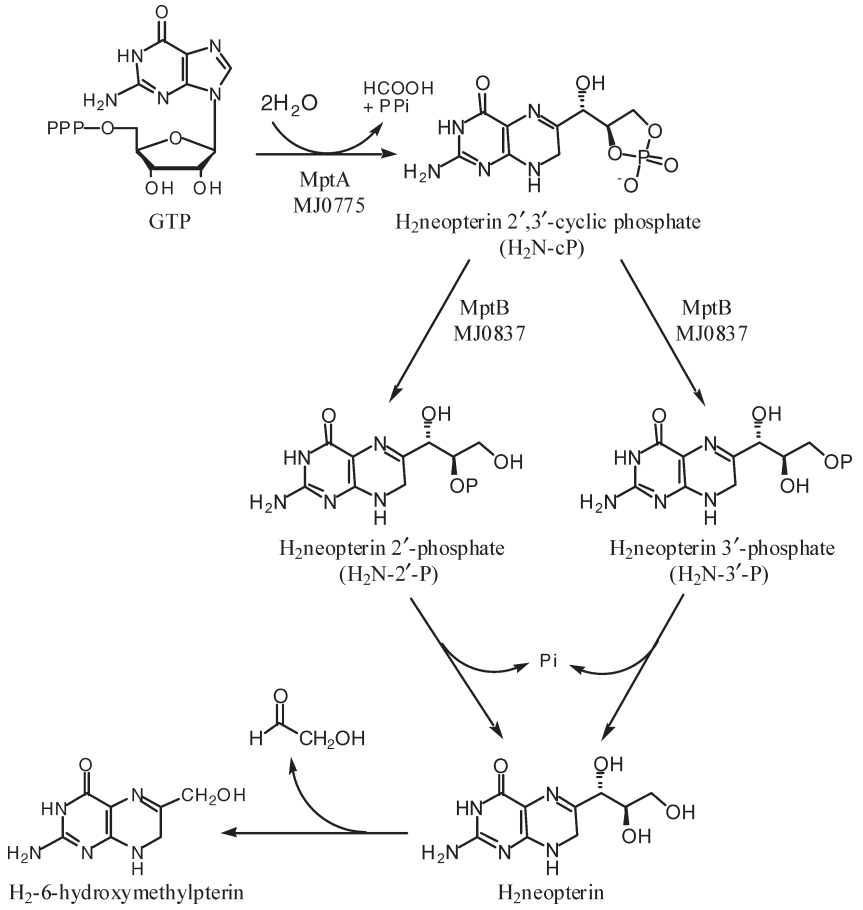

FIGURE 1: The early steps of the methanopterin biosynthetic pathway. GTP is the precursor for the synthesis of the pterin portion of the methanopterin. MptA, which is a GTP cyclohydrolase, catalyzes conversion of GTP to H_2N-cP. MptB catalyzes the second step, hydrolysis of the cyclic phosphate into two products, H_2N-2′-P and H_2N-3′-P. One or two phosphatase(s) and an aldolase, that have yet to be identified, catalyze the next two steps to give H_2-6-hydroxymethylpterin.

in plasmid pT7-7. Recombinant plasmid, pMJ0837, was transformed into *Escherichia coli* strain BL21-Codon Plus (DE3)-RIL. Expression of the MptB was performed as described previously (*12*). Induction of MJ0837 was confirmed by SDS−PAGE analysis of the cellular proteins. The plasmid DNA sequence was verified by sequencing.

Generation of Site-Directed Mutants. Three mutants of MJ0837 (H61N, H96N, and D167N) were generated using the QuikChange site-directed mutagenesis kit (Stratagene) using template pMJ0837. The H61N primers were 5′-GAAGGTGGGTTAATAGAAAATACAATACAATATCAGTAAC-3′ (forward) and 5′-GTTACTGATATTGTATTTTCTATTAACCCACCTTC-3′ (reverse). The H96N primers were: 5′-CGCTGGAGCTTTATTAAATGATATTATGAAGCCATAC-3′ (forward) and 5′-GTATGGCTTCATAATATCATTTAATAAAGCTCCAGCG-3′ (reverse). The D167N primers were 5′-CATATATTGTCCATTATGCTAATGAAGCAGATTCAAAG-3′ (forward) and 5′-CTTTGAATCTGCTTCATTAGCATAATGGACAATATATG-3′ (reverse). Expression of the mutants was the same as with the wild type. The mutations were confirmed by sequencing of plasmid inserts.

Purification of Recombinant MptB and Its Mutants. The frozen *E. coli* cell pellet (~0.5 g wet weight) was suspended in 3 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. MptB and its mutants were found to remain soluble after heating the cell extracts for 10 min at 80 °C. This process allowed for their purification from the majority of *E. coli* proteins, which denature and precipitate under these conditions. In the second step of purification, MptB and its mutants were purified by anion-exchange chromatography of the 80 °C soluble fraction on a MonoQ HR column (1 × 8 cm; Amersham Bioscience) using a linear gradient of NaCl from 0 to 1 M in 25 mM TES buffer, pH 7.5, over 55 mL at 1 mL/min flow rate. MptB elutes at about 0.5 M NaCl. All protein concentrations were determined by Bradford analysis (*13*).

Measurement of Native Molecular Weight of MptB. The native molecular weight of MptB was determined by size exclusion chromatography on a Superose 12HR column (10 mm × 300 mm) separated with aerobic buffer containing 50 mM HEPES, pH 7.2, and 50 mM NaCl 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).

