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An Iron(II) Dependent Formamide Hydrolase Catalyzes the Second Step in the Archaeal Biosynthetic Pathway to Riboflavin and 7,8-Didemethyl-8-hydroxy-5-deazariboflavin†

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ABSTRACT: The early steps in the biosynthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (Fo) and riboflavin in the archaea differ from the established eukaryotic and bacterial pathways. The archaeal pathway has been proposed to begin with an archaeal-specific GTP cyclohydrolase III that hydrolyzes the imidazole ring of GTP but does not remove the resulting formyl group from the formamide [Graham, D. E., Xu, H., and White, R. H. (2002) *Biochemistry 41*, 15074−15084]. This enzyme is different than the bacterial GTP cyclohydrolase II which catalyzes both reactions. Here we describe the identification and characterization of the formamide hydrolase that catalyzes the second step in the archaeal Fo and riboflavin biosynthetic pathway. The *Methanocaldococcus jannaschii* MJ0116 gene was cloned and heterologously expressed, and the resulting enzyme was shown to catalyze the formation of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (APy) and formate from 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-monophosphate (FAPy). The MJ0116-derived protein has been named ArfB to indicate that it catalyzes the second step in archaeal riboflavin and Fo biosynthesis. ArfB was found to require ferrous iron for activity although metal analysis by ICP indicated the presence of zinc as well as iron in the purified protein. The identification of this enzyme confirms the involvement of GTP cyclohydrolase III (ArfA) in archaeal riboflavin and Fo biosynthesis.

Studies on the biochemistry of the Archaea have revealed numerous new as well as modified metabolic pathways. Many of these pathways were suggested following the availability of large numbers of genomic sequences which showed that homologues of many known metabolic pathway enzymes were not present in the archaea. The absence of canonical pathways suggested the presence of either unique, nonorthologous enzymes or alternate pathways. Among the numerous pathways for which some of the canonical enzymes were not present was the riboflavin biosynthetic pathway. In the methanogenic archaea the early part of this pathway was also proposed to be involved in the biosynthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (Fo),[1] a component of coenzyme F_{420}. The hydride carrier coenzyme F_{420}, as $F_{420}H_2$, is a central coenzyme in methanogenesis and is also involved in a variety of biological transformations including the reduction of toxic sulfite to sulfide in *Methanocaldococcus jannaschii* (*1*) and the reduction of toxic oxygen to water catalyzed by $F_{420}H_2$ oxidase (FprA) (*2*, *3*). The core redox-active moiety of F_{420}, Fo, serves as a coenzyme of the DNA photolyases used to repair DNA (*4*) and can function in place of F_{420} in some of the enzymes using F_{420} (*5*−*7*).

Work in this and other laboratories has unraveled many of the steps in archaeal riboflavin and F_{420} biosynthesis. In the methanogens the biosynthesis of coenzyme F_{420} and riboflavin proceeds from a common intermediate, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ARP). It has been shown that the biosynthesis of ARP in the archaea follows an alternate pathway from that found in bacteria and eukarya (Figure 1) (*8*, *9*). The proposed archaeal pathway would begin with the opening of the imidazole ring of GTP by the action of an archaeal GTP cyclohydrolase III (MJ0145) (*10*). The product of this reaction is 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-monophosphate (FAPy). FAPy was

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[1]Abbreviations: GTP, guanosine 5′-triphosphate; GMP, guanosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; FAPy, 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-monophosphate; APy, 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate; ARP, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; Fo, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; coenzyme F_{420}, the *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazaflavin.

