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Identification and Characterization of an Archaeon-Specific Riboflavin Kinase▽

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The riboflavin kinase in *Methanocaldococcus jannaschii* has been identified as the product of the MJ0056 gene. Recombinant expression of the MJ0056 gene in *Escherichia coli* led to a large increase in the amount of flavin mononucleotide (FMN) in the *E. coli* cell extract. The unexpected features of the purified recombinant enzyme were its use of CTP as the phosphoryl donor and the absence of a requirement for added metal ion to catalyze the formation of FMN. Identification of this riboflavin kinase fills another gap in the archaeal flavin biosynthetic pathway. Some divalent metals were found to be potent inhibitors of the reaction. The enzyme represents a unique CTP-dependent family of kinases.

Work on the biosynthesis of riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) in the archaea has revealed a number of surprises in terms of both the genes encoding the pathway enzymes and the pathway itself. Analysis of archaeal genomes has generally shown the absence of *ribA*, the gene encoding GTP cyclohydrolase II, the first enzyme in the presently established pathways to riboflavin in bacteria. In the archaea, this reaction may be catalyzed by at least two separate enzymes. The first enzyme, GTP cyclohydrolase III, produces 2-amino-5-formylamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-phosphate (compound 2 in Fig. 1) and inorganic pyrophosphate by hydrolysis of GTP (11). This intermediate is subsequently hydrolyzed to 2,5-diamino-6-ribofuranosylamino-4(*3H*)-pyrimidinone 5′-phosphate (compound 3) by a currently unknown enzyme. Compound 3 is then converted into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ARP, compound 6 in Fig. 1) by a dehydrogenase (MJ0671) (5), a deaminase, and a phosphatase following the eukaryotic pathway (13). The involvement of compound 2 does not occur in either the bacterial or the eukaryotic pathway, and the dehydrogenase and deaminase steps are in the same order as in the eukaryotic pathway, which is the reverse of that found in the bacterial pathway. The product of this series of reactions, ARP, is the precursor of F_{420} and riboflavin (10). In the conversion of ARP to riboflavin in the archaea, there is a fundamental difference in the stereochemistry of the pentacyclic intermediate involved in the dismutation of 6,7-dimethyl-8-ribityllumazine into riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione in the last step of riboflavin biosynthesis (17).

Two different groups of enzymes are known to be involved in the conversion of riboflavin first to FMN and then to FAD. In the first group, the reaction is catalyzed by FAD synthetase, a bifunctional enzyme (RibF or RibC) that first acts as a kinase converting riboflavin to FMN in the presence of ATP and then acts as a nucleotidyltransferase by using a second ATP to convert the FMN to FAD and PP_i (8, 21). In the other group, these reactions are catalyzed by two separate enzymes, riboflavin kinase (RibR, flavokinase, or FMN1) and FAD synthetase (FAD1 in yeast) (32). Enzymes homologous to the yeast flavokinase (FMN1) (27) are widely distributed (29). Aside from the differences in the enzymes used to generate FAD, other differences in the pathways and/or enzymes have been noted. It has been reported that the *ribF* gene in *Bacillus subtilis* uses reduced riboflavin as the substrate (19, 28), *Corynebacterium ammoniagenes* RibF can use *meta*-phosphate as the phosphoryl donor (23), and the macrolide resistance gene *mreA* of *Streptococcus agalactiae* has also been shown to function as a riboflavin kinase (6). The absence of identifiable genes encoding any of these enzymes in the archaea has prompted a search for the genes and reactions required to carry out these last two steps of FAD biosynthesis in the methanogens. Here we report that the MJ0056-derived protein is an archaeon-specific riboflavin kinase that catalyzes the reaction shown in Fig. 1. We designate it RibK to indicate the unique character of this archaeal riboflavin kinase. To date, orthologs of MJ0056 have been found only in archaea.