Metal Ion Analysis of MptB and Its Mutants. Metal analysis of MptB and its mutants was performed at the Virginia Tech Soil Testing Laboratory using inductively coupled plasma emission spectrophotometry (ICPES). The ICPES was a Spectro CirOS VISION (Spectro Analytical Instruments) equipped with a cross-flow nebulizer with a modified Scott spray chamber. A 50 mg/L yttrium internal standard was introduced by peristaltic pump. Protein solutions, which were eluted from MonoQ, were diluted in elution buffer to give a final calculated metal concentration of 5 ppm, assuming 1 equiv of metal/protomer. Samples were analyzed for iron, manganese, zinc, magnesium, and nickel.

Analysis of Enzymatic Activity of MptB. The standard HPLC assay for MptB with H_2N-cP as substrate was performed in two steps. The first step includes the incubation of 3 μg of MptA in 39 mM TES (Na^+) buffer containing 15 mM DTT, pH 7.2, 1.4 mM $Fe(NH_4)_2(SO_4)_2$, and 2.4 mM GTP in total volume of 42 μL under Ar gas at 70 °C for 30 min to make the H_2N-cP substrate for MptB. As the second step, the assay was continued by adding 12 μg of MptB and incubating at 70 °C for 30 min. Methanol (60 μL) was added to stop the reaction. The dihydropterin reaction products were oxidized to pterins by the addition of 5 μL of iodine in MeOH (50 mg/mL), and the samples were incubated for 30 min at room temperature. $NaHSO_3$ (5 μL, 1 M) was added to reduce excess iodine. HPLC analysis of the reaction mixtures is described below.

The standard spectrophotometric assay for MptB was performed by incubation of 8.4 μg of MptB in 11 mM TES buffer, pH 7.2, 0.75 mM $MnCl_2$, and 1.5 mM bis-pNPP in total volume of 134 μL at 70 °C for 10 min. The incubation was quenched at the end of incubation by adding 1 mL of 0.02 M NaOH. UV−vis absorption at 405 nm was used to detect the generated 4-nitrophenolate.

Testing Alternative Substrates. MptB was tested for hydrolysis of alternative substrates such as ATP, 2′,3′-cAMP, 3′,5′-cAMP, GTP, and 3′,5′-cGMP by incubation of 13 μg of MptB in 29 mM TES buffer, pH 7.0, 5.8 mM $MnCl_2$, and 8.8 mM substrate in a total volume of 17 μL at 70 °C for 10 min. Thin-layer chromatography was used for product analysis as described below.

4′,5′-cFMN was also tested as an alternative substrate. The assay for the hydrolysis of the phosphodiester bond of 4′,5′-cFMN was performed by incubation of 34 mM TES buffer, pH 7.0, 13 μg of MptB, 2.3 mM $MnCl_2$, and ~3 mM cFMN in a total volume of 44 μL at 70 °C for 20 min. The HPLC method was used to separate and detect FMN, FAD, and 4′,5′-cFMN.

The standard spectrophotometric assay condition was used for the hydrolysis of the 4-nitrophenylphosphorylcholine (pNPPC) phosphodiester bond.

HPLC Analysis of Products. A Shimadzu HPLC system with a C18 reverse-phase column (Varian PursuitXRs; 250 × 4.6 mm, 5 μm partical size) was used for analysis of products in some of our assays. The elution profile included 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% MeOH for 5 min followed by a linear gradient to 20% sodium acetate buffer and 80% MeOH over 40 min. The flow rate was 0.5 mL/min. In all assays using HPLC analysis, 80 μL of methanol was added to the assay mixture at the end to precipitate the proteins. After centrifugation (14000*g*, 10 min), 600 μL of H_2O was added to the supernatant for HPLC analysis.

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 and indicated retention times: neopterin 3′-phosphate, 6.2 min; neopterin 2′-phosphate, 6.8 min; D-neopterin 2′,3′-cyclic phosphate, 7.9 min; neopterin, 10.8 min. Flavins were detected by fluorescence using λ_{max} of excitation of 450 nm and λ_{max} of emission of 520 nm. The flavins were eluted in the following order: FAD, 26.5 min, cFMN, 28 min, and FMN, 29 min.

Thin-Layer Chromatography Analysis. In studies using cyclic nucleotides as substrates, products were separated on silica TLC plates with a solvent system consisting of 0.2 M ammonium bicarbonate in 70% ethanol. This solvent system was used previously to separate the cyclic nucleotides from tri-, di-, and monophosphonucleotides (*14*). Under this condition the retention factors (R_f) for the following compounds are as follows: ATP, 0.11; ADP, 0.35; 5′-AMP, 0.44; 2′,3′-cAMP, 0.84; 3′,5′-cAMP, 0.77; GTP, 0.038; 5′-GMP, 0.36; 3′,5′-cGMP, 0.78. Also, cellulose plates eluted with a solvent system consisting of saturated ammonium sulfate, 3 M sodium acetate, and 2-propanol (80:6:2 v/v/v) was used to separate 2′- from 3′-NMPs (*15*). Under this condition the R_f's are as follows: 2′,3′-cAMP, 0.08; 2′-AMP, 0.23; 3′-AMP, 0.15.

Metal-Ion Dependency of the MptB Reaction. Assay for metal dependency of MptB was performed in the presence of either 0.75 mM $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $MgCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $FeNH_4(SO_4)_2 \cdot 12H_2O$, or no metal under standard spectrophotometric assay conditions with bis-pNPP as substrate.