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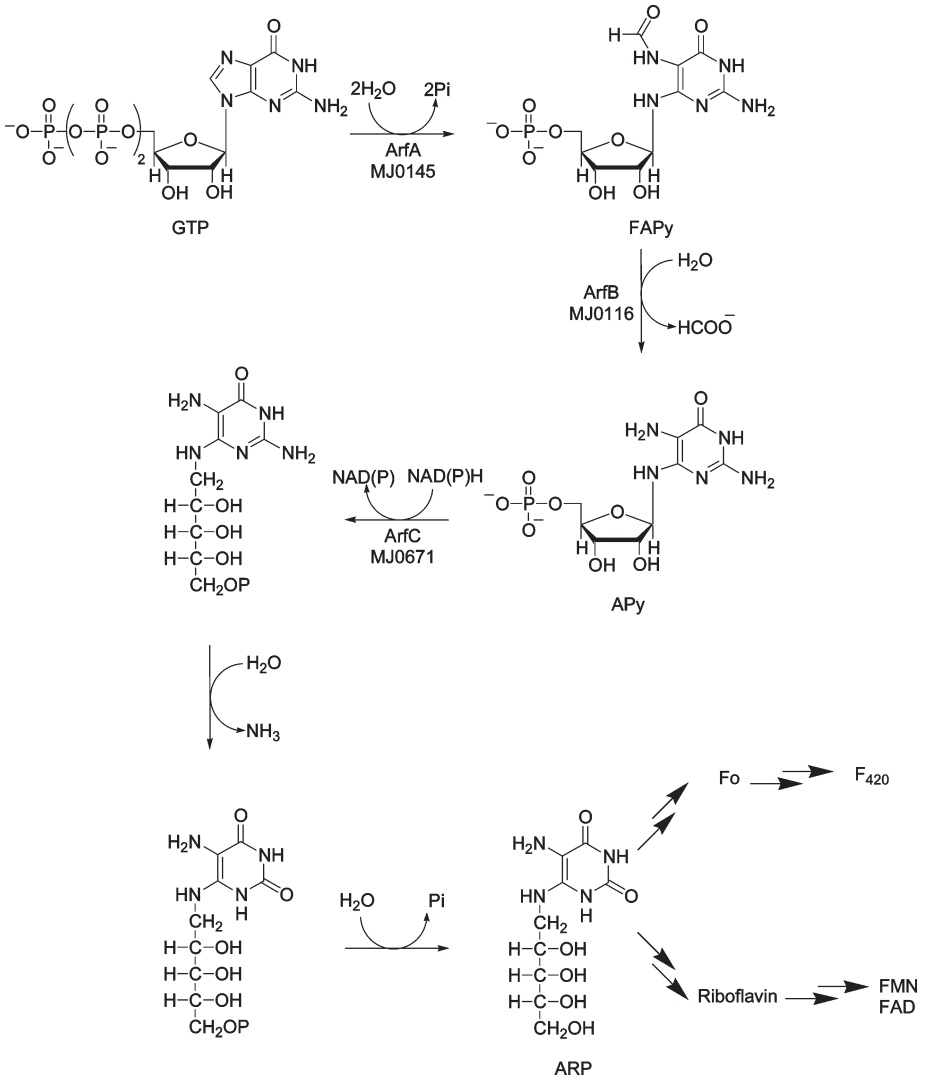

FIGURE 1: The archaeal biosynthetic pathway from guanosine 5′-triphosphate (GTP) to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ARP) . The MJ0116 gene product (ArfB) catalyzes the hydrolysis of the formamide of 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-monophosphate (FAPy) to form 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (APy); Fo, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; F_{420}, the *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazaflavin.

proposed to be converted to 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (APy) by an unknown hydrolase. APy is then reduced to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione phosphate by the MJ0671 gene product (*8*). A deamination followed by a final dephosphorylation would then produce the common intermediate, ARP.

Although a pathway for these early steps in archaeal Fo and riboflavin biosynthesis has been proposed, an essential step linking the product of GTP cyclohydrolase III with the remainder of the pathway had not previously been identified. Here we report the identification and characterization of the formamide hydrolase involved in the second step of archaeal Fo and riboflavin biosynthesis.

MATERIALS AND METHODS

Chemicals. FAPy was prepared by incubation of GTP with ArfA (MJ0145, GTP cyclohydrolase III). The reaction mixture contained 12 μg of ArfA, 10 mM GTP, 6 mM $MgCl_2$, 1 mM KCl, 0.4 mM Fe(II) as ferrous ammonium sulfate, 10 mM dithiothreitol (DTT), and 25 mM TES (K^+), pH 7.2, and was incubated at 60 °C for 4−6 h. ArfA was then precipitated with 2 volumes of methanol and removed by centrifugation. After the methanol was removed by evaporation under a stream of nitrogen gas, FAPy 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(K^+), pH 7.5, over 100 mL at 1 mL/min. Under these conditions FAPy eluted at 280 mM NaCl. The UV spectrum of purified FAPy was indistinguishable from published spectra of the authentic compound (*11*) with a λ_{max} of 274 nm. The concentration of FAPy was determined after chemical hydrolysis of the formamide in 1 N HCl for 10 min at 100 °C, cooling on ice, and neutralizing the solution with an equal volume of 1 N NaOH. The formed APy was then derivatized and the concentration determined by fluorescence as described below. All other chemicals and reagents were obtained from Sigma/Aldrich.