It was noticed that the crystal structure of the MJ0056 gene product recently deposited in the Protein Data Bank (code 2oyn) has a structural fold similar to that found in the human and yeast riboflavin kinases (4, 18), as well as the riboflavin kinase domain of the flavin-binding protein from *Thermotoga maritima*, annotated as an FAD synthetase (30). This crystal structure also contains a bound CDP in the same position as the ATP bound in the human enzyme. In addition, in the archaeal genomes of *Archaeoglobus fulgidus* DSM 4304, *Haloarcula marismortui* ATCC 43049, *Halobacterium* sp. strain NRC-1, *Methanothermobacter thermautotrophicus* strain Delta H, *Methanococcus maripaludis* S2, *Methanococcoides burtonii* DSM 6242, *Methanosarcina acetivorans* C2A, *Methanosarcina mazei* Go1, and *Methanopyrus kandleri* AV19, the MJ0056 gene (*ribK*) is next to the MJ0055 gene (*ribB*), which encodes 3,4-dihydroxy-2-butanone-4-phosphate synthase, which is involved in the biosynthesis of riboflavin. These findings indicated that

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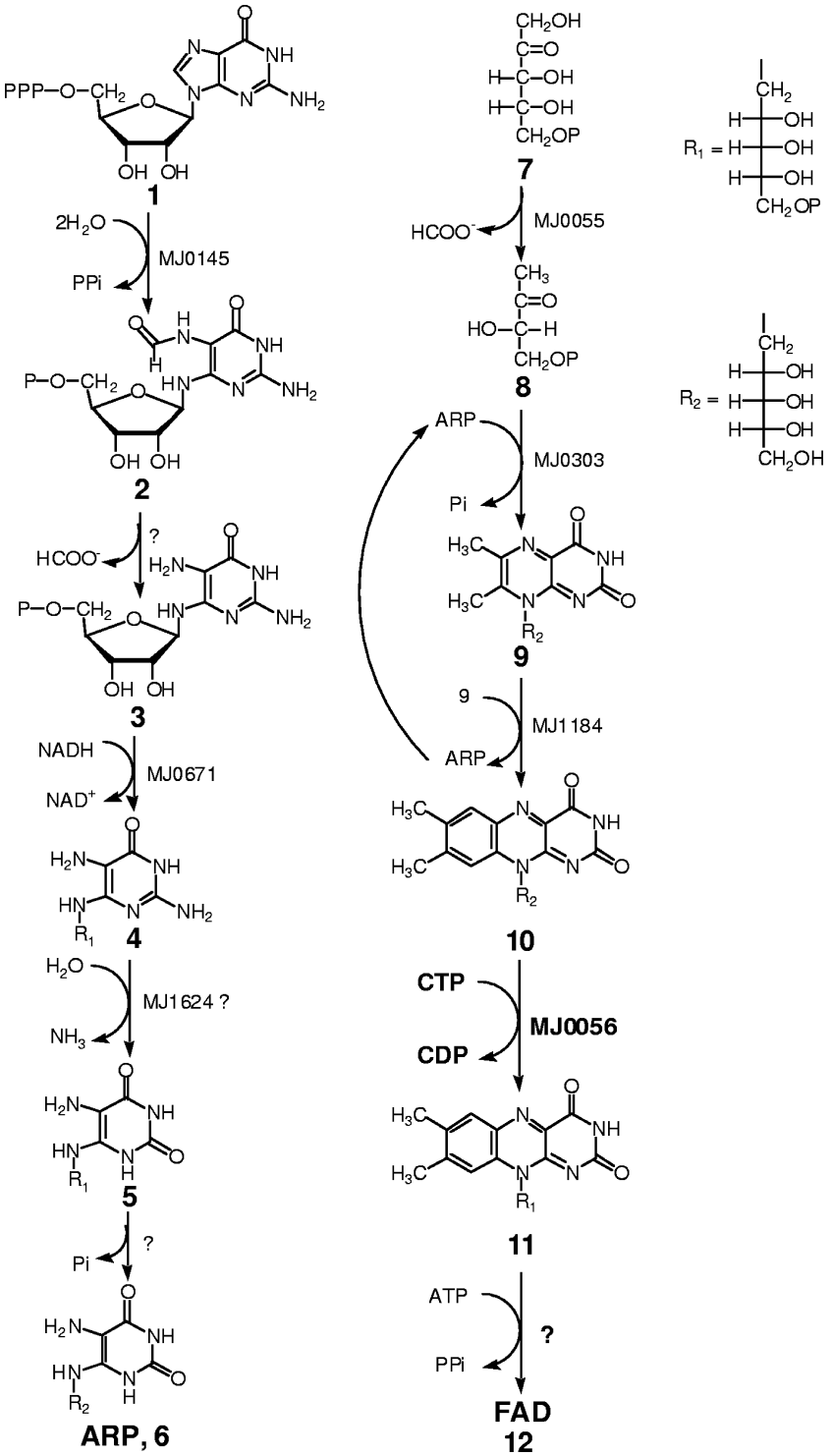

FIG. 1. Riboflavin biosynthetic pathway in archaea.

this gene product could be the archaeal kinase despite the fact that it has no detectable sequence similarity to any known kinase.