Temperature Stability of MptB. The temperature stability of MptB was determined by treatment of the enzyme under different conditions. These included incubation of 13 μg of enzyme in standard spectrophotometric assay buffer in a total volume of 34 μL containing 0.73 mM $MnCl_2$ at 70, 80, 90, and 100 °C for either 45 min or 2.5 h in sealed tubes. A sample that did not contain $MnCl_2$ was also heated for 45 min at these different temperatures. Following heating, the enzyme mixture was cooled on ice and centrifuged, and bis-pNPP was added as substrate. The final composition, volumes of the reaction mixture, and incubation condition were as described for the bis-pNPP standard assay above. The samples were incubated at 70 °C for 10 min and analyzed as described above for standard spectrophotometric assay.

pH Optimum of the Recombinant Enzyme. The activity of MptB was determined at 0.5 pH increments between pH 5 and pH 9 using a three-component buffer system consisting of BisTris, HEPES, and CHES (*16*). The assay was performed by the incubation of 33 mM BisTris, 17 mM HEPES, 17 mM CHES, 8.4 μg of MptB, 0.75 mM $MnCl_2$, and 1.5 mM bis-pNPP at the indicated pHs in a total volume of 134 μL at 70 °C for 10 min. After incubation 1 mL of 0.02 M NaOH was added to all samples followed by measuring the absorbance at 405 nm.

Titration of MptB with Metals. The enzymatic activity of MptB was assayed by incubation with different concentrations of Mn^{2+} and Fe^{2+} and using the bis-pNPP standard spectrophotometric assay. The ratio of Mn^{2+} and Fe^{2+} to MptB protomer ranged from 0 to 682 and from 0 to 3.4, respectively. The assays were performed in a total volume of 134 μL in the presence of 2.2 μM MptB at 70 °C for 10 min. Experiments using $Fe(NH_4)_2(SO_4)_2$ were done in the presence of 4 mM DTT and under Ar gas.

Oxygen Sensitivity of MptB Activated with Fe^{2+}. The inactivation of Fe^{2+}-activated MptB by air was measured by exposing an Fe^{2+}-activated sample to air and following the loss of activity with time. Thus to a stirred 10 μM anaerobic solution of MptB in 40 mM TES buffer, pH 7.2, was added 20 μM $Fe(NH_4)_2(SO_4)_2$. At times 0, 2, 10, 30, 60, and 120 min, 37 μL of the mixture was transferred to a sealed tube containing Ar gas, and 100 μL of 2 mM bis-pNPP was added. After 10 min at 70 °C the released p-nitrophenolate was measured as described above.

RESULTS

Identification of MptB. On the basis of the information that the HD domain superfamily of enzymes has phosphohydrolase activity (*17*), we first considered the MJ0778-derived protein, which is a member of this superfamily, to be our desired enzyme

since it is clustered with the MptA coding gene in several methanogenic archaeal genomes. This protein could generate H_2neopterin by hydrolyzing the H_2N-cP to H_2N-3′-P. We recombinantly expressed the MJ0778-derived enzyme and found that it did not catalyze the hydrolysis of H_2N-cP. Several other homologues to MJ0778, containing the HD motif, are also found in the *M. jannaschii* genome by Aravind and Koonin (*17*). We then checked another proposed member of this superfamily, MJ0837. The protein product of the MJ0837 gene readily catalyzed the hydrolysis of H_2N-cP to H_2N-P as shown in Figure 1.

Purification and Characterization of MptB. The MJ0837 gene from *M. jannaschii* was cloned and overexpressed in *E. coli*. The resulting protein was purified first by heating the cell extract at 80 °C followed by anion-exchange chromatography of the soluble proteins. The SDS−PAGE analysis of the purified MptB with Coomassie staining showed a single band corresponding to a mass of about 29−30 kDa with a purity of >95%. This molecular mass is consistent with the predicted monomeric molecular mass of 28.5 kDa. Size exclusion chromatography showed a molecular mass of 370 kDa, consistent with the MptB existing as a dodecamer.

The MonoQ-purified recombinant MptB showed no activity with the range of substrates discussed above presumably due to the oxidation of Fe^{2+} to Fe^{3+}. This activity could be restored by the addition of either Fe^{2+} or Mn^{2+} to the purified enzyme. After the addition of 1.4 mM Fe^{2+}, the enzyme showed a specific activity of 29 ± 3 and 360 ± 30 nmol min^{-1} mg^{-1} for the hydrolysis of H_2neopterin 2′,3′-cyclic phosphate (H_2N-cP) and bis-pNPP, respectively (Table 1). Experiments with the Fe^{2+}-reconstituted enzyme acting on H_2N-cP greatly complicated the enzymatic assay due to both the inactivation of the enzyme and destruction of this substrate by oxygen. Both of these problems were eliminated by the use of 0.75 mM Mn^{2+} to activate the enzyme and the use of bis-pNPP as substrate. With these changes the enzyme had a specific activity of 430 ± 20 nmol min^{-1} mg^{-1} with bis-pNPP as substrate.

Efficiency of Oxidation of 7,8-Dihydroneopterin to Neopterin. 7,8-Dihydropterins are not fluorescent whereas pterins are, so it is necessary to oxidize the products of the reaction to pterins so that they could be analyzed by fluorescence. It is well-known that dihydropterins can be oxidized to pterins with I_2 in dilute HCl (*18*). However, such acidic conditions cannot be used in our assay, because it will result in acid-catalyzed opening of the five-membered cyclic phosphate. Iodine oxidation in basic solution, which will prevent this hydrolysis of the cyclic phosphate, can also do the desired oxidation (*19*); however, basic solution cannot be used in our assay because it is known to result in removal of the chain from the pterin ring during oxidation (*19*). In this work we used I_2 in methanol at neutral pH to oxidize the dihydroneopterins to pterins. To determine the efficiency of this oxidation, known concentrations of H_2pterin and H_2neopterin were oxidized with I_2 in methanol, and the efficiency of the conversion to pterin and neopterin, respectively, was measured by HPLC. The efficiency of oxidation with I_2 in methanol for 30 min at room temperature was determined to be 80% for H_2pterin and 90% for H_2neopterin.