Cloning and Expression of MJ0116 and Purification of the Recombinant Gene Product, ArfB. The *M. jannaschii* MJ0116 gene (SwissProt Q57580) was amplified by PCR

from genomic DNA using oligonucleotide primers: MJ0116F, 5′-GGTCATATGCAACTTAGATTATC-3′; MJ0116R, 5′-GCTGGATCCTCACCTTATCATTTC-3′. PCR was performed as described previously using a 50 °C annealing temperature (*12*). The amplified PCR product was purified by a QIAquick spin column, digested with restriction enzymes *Nde*I and *Bam*HI, and ligated into compatible sites in plasmid pT7-7. The plasmid DNA sequence was verified by sequencing. The resulting plasmid, pMJ0116, was transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). The transformed cells were grown in Luria−Bertani medium (200 mL) supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an OD_{600} of 1.0. Recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional 6 h of culture at 47 °C, the cells were harvested by centrifugation (4000*g*, 5 min) and frozen at −20 °C. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of total cellular proteins confirmed induction of the desired protein. *E. coli* cells expressing MJ0116 were resuspended in 4 mL of extraction buffer (50 mM TES(K^+), pH 7.0, 10 mM $MgCl_2$, 20 mM DTT) and lysed by sonication. After precipitating the majority of *E. coli* proteins by heating the cell lysate to 80 °C for 10 min, the MJ0116-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, over 55 at 1 mL/min. The MJ0116 gene product eluted at 240 mM NaCl. Protein concentration was determined by Bradford analysis (*13*).

Size Exclusion Chromatography. The native molecular weight of ArfB was determined by size exclusion chromatography on a Superose 12HR column (10 × 300 mm) with a buffer containing 50 mM HEPES, pH 7.0, and 150 mM NaCl at 0.5 mL/min with detection at 280 nm. Protein standards used to generate the standard curve included apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), conalbumin (77 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and B_{12} (1.4 kDa).

Preparation of Apo-ArfB. Apo-ArfB was generated by removing the metals with a Chelex resin. Chelex resin (Bio-Rad) was prewashed sequentially with 6 N HCl, H_2O, and 1 N NaOH and then equilibrated with 100 mM TES, pH 7.0. Equilibrated resin (1.5 mL) was transferred to a glass column. ArfB (370 μg in 1 mL of 25 mM TES, pH 7.5, and 240 mM NaCl) was then passed through the column and collected in the flow-through. ArfB activity with various metals was determined by incubation under the standard assay conditions with the exception that no $MgCl_2$ was added to the reaction buffer.

Assay of ArfB. The typical ArfB reaction mixture included 8 μg of ArfB, 0.1−3 mM FAPy, 2 mM Fe(II) as ferrous ammonium sulfate or Mn(II) as $MnCl_2$, 5 mM $MgCl_2$, 10 mM DTT, and 25 mM TES, pH 7.2, and was incubated at 70 °C for 15 min. Incubation mixtures containing Fe(II) were conducted under nitrogen gas. Following incubation, the formed APy was converted to 6,7-dimethylpterin by the addition of an equal volume (typically 100 μL) of 35 mM 2,3-butanedione in 0.9 M Tris-HCl, pH 8.0, and heating at 100 °C for 40 min. The samples were then cooled on ice and centrifuged before diluting with H_2O to a final volume of 1 mL. The amount of formed 6,7-dimethylpterin was measured by fluorescence spectroscopy with an excitation wavelength of 365 nm and emission wavelength of 445 nm. A Shimadzu RF1501 spectrofluorophotometer was used for quantification of APy as the 6,7-dimethylpterin derivative in the standard assays. As the product of the ArfB reaction is unstable, the concentration of product was determined from a standard curve generated by incubating known concentrations of 6-hydroxy-2,4,5-triaminopyrimidine sulfate (Aldrich H5,920-6) under standard assay conditions for 30 min. Following incubation the remaining 6-hydroxy-2,4,5-triaminopyrimidine was converted to 6,7-dimethylpterin as described above and used to generate the standard curve.

Chromatographic separation and analysis of 6,7-dimethylpterin were performed on a Shimadzu HPLC system with a C18 reverse-phase column (Varian PursuitXRs, 250 × 4.6 mm, 5 μm particle size). The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% methanol followed by a linear gradient to 20% sodium acetate buffer/80% methanol over 40 min at 0.5 mL/min. The formed 6,7-dimethylpterin was detected by fluorescence using an excitation wavelength of 356 nm and an emission wavelength of 450 nm. Under these conditions 6,7-dimethylpterin had a retention time of 27.0 min.

pH Profile of ArfB Activity. The optimal pH for ArfB activity was determined following the standard assay conditions, with 50 mM TES buffer, pH 6.5−8.5, 5 mM DTT, and 2 mM Mn(II). The pH of the respective TES buffers was adjusted at room temperature and found to be constant up to 70 °C, the ArfB assay temperature. Manganese was used in place of Fe(II) in order to avoid the required maintenance of anaerobic conditions.