The *M. jannaschii* gene at locus MJ0056 (Swiss-Prot accession number Q60365) was amplified by PCR from genomic DNA with oligonucleotide primers pMJ0056Fwd (5′-GGTCA TATGATTATTGAGGGAGAAG-3′) and pMJ0056Rev (5′-GATCGGATCCTTATTCATCTTTATCTCCC-3′). The PCR was performed as described previously, with a 55°C annealing temperature (12). The primers introduced an NdeI and a BamHI site at the 5′ and 3′ ends, respectively. The amplified PCR product was purified with a QIAquick spin column (Qia-

gen), digested with restriction enzymes NdeI and BamHI, and then ligated into the compatible sites in plasmid pT7-7 to make recombinant plasmid pMJ0056. DNA sequences were verified by dye terminator sequencing at the University of Iowa DNA facility. The resulting plasmid, pMJ0056, was transformed into *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene). The transformed cells were grown in Luria-Bertani medium at 37°C until an absorbance at 600 nm of 1.0 was reached. The protein was induced by adding 2% (wt/vol) lactose and culturing the cells for an additional 4 h. The protein was then extracted from the cells, heated at 70°C, and purified on a MonoQ column as previously described (14). Elution of the protein was monitored by UV absorbance at 280 nm. One major UV-absorbing peak eluting at 0.37 M NaCl contained the desired protein, based on activity measurement and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The protein purified by this method was >95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie staining and showed a molecular mass of 15 kDa. Electrospray mass spectrometry (MS) measured a mass of 15,218.1 ± 10 Da, which agrees with the mass of 15,213.8 Da calculated from the gene sequence for a protein of 132 amino acids. All of the kinetic analyses were done with protein purified in this manner.

The FMN eluting from MonoQ-separated cell extractions was used to measure the amount of FMN present in the extracts. The MonoQ fractions containing FMN were identified by fluorescence, and the total amount was quantified by high-performance liquid chromatography (HPLC). Extracts derived from cells expressing the MJ0056 gene were found to have 8.7 μM FMN. This value was sixfold higher than that observed in extracts from cells expressing other enzymes not related to FMN production.

The standard assay for RibK was performed by incubation of 47 mM TES buffer (pH 7.2), 1.2 μg of RibK, 1.7 mM CTP, and 0.37 mM riboflavin in a total volume of 60 μl at 70°C for 10 min. Under this assay condition, 42% of the riboflavin was consumed and converted into FMN. Analysis of the enzyme-catalyzed reaction was conducted by HPLC with detection of riboflavin and FMN with a Shimadzu HPLC system with a C_{18} reverse-phase column (Varian PursuitXRs; 250 by 4.6 mm, 5-μm particle size) and detection by fluorescence with an excitation λ_{max} of 450 nm and an emission λ_{max} of 520 nm. The elution profile consisted of 5 min in 95% sodium acetate buffer (25 mM [pH 6.0], 0.2% NaN_3)–5% methanol, followed by a linear gradient to 20% sodium acetate buffer–80% methanol over 40 min at 0.5 ml/min. Specifically, the ratio of riboflavin to FMN was measured from the areas of the HPLC fluorescence signals generated by the separated compounds. Under these conditions, riboflavin was eluted at 31.1 min, FMN at 29 min, and FAD at 26.5 min. The FMN product gave the same electrospray MS spectrum as authentic FMN with an MH^+ ion at 457.0 *m/z* and an MNa^+ ion at 479.0 *m/z* in the positive mode and an M^- ion at 454.9 *m/z* in the negative mode. The MS-MS of the 457.0 *m/z* ion for both the known and enzymatically generated FMN gave the same ions at 439.0 *m/z* for $[M\text{-}H_2O]^+$, 377.1 *m/z* for [riboflavin]$^+$, 359.1 *m/z* for [riboflavin-H_2O]$^+$, and 243.1 *m/z* for [flavin ring system]$^+$. The MS-MS of 454.9 *m/z* ion for both the known and enzymatically generated FMN gave the same ions at 241.0 *m/z* for [flavin ring system]$^-$, 212.9 *m/z* for [M-flavin ring system]$^-$, and 96.9 *m/z* for [phosphate]$^-$.