Use of a Coupled Assay. Since 6,7-dihydroneopterin 2′,3′-cyclic phosphate (H_2N-cP) was not easily prepared synthetically, we generated it enzymatically from GTP catalytically using MptA. During this assay about 0.4 mM H_2N-cP was made before the MptB enzymatic reaction was started.

Identification of MptB Reaction Product(s). Hydrolysis of 6,7-dihydroneopterin 2′,3′-cyclic phosphate with MptB at 70 °C for 30 min produced two products when assayed by HPLC using fluorescence detection at the pterin λ_{max} of excitation and λ_{max} of emission wavelengths. These two peaks were identified based on their elution times as D-neopterin 2′-phosphate (about 40% of the total) and D-neopterin 3′-phosphate (about 60% of the total). Both showed fluorescent spectra consistent with the presence of pterins. Acid hydrolysis of neopterin 2′,3′-cyclic phosphate produced the same two products with the ratio of 1:4 for H_2N-3′-P and H_2N-2′-P when analyzed by HPLC (*8*). Treatment of the MptB reaction mixture with alkaline phosphatase resulted in the removal of the phosphate from both isomers with the resulting formation of D-neopterin that was confirmed by HPLC.

Substrate Specificity of the MptB Reaction. MptB was able to utilize a variety of phosphodiesters as substrates: H_2N-cP produced H_2N-2′-P and H_2N-3′-P; bis-pNPP and pNPPC produced nitrophenyl phosphate; and 2′,3′-cAMP produced 3′-AMP. Other phosphate ester containing compounds including ATP, 3′,5′-cAMP, GTP, 3′,5′-cGMP, and 4′,5′-cFMN did not serve as substrates for MptB. Bis-pNPP was used as substrate in all characterization experiments since the assay can be done under aerobic condition. The specific activity of the Mn^{2+}-activated enzyme was 430 ± 20 nmol min^{-1} mg^{-1} for bis-pNPP and 710 nmol min^{-1} mg^{-1} for 2′,3′-cAMP (Table 1).

Attempt To Determine the Kinetic Values. H_2N-cP is not commercially available, which makes it hard to determine the kinetic values of MptB with H_2N-cP as a substrate. Even by producing H_2N-cP by the MptA-catalyzed transformation of GTP, high sensitivity of the H_2N-cP to oxygen makes working with this substrate almost impossible without anaerobic facilities. The activity of MptB measured with different concentrations of either bis-pNPP or pNPPC as substrate increased in a linear manner up to 15 mM. Even at high concentration of bis-pNPP or pNPPC, MptB's specific activity continued to increase up to the

Table 1: Metal Content and Specific Activities of MptB and Its Mutants with either H_2N-cP, Bis-pNPP, or 2′,3′-cAMP as a Substrate

	specific activity[a] (nmol min^{-1} mg^{-1})			metal/protomer[b]			
protein	H_2N-cP + Fe^{2+}	bis-pNPP + Mn^{2+}	2′,3′-cAMP + Mn^{2+}	Fe	Zn	Ni	Mn
MptB wild type	29 ± 3	430 ± 20	710	0.8	1.0	< 0.36	< 0.03
MptB H61N	3.8 ± 0.2	74;	54	< 0.05	0.6	< 0.25	< 0.02
MptB H96N	19 ± 7	800;	350	0.4	0.77	< 0.3	< 0.02
MptB D167N	1 ± 0.3	75;	15	0.76	0.47	< 0.07	0.05

[a]The specific activity measurement with H_2N-cP as the substrate was done at an Fe^{2+} concentration of 1.4 mM. The concentrations of Mn^{2+} in the bis-pNPP and 2′,3′-cAMP experiments were 0.75 and 5.8 mM, respectively. [b]The metals in the proteins were measured from MonoQ purified samples without the addition of any metals.

Table 2: Dependency of the Activity of MptB on Different Divalent Metal Ions[a]

	no metal	Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Mg^{2+}	Zn^{2+}	Fe^{3+}
specific activity (nmol mg^{-1} min^{-1})	2.0	430 ± 20	370 ± 30	12.0	3.0	0.0	0.0	0.0

[a]The assay was done in the presence of either 0.75 mM $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_2$, $Fe(NH_4)_2(SO_4)_2$, $FeNH_4(SO_4)_2$, or no added metal with bis-pNPP as substrate.

solubility limit of these compounds. As a result we were not able to determine the kinetic values of MptB with either bis-pNPP or pNPPC as substrates.

Metal-Ion Dependency of the MptB Reaction. As isolated, MptB is inactive in the absence of added metal ions. By adding different metal ions to the assay mixture, it was revealed that MptB hydrolyzes the phosphodiester bond of bis-pNPP most efficiently in the presence of added Mn^{2+} or Fe^{2+} but not Fe^{3+} (Table 2). Activity of MptB was restored by using Fe^{2+} at the physiologically relevant concentrations of about 4 μM although higher concentrations of Mn^{2+} (750 μM) were able to restore activity comparable to that seen with Fe^{2+}. Also, Co^{2+} was found to restore some activity at 0.75 mM, but the level was only 3.2% of that of Fe^{2+} (Table 2).

Since high concentrations of Mn^{2+} were able to restore most of the activity of MptB, and it is more convenient to work with Mn^{2+} than with Fe^{2+} under aerobic condition due to air oxidation of Fe^{2+} to Fe^{3+}, most of the experiments were done with Mn^{2+}.