Kinetic Studies on ArfB. Kinetic studies on ArfB were conducted under the standard assay conditions with the following modifications: the incubations included 0.4−4 μg of ArfB, 0.3−2.5 mM FAPy, and 2 mM Fe (II) as ferrous ammonium sulfate and were incubated at 70 °C for 15 min. Following incubation the reaction products were derivatized and measured by fluorescence as described above.

Metal Ion Analysis of ArfB. Metal analysis of ArfB was performed at the Virginia Tech Soil Testing Laboratory using an inductively coupled plasma emission spectrophotometer (ICP). Instrumentation included a Spectra Ceros VISION made by Spectra 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, magnesium, cobalt, and zinc.

RESULTS AND DISCUSSION

The hydrolysis of the formamide of FAPy to produce APy was predicted to be the second step in archaeal Fo/riboflavin biosynthesis (*10*). A survey of the *M. jannaschii* genome identified a number of predicted amidohydrolases

Table 1: Occurrence of Putative Fo and Riboflavin Biosynthetic Genes in Archaeal Genomes[a]

	enzyme, gene			
	ArfA, MJ0145	ArfB, MJ0116	unknown, MJ0699	RibA, GTPCHII
Archaeoglobaceae				
Archaeoglobus fulgidus				X
Halobacteriaceae				
Haloarucula marismortui	X		x	
Halobacterium sp.	X		x	
Haloquadratum walsbyi	X		x	
Natronomonas pharanis	X		x	
Methanobacteriaceae				
Methanbrevibacter smithii	X	X	X	
Methanosphaera stadtmanae	X	X	X	
Methanothermobacter thermautotrophicus	X	X	X	
Methanocaldococcaceae				
Methanocaldococcus jannaschii	X	X	X	
Methanococcaceae				
Methanococcus aeolicus	X	X	X	
Methanococcus maripaludis C5	X	X	X	
Methanococcus vannielii	X	X	X	
Methanosarcinaceae				
Methanococcoides burtonii			X	
Methanosarcinaceae			x	
Methanosaetaceae				
Methanosaeta thermophila PT			x	X
Picrophilaceae				
Picrophilus torridus			x	X

[a] An X indicates strong homology to the indicated genes while an x indicates lower sequence homology.

that could catalyze this hydrolysis. Of these, four hypothetical proteins did not have a demonstrated function, including the products of the MJ0116, MJ0699, MJ1459, and MJ1541 genes. Two of these, MJ0116 and MJ0699, were found to be genomically associated with genes of the riboflavin biosynthetic pathway. The distribution of MJ0699, RibA, MJ0116, and MJ0145 (ArfA) homologues among the archaea is shown in Table 1. Homologues of MJ0699 are found throughout the archaea including several species whose genomes contain homologues of GTP cyclohydrolase II (RibA). The MJ0116 gene homologues are present in the Methanobacteriaceae, Methanocaldococcaceae, and Methanococcaceae; they are absent in the Halobacteriaceae which also contain ArfA homologues. This would suggest that another, unidentified formamide hydrolase may be operating in the Halobacteriaceae. The fact that ArfA and ArfB are not found in all archaea indicates that additional unknown enzyme(s) processing GTP, like the GTP cyclohydrolases, are still to be found. Like ArfA, MJ0116 was identified as an archaeal signature gene (*14*).

We cloned and expressed both MJ0116 and MJ0699 to test for the ability of their gene products to hydrolyze the formamide of FAPy. Overexpression of the MJ0116 gene in *E. coli* produced a protein with a subunit molecular mass of approximately 25 kDa, consistent with the predicted mass of the protein. A higher expression temperature of 47 °C was used in order to increase protein solubility based on the work of Koma et al. (*15*), as a previous 37 °C expression resulted in mostly insoluble protein. The MJ0116 gene product was thermally stable to 80 °C, and heating the *E. coli* extract containing ArfB to this temperature for 10 min produced the MJ0116 gene product that was >90% pure, as measured by SDS−PAGE with Coomassie staining. Additional purification by anion-exchange chromatography produced the MJ0116 gene product that was >98% pure as judged by SDS−PAGE with Coomassie staining.