Identification of Catalytically Important Residues. Alignment of MptB homologues from the HD superfamily allowed for the identification of two conserved histidine and two conserved aspartate residues, H61, H96, D97, and D167 (*17*). With this knowledge, three of those residues in MptB, H61, H96, and D167 were chosen to change to asparagines, the most conservative changes possible. ICPES was used to determine the identity and quantity of metal ions present in the purified recombinant enzymes. Isolated pure recombinant MptB was found to contain 0.8 mol of iron and 1.0 mol of zinc per protomer using the Bradford protein assay to measure the protein concentration. The metal content of the wild type and mutants and their specific activity with H_2N-cP, bis-pNPP, and 2′,3′-cAMP are summarized in Table 1. With one exception, all of the mutant enzymes showed reduced activities when assayed with each of the three substrates. The exception was the H96N mutant that showed about 1.9-fold higher specific activity with bis-pNPP than that of the wild type (Table 1).

Temperature Stability of Recombinant MptB. Heating of MptB at 70, 80, 90, and 100 °C, in either presence or absence of Mn^{2+} reveals interesting results. In the first case by heating MptB at 70, 80, 90, and 100 °C in the absence of Mn^{2+} for 45 min, the activity of the enzyme drops about 30% from the 70 °C heated sample to the 100 °C heated sample. In the presence of Mn^{2+}, however, in the case of 45 min heating the activity of the enzyme increases about 53% from the 70 °C heated sample to the 100 °C heated sample. But in the case of 2.5 h heating the activity of the enzyme drops about 26% from the 70 °C heated sample to the 100 °C heated sample. Adding Mn^{2+} to the enzyme was observed to increase the temperature stability of the protein.

pH Optimum of the Recombinant Enzyme. Using a three-component buffer system consisting of BisTris, HEPPS, and CHES allowed a pH range from 5 to 9 to be determined in one buffer system. The best activity of MptB with bis-pNPP in this buffer system was observed at pH 7.5 (Figure 2).

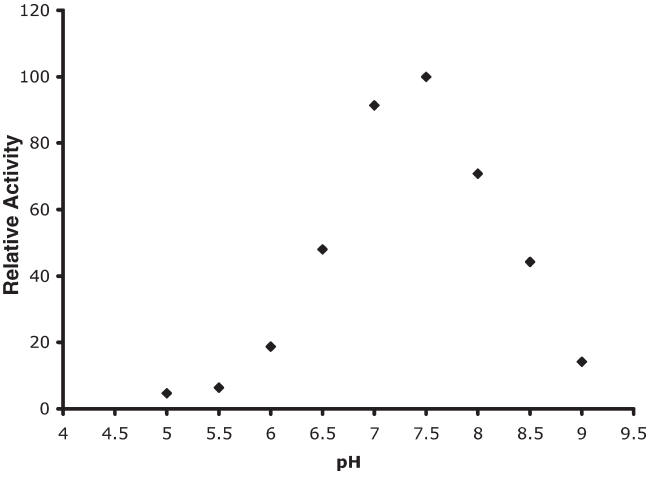

FIGURE 2: Effect of pH on hydrolysis of bis-pNPP by MptB activity. The best activity for MptB was observed at pH 7.5.

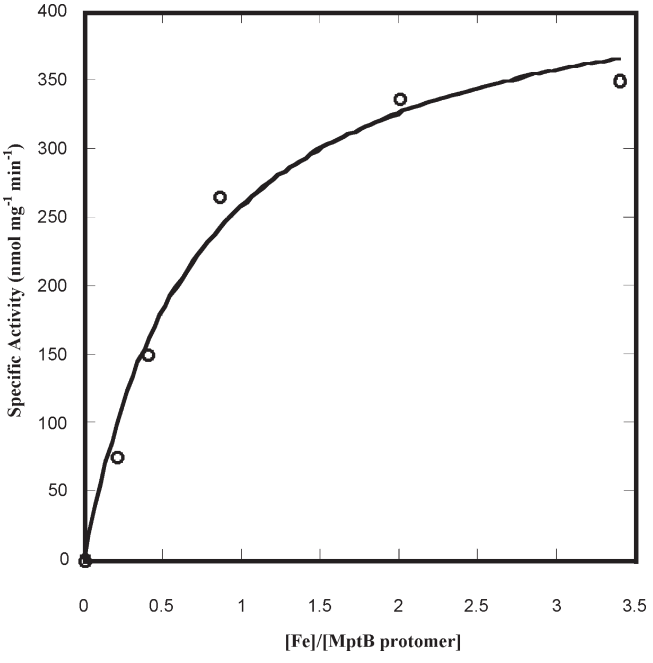

FIGURE 3: Titration of MptB with Fe^{2+}. The activity of MptB toward bis-pNPP was measured while the ratio of $Fe(NH_4)_2(SO_4)_2$ to MptB was varied from 0 to 3.4 in the presence of 2.2 μM MptB.

Titration of MptB with Metals. The activity of MptB increased by increasing the ratio of either Fe^{2+} to MptB from 0 to 2.1 (Figure 3) or Mn^{2+} to MptB from 0 to 682 (Figure 4). When doing these experiments, it was found that DTT is required in the activation experiments using Fe^{2+}. It is not clear why DTT was required for the Fe^{2+} activation of MptB, but there are several examples of where thiol compounds are required to remove Fe^{3+} from a protein so it can be replaced with Fe^{2+} (*20*).

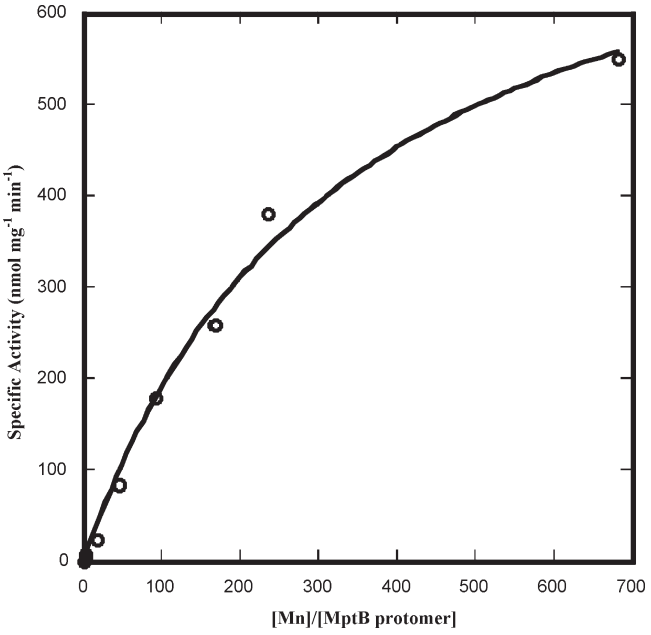

FIGURE 4: Titration of MptB with Mn^{2+}. The activity of MptB bis-pNPP was measured while the ratio of $MnCl_2$ to MptB was varied from 0 to 682 in the presence of 2.2 μM MptB.

Since we are using the enzymatic rate to determine Fe^{2+} binding to the enzyme, KeleidaGraph 4.0 was used to fit the metal titration data (Figures 3 and 4) to a rectangular hyperbolic equation to determine the dissociation constants (K_D) of about 1.6 ± 0.35 μM for iron and 739 ± 0.1 μM for manganese.

Oxygen Inactivation of MptB. The specific activity of Fe^{2+}-activated MptB toward hydrolysis of bis-pNPP upon exposure to air assuming an exponential decay was determined to have a half-life of about 5 min.

DISCUSSION

MptB contains the HD motif and is annotated as a member of the metal-dependent phosphohydrolase superfamily (*17*). The members of this superfamily are very different in sequence, but they all have conserved histidines and aspartates, which are involved in divalent metal binding and in forming the catalytic site (*17*). Some of the HD domain enzymes have been crystallized, and according to their structures, HD domain proteins even have different folds. By using the DALI-structural similarity search program, it is revealed that the local structures around the putative active sites in all HD domain enzymes, however, are similar (*21*). Based on the secondary structure threading, the most similar known structure to MptB is a protein with the HD domain that is predicted to be a hydrolase. This structural analogous protein to MptB was produced from uncultured thermotogales bacterium and was also found to contain two irons (PDB id 2pq7).

Characterized HD domain enzymes catalyze many different reactions, but the functions of many are presently unknown. There are 11 known families of phosphodiesterases (PDEs), which are members of the HD domain superfamily (*22*). All 11 families contain two histidine and two aspartate metal binding residues that are conserved (*23*). All of the known enzymes in PDE's family that use cyclic nucleotides 3′,5′-cAMP or 3′,5′-cGMP as substrates produce 5′-AMP or 5′-GMP as products (Table 3) (*24*−*32*). All of these PDEs function in signaling pathways to regulate the concentration of intracellular secondary messengers 3′,5′-cAMP or 3′,5′-cGMP. Also, there are some cyclic phosphodiesterases which hydrolyze 2′,3′-cNMP to produce either 2′-NMP (CthPnkp) (*33*), 3′-NMP (DR1281) (*34*), or both (λ-phage and Rv0805) (*33*, *35*). It is proposed that the role of these 2′,3′-cNMP phosphodiesterases is to repair RNA 2′,3′-cyclic ends (*33*), which are intermediates in RNA processing. MazF, an mRNA interferase in *E. coli*, is an example of these cyclic phosphodiesterases that is highly conserved among prokaryotes. MazF is a endonuclease that produces a 2′,3′-cP at the 3′ end of RNA and a hydroxyl group on the 5′ end (*36*). tRNA splicing endonuclease in yeast is another characterized endonuclease, which converts pre-tRNA to mature tRNA, by producing 2′,3′-cyclic nucleotides (*37*). An archaeal tRNA endonuclease, which is homologous to yeast tRNA splicing gene, has also been identified (*38*). There are even members of the HD domain family that have both 2′,3′-cyclic phosphodiesterase and phosphomonoesterase activities (*39*).

On the basis of this information we first considered the MJ0778, which is a HD domain enzyme that is also clustered with the MptA coding gene in several methanogenic archaeal genomes. We recombinantly expressed the MJ0778-derived enzyme and found that it did not catalyze the hydrolysis of H_2N-cP. Several other homologues to MJ0778, containing the HD motif, are also found in the *M. jannaschii* genome (*17*). Among these is MJ0837. The protein product of the MJ0837 gene readily catalyzed the hydrolysis of H_2N-cP to H_2N-P as shown in Figure 1. Unlike most enzymes that produce a single enzymatic product, this enzyme catalyzed the formation of both H_2N-2′-P (about 40% of the total) and H_2N-3′-P (about 60% of the total) positional isomers from H_2N-cP. The formation of both isomers is of no consequence since we have shown that both isomers are readily metabolized to H_2neopterin by *M. jannaschii* cell extracts (unpublished data). MptB is also able to hydrolyze the cyclic phosphate of 2′,3′-cAMP but not that of 3′,5′-cAMP, 3′,5′-cGMP, or 4′,5′-cFMN. Interestingly, only 3′-AMP was formed from 2′,3′-cAMP. We propose that the production of the two isomers results from the binding of the H_2N-cP in two different conformations that allows for two different orientations of the cyclic phosphate as shown in Figure 5. As the adenine ring binds to its binding pocket in MptB, the 2′,3′-cyclic phosphate of the cAMP is fixed in place because of the presence of the ribose ring, which causes strict orientation of the cyclic phosphate in the active site containing the nucleophilic Fe^{2+}-bound water (Figure 5A). In the case of the H_2N-cP, that does not contain the ribose ring, two different orientations of the cyclic phosphate can occur upon binding to MptB, thus allowing for the hydroxide to attack the phosphate from either apical position, one producing H_2N-3′-P (Figure 5B) and the other producing H_2N-2′-P (Figure 5C). This can occur because H_2N-cP does not have the β-substituted ribose ring in its structure so the cyclic phosphate may rotate freely around the C1′−C2′ bond ("b" bond in Figure 5), and as a result H_2N-cP can have two different orientations. The nucleophilic attack of a hydroxide at the phosphorus by applying the "apical entry apical exit" rule, which is applied in all phosphoryl exchange reactions involving a pentavalent intermediate, forms only 3′-AMP in the case of consuming 2′,3′-cAMP as substrate.