Incubation of the purified MJ0116 gene product with ArfA and GTP resulted in the production of low amounts of APy, detected after conversion into the 6,7-dimethylpterin derivative. This indicated that the MJ0116 gene product was cleaving the formamide of FAPy, forming the diamino product (APy) that is capable of reacting with 2,3-butanedione to form 6,7-dimethylpterin. The formed 6,7-dimethylpterin had identical UV/vis, fluorescence, and mass spectra as authentic material and also had identical retention time by C18 HPLC under the conditions described in Materials and Methods. The formation of APy was found to increase linearly with increased enzyme concentration and was also linear up to 40 min under the standard assay conditions. We did not observe this activity with cell-free extracts of *E. coli* harboring the MJ0699 gene; however, expression of this protein was not sufficient to verify that it does not catalyze the conversion of FAPy to APy (data not shown). Based on the demonstrated activity, the MJ0116-derived protein has been named ArfB, to indicate that it catalyzes the second step in archaeal riboflavin and Fo biosynthesis. The previously reported GTP cyclohydrolase III would then be named ArfA.

Considering that the enzyme was likely a metallohydrolase, we tried to increase the activity of the enzyme by adding either Zn(II), Mn(II), or Fe(II) to the assay mixture and found that addition of either Mn(II) or Fe(II) increased the rate of APy production by >10 fold. We believe that this increase in activity can be attributed to

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M.jannaschii              ------------------------------MQLRLSSGNVLNEKVHKVGIIALGSFLEN 29
M.vannielii               -------------------------------MDKMELRYNSGNILNEEVHKIGIIALGSFLEN 32
M.thermoautotrophicum     ------------------------------MLVELNLDAGNIISEDVHRIGILAVGSHLEN 31
M.stadtmanae              -------------------------MSSNNIKLKYDSGNILSKDVHSIGIIALGSHREN 34
P.pudita                  -------------MSKS-----------VFVGELTWKEYEARVAAGDCVLMLPVG-ALEQ 35
Rosevarious sp217         --MNFAHEASCSRISQSEKKGNEMAQDTVFAAELAWPEYQRRVARGAVPILIPLG-SMEQ 57
B.phymatum STM815         MQNNATRNNEETVMKQS-----------VVIGELAAPEYARRIGDG-WPVLIPLG-ALEQ 47
                                                                              *

M.jannaschii              HGAVLPIDTDIKIASYIALKASILTGAKFLG-VVIPSTEYEYVKHGIHNKPEEVYS---- 84
M.vannielii               HGSALPIDTDAKIASYIGLNVSILTGAKFLG-VVIPSTEYSYVKHGIHNSPNEVVE---- 87
M.thermoautotrophicum     HGPALPIDTDAKIASYVALEAALRTGARFLG-VLYAASEFPYVKHGIHMDRDELVERE-- 88
M.stadtmanae              HGAALPIDTDSKIAANVALNVATKTGATFLG-IFYGATEYDYIKHGHHLKKDDLVNKQ-- 91
P.pudita                  HGHHMCMNVDVLLPTAVCKRVAERIGALVMPGLQYGYKSQQKSGGGNHFPGTTSLDGATL 95
Rosevariousp217           HGHHMPMHVDVLLPTEFARRAAVEVGGLVAPPFTYGYKSQQKSGGGNFFPGTTSLDGASL 117
B.phymatum STM815         HGPHMSMNPDVLLPTAIGIAVARNIEALVAPAIAYGYKSQQKSGGGNHLCGTTSLDGHTL 107
                          *        *

M.jannaschii              --YMRFLINEGKKIGVEKFLIVNCHGGNILVESFLKDLEY------EFDIKVEMINITFT 136
M.vannielii               --YIKIMIEHSKKIGINKFLIINCHGGNTLIKDLISELN-------DKKTSVILENVCFT 138
M.thermoautotrophicum     --LKPVLRKARRLLNLEAAVIVNGHGGN-KLEDCMDDLEE------ELGLEIAWNNRIVE 139
M.stadtmanae              --IIPQLINIKKQLNIKSVIIVNGHGGNNLIIEDIDKISK------KTELKIIFNNSIIE 143
P.pudita                  TGTVQDIIRELARHGARRLVLMNGHYENSMFIVEGIDLALRELRYAGIQDFKVVVLSYWD 155
Rosevarious sp217         VNALKDVIREFARHGNRQICIVNGHFENSWFIAEGIDLALRELGWSGIHDTKVVVLSYWD 177
B.phymatum STM815         TSTIKDILKEFARHGARSICLINGHFENSMFAVEGIDLALRELKWEGTCDLQVVMLSYWD 167
                                                  *