Another possible explanation for the production of two different isomers is by the binding of the H_2N-cP in two different binding sites that results in the orientation of the cyclic phosphate in the active site to be in the two different orientations as described above. The resolution of this issue must await the structural determination of the enzyme.

Table 3: List of the Known Phosphodiesterases and Phosphomonoesterases Where Their Metal Dependency and Substrates Are Known

enzyme	metal	substrate	ref
PDEs 2	Zn^{2+}, Mg^{2+}	cAMP, cGMP	*31*
PDEs 3	Fe^{2+}, Fe^{3+}	cAMP	*29*
PDEs 3B	Mg^{2+}, Mg^{2+}	cAMP, cGMP	*28*
PDEs 4	Zn^{2+}, Mn^{2+}	cAMP	*30*
PDEs 5	Zn^{2+}, Mg^{2+}	cGMP	*26*
PDEs 7	Zn^{2+}, Mg^{2+}	cAMP	*25*
PDEs 10	Zn^{2+}, Mg^{2+}	cAMP, cGMP	*24*
cNMP PDEs	Zn^{2+}	cGMP	*32*
cNMP PDE (Rv0805) from *M. tuberculosis*	Mn^{2+}, Fe^{3+}	cAMP, cGMP	*27*
mammalian PAPs	Fe^{2+}, Fe^{3+}	nonspecific phosphomonoesterase	*40*
plant PAPs	Zn^{2+}, Fe^{3+}	nonspecific phosphomonoesterase	*40*
YfcE	Zn^{2+} (PDB 1SU1), requires Mn^{2+}	no natural substrate detected	*41*
*Cth*Pnkp	Ni^{2+}, Mn^{2+}	2′,3′-cAMP	*35*
bacteriophage λ phosphatase (λ-Pase)	Mn^{2+}, Mn^{2+}	2′,3′-cAMP	*35*
MJ0936	Mn^{2+}, Mn^{2+}	phosphodiesters but not cyclic phosphodiesters	*42*
DR1281	Mn^{2+}, Fe^{2+}, or Co^{2+}	2′,3′-cAMP	*34*
Rv0805	Mn^{2+}, Mn^{2+}	2′,3′-cAMP	*33*

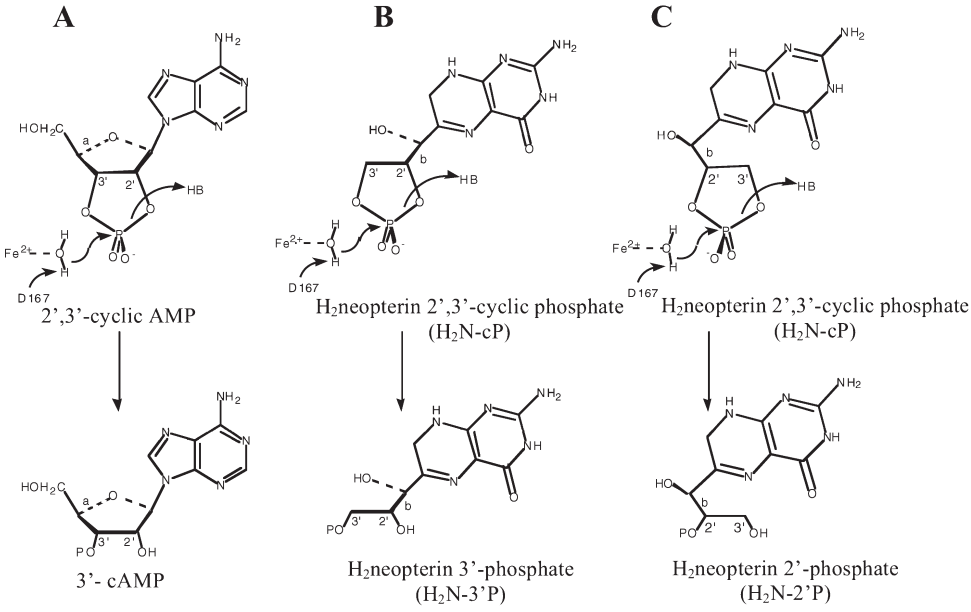

FIGURE 5: A proposed MptB cyclic phosphodiesterase mechanism. When 2′,3′-cAMP is used as substrate (A), the presence of the ribose ring allows only one orientation of the cyclic phosphate when bound to the enzyme. Under these conditions the nucleophilic attack of an Fe^{2+}-bound hydroxide at the phosphorus allows for the formation of only 3′-AMP. In the case of H_2N-cP, because of the absence of the ribose ring, the cyclic phosphate ring can easily rotate around bond "b". H_2N-cP can have one orientation as shown in (B), and nucleophilic attack of a hydroxide at the phosphorus produces H_2neopterin-3′-P. By rotating the cyclic phosphate around bond "b", H_2N-cP will produce another orientation as shown in (C), and nucleophilic attack of a hydroxide at the phosphorus produces H_2neopterin-2′-P.

The metal dependency of phosphodiesterases varies widely. We have summarized in Table 3 the list of metals, which are either observed in the crystal structure of some of the phosphodiesterases (*24*−*27*, *31*, *32*, *40*) or have been observed to be required for the activity of these enzymes (*29*, *33*−*35*, *41*, *42*). ICPES analysis of purified inactive MptB indicated the presence of 0.8 mol of iron and 1.0 mol of zinc per protomer. Although ICPES analysis was able to detect the presence of iron, the exact location and ligands of both the iron and zinc are unknown. Since the enzyme is inactive when purified aerobically on MonoQ or when incubated with Fe^{3+} and the enzymatic activity was restored by the addition of Fe^{2+}, we can conclude that the iron needs to be in the reduced state. Also, the time course assay of the O_2-dependent loss of activity of the MptB in the presence of Fe^{2+} emphasized that this reaction is Fe^{2+}-dependent. An Fe^{2+}−aquo complex has an acid dissociation constant that would allow for the production of the required nucleophilic hydroxide (*43*). Although the Fe^{3+} enzyme was expected to have an even lower pK_a, the additional positive charge on the metal to which the hydroxide is bound will not allow the hydroxide to function as a nucleophile. The utilization of Fe^{2+} by the enzymes from anaerobic methanogens may relate to the higher availability of the soluble, reduced form of iron present in the habitats of the methanogens.

Adding either DTT or dithionite to the enzyme-containing assay mixture was not able to restore the activity of the enzyme by reducing the Fe^{3+} bound to MptB back to Fe^{2+} (unpublished data). As shown here, adding either Fe^{2+} or Mn^{2+} was able to restore the MptB activity. This regeneration of activity could result either from the replacement of the bound Fe^{3+} by either Fe^{2+} or Mn^{2+} or by reduction of the bound Fe^{3+} with the added Fe^{2+}. However, since other reducing agents failed to restore the

enzymatic activity and the nonreducing Mn^{2+} did restore activity, the replacement of the Fe^{3+} by Fe^{2+} is the most likely scenario. This replacement is also expected considering that catalytically active Fe^{2+} is likely bound more tightly than catalytically inactive Fe^{3+}.

Having metal ions in the catalytic site of phosphodiesterase can also stabilize the transition state of hydrolysis by neutralization of negative charge on phosphate and interaction with the oxygen of the leaving alcohol (*44*). Metal ions can accelerate the hydrolysis of phosphate diesters due to three factors: Lewis acid activation ($<10^2$-fold), intermolecular nucleophile activation (10^8-fold), and leaving group stabilization (10^6-fold). In some cases these factors can combine to give the overall rate acceleration more than 10^{16} (*45*).

In the site-directed mutant H61N, MptB loses almost all of the iron, and its specific activity is about 13% of that of wild type for H_2neopterin-cP, 7.6% for 2′,3′-cAMP, and about 17% for bis-pNPP. In the case of H96N, MptB loses half of the iron, and so its specific activity is about half of that of wild type for 2′,3′-cAMP, 65% for H_2neopterin-cP, but 186% for bis-pNPP. The reason for the higher activity of H96N in the case of using bis-pNPP as substrate is not clear, but the same result was observed for a phosphodiesterase from *Mycobacterium tuberculosis* (*33*). In the case of the site-directed mutant D167N, MptB has almost all of the iron, but its specific activity is only about 3.5% of that of wild type for H_2neopterin-cP in the presence of added Fe^{2+}, 3% for 2′,3′-cAMP, and about 17% for bis-pNPP in the presence of added Mn^{2+}. We propose that the reason for this decrease in the specific activity without losing any iron is that D167 is the base (Figure 5) that abstracts the proton of the Fe^{2+}- or Mn^{2+}-bound hydroxide ion that facilitates its nucleophilic attack on phosphate.

Because adding Zn^{2+} plus Fe^{2+} caused lower activity compared to adding Fe^{2+} alone in the assay (unpublished data), we can speculate that Zn^{2+} replaces Fe^{2+}, resulting in decreased activity. This result is consistent with a proposed structural role for Zn^{2+} and a catalytic role for Fe^{2+} in MptB.

The only well-studied example of the enzymatic hydrolysis of five-membered cyclic phosphodiesters is concerned with their involvement as proposed intermediates in the hydrolysis RNA by ribonuclease. Nucleoside 2′,3′-cyclic phosphates can serve as substrates for RNase (*46*). The absolute stereochemistry of this hydrolysis has been studied and shown to proceed by an in-line mechanism (*47*, *48*). The same stereochemistry has been found in ribonuclease-T_1 (*49*) and by a nonspecific phosphohydrolase (*50*) and is likely to be the same for MptB.

MptA is present in the genomes of almost all the archaea except in the class Thermoprotei, while MptB is present in members of the class Methanococcales as well as in only a few other archaea. Since the other methanogens contain methanopterin or one of its many derivatives, then there should be at least one other gene in the other methanogens that encodes for a protein to hydrolyze the cyclic phosphate intermediate in their methanopterin biosynthetic pathway. Since the halobacteria contain folate but no methanopterin, then it is likely their MptA is producing the folate pterin without the help of MptB.

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