M.jannaschii              ----HASTEEVSVGYIIGIAKADEETLKEHNNFEKYPEVGMVG--LKEARENNKAIDKEA 190
M.vannielii               ----HAAFEEIAIGYAVGILS--EDKMKTHS-FKTYPEIGMIG--LTEARLKNTDIDNEA 189
M.thermoautotrophicum     IEGPHAGSGELSAGLILGIAD--LRRLDECIPEL-YPEIGMIG--LKEAREANREIDKAA 194
M.stadtmanae              SEGPHACTGELSMGAVLGITD--MTSLKEHENFIKHPEVGMVG--LKEARDNEPIINKEA 199
P.pudita                  FVKDPAVIQQLYPEGFLGWDIEHGGVFETSLMLALYPDLVDLDRVVDHPPATFPPYDVFP 215
Rosevarious sp217         FV-DQAAIAKLYPEGFLGWDIEHGGVLETSLMLRLYPDLVSLERAVDHAPASFPPYDVYP 236
B.phymatum STM815         FV-TETTIARIYPDGFPGWAVEHGGVLETSLMLHLHPHLVDMSQVPTHAAVTFPPYDVFP 226
                                                    *

M.jannaschii              KVVKRFG--------VKLDKKLGEKILNNAIEKVVEKIKEMIR------------- 225
M.vannielii               KILEEKGA-------IFLDKNYGKTLLKNLINNHVEIVKKMSEGDSNVGRLPITRL 238
M.thermoautotrophicum     RICERDG--------VNPDPVLGQRILDDAVESVISDVKELLKIIQEF-------- 234
M.stadtmanae              LTIEKEG--------FEVNMILGQDMLENAQKEIINEVKKLL-------------- 233
P.pudita                  VDPARTPAPGTLSSAKTASREKGELILEVCVQGIADAIREEFPPT----------- 260
Rosevarious sp217         PHPEWTPASGTLSSPKNASAEKGDILIDVCTRGIVAALRTEFATRDRVAAE----- 287
B.phymatum STM815         PIPEWTPASGCLSSAANASAEKGKLLFDVCVEGMSQALGEAWQARAKASRA----- 277
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FIGURE 2: Alignment of archaeal FAPy formamide hydrolases with bacterial creatininase. Residues that are conserved in both Archaea FAPy formamide hydrolases and Bacteria creatininase are highlighted in gray. Residues shown to be involved in metal binding in creatininase are indicated with an asterisk. Sequences (with accession numbers) include ArfB homologues: *Methanocaldococcus jannaschii* (Q57580), *Methanococcus vannielii* (YP_001323522), *Methanothermobacter thermoautotrophicum* (NP_275793), and *Methanosphaera stadtmanae* (YP_448201). Creatininase homologues include *Pseudomonas pudita* ps-7 (BAA08265), *Rosevarious* sp217 (ZP_01035841), and *Burkholderia phymatum* STM815 YP_001862362).

either the replacement of the Fe(III) in the enzyme by Fe (II) (or Mn(II)) or by the reduction of the Fe(III) in the enzyme by the added Fe(II). The Fe(III) would have been produced by air oxidation of the Fe(II) during the aerobic isolation of the enzyme. These data indicated that the MJ0116 gene product is Fe(II) dependent. ArfB was not active in the absence of DTT (2 mM), even when Mn(II) was provided as the divalent metal, indicating that reduction of Fe(III) still bound to the enzyme may be occurring.

The MJ0116 protein is a member of COG1402, a family of uncharacterized putative amidases. Aptly, the structure of ArfB is predicted by threading to be like creatinine amidohydrolase (creatininase; EC 3.5.2.10). Despite the predicted structural similarity, ClustalW alignment of the MJ0116 protein and creatininase from *Pseudomonas putida* ps-7 revealed low sequence similarity with less than 16% identity. Most of the conserved residues between the two proteins are the predicted metal binding residues identified in the crystal structure of creatininase (Figure 2) (*16*, *17*). Creatininase from *P. putida* contains a binuclear zinc center (*18*, *19*) that has E34, D45, and H120 as ligands for metal 1 and H36, D45, and E183 as ligands for metal 2, with D45 bridging the two metals. Yosimoto observed that the Zn(II) in the metal 1 binding site can be replaced with Mn(II); however, the second zinc in metal binding site 2 was much more tightly bound and was not replaced (*19*). Archaeal ArfB homologues have six conserved histidine, four conserved glutamate, and two conserved aspartate residues that could serve as metal binding ligands. However, based on alignment with the metal binding site of creatininase, we predict that conserved ArfB residues E28, D39, and H107 of *M. jannaschii* ArfB form the metal 1 binding site and may be involved in Fe(II) binding. Two of the three residues involved in binding the second metal in creatininase are conserved in the ArfB homologues (H30 and D39 in *M. jannaschii*). However, the third ligand, a glutamate residue in creatininase, is not conserved. Among the archareal ArfB homologues there are three conserved glutamate residues (E142, E170, and E178) that are not conserved in creatininase. In addition, a fourth glutamate (E161) is partially conserved in the archaea. One of these glutamates may be serving as the third ligand for

the putative second metal. Despite the conservation of binding ligands for metal 2, the presence and the identity of the proposed second metal could not been confirmed.

The canonical GTP cyclohydrolase I and II enzymes, that catalyze both the opening of the purine imidazole ring and the hydrolysis of the formamide, are known to be zinc-dependent, and mechanisms have been proposed in which the zinc ion participates in both steps of the reaction (*20*). This, as well as the predicted structural similarity of ArfB with creatininase and the observed increased activity in the presence of Mn(II) and Fe(II), prompted us to investigate the metal content of this enzyme. ICP analysis of the purified enzyme showed that it contained both iron and magnesium (1.4 and 6.2 mol/mol of protomer, respectively). A large amount of zinc was also found to be associated with the purified protein (1.5 mol/protomer). No cobalt (<0.1 mol/protomer) and very low amounts of manganese (0.08 mol/protomer) were found in the enzyme. ICP analysis of Chelex-treated ArfB revealed much lower amounts of iron and zinc associated with the protein (0.2 and 0.4 mol/protomer, respectively). Manganese, cobalt, and magnesium concentrations were below the detection limits (<0.05, <0.1, and <2 mol/protomer, respectively) in Chelex-treated enzyme. Despite the presence of zinc in the protein, addition of Zn(II) to the incubation mixture containing purified or apo (Chelex-treated) enzyme did not activate ArfB. Conversely, ArfB was found to be activated by the addition of Fe(II) or Mn (II) to the incubation mixture of both untreated enzyme (Figure 3) and Chelex-treated enzyme. At low (<0.1 mM) metal concentrations the enzyme had higher activity with Fe(II) rather than Mn(II); however, the activity was similar at high metal concentrations (>1 mM). The maximum activity of Chelex-treated enzyme, with added Fe(II) or Mn(II), was only 30% that seen with the untreated enzyme. This indicates that the apoenzyme cannot be fully reconstituted with the addition of just one metal. However, addition of a second metal, such as Zn(II) or Mg(II), did not improve activity. It is possible that the residual activity is from the portion of enzyme molecules that were found to still contain some bound Zn and/or Fe following Chelex treatment (0.4 and 0.2 mol/protomer, respectively). A possible explanation of this would be that two divalent metals, such as Fe(II) and Zn(II) or Fe(II) and Fe(II), are required for catalysis, where one site can be replaced with Mn(II).

Unlike creatinase, which is a hexameric protein, ArfB was found to be a dimeric protein by size exclusion chromatography. ArfB had a broad optimal pH range, between pH 7 and pH 8, which is lower than that reported for *E. coli* GTP cyclohydrolase II (8.5) (*21*); however, it is similar to the broad optimal pH range observed for creatininase (*22*). ArfB had approximately 80% maximal activity at pH 6.5 and 40% activity at pH 8.5.

A plot of ArfB specific activity with increasing substrate concentration produced a sigmoidal curve (Figure 4). From the curve, the kinetic parameters were approximated as follows: $V_{max} \approx 6\,\mu$mol min^{-1} mg^{-1}, $K_M^{app} \approx 1$ mM, and $k_{cat}/K_M^{app} \approx 3 \times 10^3$ M^{-1} s^{-1}. This type of curve is typically indicative of homomeric cooperativity and suggests that ArfB may exhibit positive cooperative substrate binding. This type of cooperatively is often seen in enzymes that

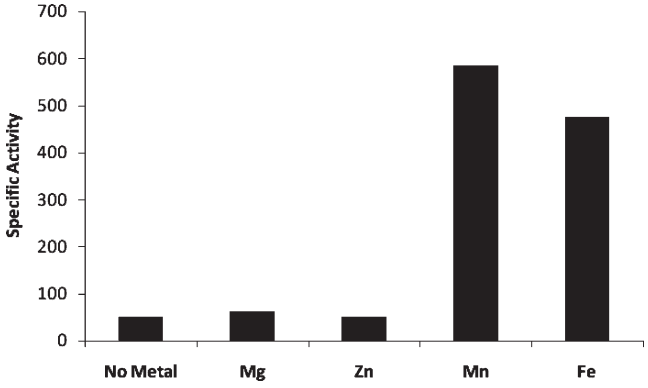

FIGURE 3: Restoration of purified ArfB activity with various divalent metals. The concentration of metal is 2 mM, and the concentration of FAPy was 0.2 mM. The *Y* axis is specific activity in nmol min^{-1} mg^{-1}.

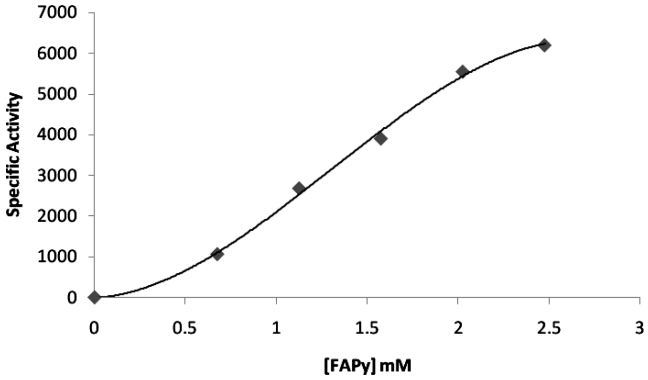

FIGURE 4: Specific activity of ArfB with increasing concentrations of FAPy. Specific activity is in nmol min^{-1} mg^{-1} and is based on initial rate data.

catalyze the first committed step of biochemical pathways (*23*). Positive cooperativity was observed for the GTP cyclohydrolases I and II respectively involved in the first steps of bacterial folate and riboflavin biosynthesis. GTP cyclohydrolase II (RibA) from *E. coli* was also found to exhibit non-Michaelis−Menten behavior that was consistent with cooperative substrate binding (*24*).

ArfA-type activity has been observed for one of the three annotated GTP cyclohydrolase II gene products in *Streptomyces coelicolor* (SCO6655) (*25*). A subsequent search of bacterial genomes for the mutations found to be responsible for the altered catalytic activity observed in the SCO6655 protein identified many GTP cyclohydrolase II homologues that are also expected to have altered function as seen in ArfA (*20*). The presence of enzymes that produce FAPy in organisms that presumably have the canonical riboflavin pathway suggests that FAPy may be a precursor to other bacterial and archaeal metabolites. This raises the possibility that ArfB and not ArfA may be first committed step in archaeal riboflavin and Fo biosynthesis.

Here we report the identification of the second enzyme in the common pathway to ARP, the last common intermediate in riboflavin and Fo biosynthesis. The identification of ArfB provides the crucial linkage between the archaeal GTP cyclohydrolase III and the rest of the riboflavin/Fo biosynthetic pathway and confirms the intermediacy of FAPy in the biosynthesis of these

coenzymes. GTP cyclohydrolases are remarkable enzymes in that they catalyze five or more distinct chemical reactions in the conversion of GTP to the pterin or APy product. In that regard, ArfA may represent a more primitive enzyme in that, unlike its bacterial counterpart, it has not evolved the ability to hydrolyze the formamide that is generated from the opening of the imidazole ring of GTP.

The data indicate that ArfB requires at least one Fe(II) ion for activity and an additional second metal ion that could be Fe(II) or Zn(II). Therefore, ArfB represents the third enzyme in archaeal methanogenic coenzyme biosynthesis that has been confirmed to require ferrous iron for activity. The first two enzymes in the methanopterin biosynthetic pathway, a GTP cyclohydrolase (MptA) and neopterin cyclophosphate hydrolase (MJ0837, MptB), have also been shown to be Fe(II)-dependent (*26*) (Mashhadi, unpublished data). We have also reassessed the activity of ArfA and now believe that it may be an Fe(II)-dependent enzyme as well. These newly discovered archaeal hydrolases more than double the total number of the currently known Fe(II)-dependent hydrolases that include cytosine deaminase (*27*), peptide deformylase (*28*), histone deacetylase (*29*), and methionine aminopeptidase (*30*).

Dupont et al. observed that the archaea and bacteria retain more predicted iron-dependent enzymes in their proteome than did eukaryotes, which evolved later in an oxygen-rich atmosphere (*31*). The characterization of new and predicted zinc-dependent metalloenzymes as Fe(II)-dependent indicates that the archaea may utilize more Fe (II) and less Zn(II) in biochemical reactions than the current genome annotations would imply. Consequently, this provides further evidence for the importance of metal geochemistry on the evolution and metal-cofactor selection of enzymes. The abundance of ferrous hydrolases in the biosynthetic pathways of methanogenic coenzymes is consistent with the evolution of the cofactors for methanogenesis occurring in anaerobic environments such as the deep sea hydrothermal vents where *M. jannaschii* was collected or in the anaerobic, ferrous-rich oceans on the early earth.

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