Size exclusion chromatography was performed on a Superose 12HR column (1 by 30 cm; Amersham Biosciences) with the same conditions and protein standards as previously described (20). The data indicated that the enzyme was a monomer (19.7 kDa). When 1.2 mg of the enzyme was heated in 40 μl of extraction buffer at different temperatures of up to 100°C for 10 min, no significant change in enzyme activity was observed. Although dithiothreitol was used in these thermal experiments, we have no indication that dithiothreitol is required to maintain the enzyme's activity. The enzyme retained 40% (0.13 nmol min^{-1}) of its activity compared to the control (0.32 nmol min^{-1}) after treatment with 0.1 M HCl (pH ~1.05) for 10 min at room temperature, followed by dilution in acetate buffer (pH 6.0). In these experiments, the detection limit was about 2% of the control activity.

The enzyme was most active with CTP as the substrate. The activities with ATP and GTP at the same concentration were 30% and 11% of that seen with CTP, respectively.

The kinetics of RibK were determined under steady-state conditions in the presence of a saturating concentration of either CTP or riboflavin. KeleidaGraph 4.0 was used to calculate the kinetic parameters from the Michaelis-Menten plot. The measured kinetic parameters for RibK with CTP as the phosphate donor are as follows for riboflavin: K_m^{app} = 159 μM, $k_{cat}/K_m = 2.1 \times 10^3\ M^{-1}\ s^{-1}$. They are as follows for CTP: K_m^{app} = 1.8 mM, k_{cat}/K_m = 190 M^{-1} s^{-1}, and V_{max} = 1.3 μmol min^{-1} mg^{-1}. There is a wide range of kinetic values reported for riboflavin kinases from different organisms. The lowest K_m values reported for riboflavin and ATP are 120 nM and 210 nM, respectively, for the flavokinase from *Neurospora crassa* (24), and the highest K_m values for riboflavin and ATP are reported as 420 μM and 4.55 mM, respectively, for the rat liver enzyme (3). Also, the range of specific activities for flavokinases is enormous at 0.00023 μmol min^{-1} mg^{-1} for the flavokinase in rat liver (3) to 2.95 μmol min^{-1} mg^{-1} for the flavokinase from *N. crassa* (24). Using the standard assay method supplemented with ether no metal ion or 1.6 mM Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, or Co^{2+}, the specific activity observed was 0.7, 0.6, 0.35, 0.12, 0.4, 0.02, or 0.27 μmol min^{-1} mg^{-1}, respectively. Rat liver riboflavin kinase was active with these cations and had the highest activity with Zn^{2+} (22).

The MonoQ-purified protein was analyzed for magnesium and zinc at the Virginia Tech Soil Testing Laboratory by inductively coupled plasma emission spectrophotometry. The result showed 4 to 5 mol of Mg and 0.0013 mol of Zn per mol of protein.

The reasons why this enzyme preferentially uses CTP as the phosphate donor instead of ATP, as is found in almost all known kinases, is not clear. ^1H nuclear magnetic resonance analysis of *M. jannaschii* cell extracts indicates that the concentration of CTP is comparable to that of ATP (R. H. White, unpublished data). Thus, the enzyme could simply be taking advantage of the high CTP concentration. The only known example of a CTP-dependent kinase is dolichol kinase (1). There are a few GTP-dependent kinases, e.g., phosphoglycerate kinase (25) and polyribonucleotide 5′-hydroxyl-kinase (31), and some kinases are probably promiscuous and can use either GTP or CTP. There are also some other non-ATP-dependent

kinases such as a few ADP-dependent and pyrophosphate kinases (16, 26).

The evolutionary lineage of this archaeal riboflavin kinase is not clear, as it is so different from all other kinases. This alternation in kinases is common in the archaeal biosynthetic pathways where we find a new shikimate kinase in the shikimate pathway (7), a new kinase in a new pathway to isoprenoids (15), and a new predicted pantothenate kinase in coenzyme A biosynthesis (9).

An important biological implication of the presence of RibK in those archaeal genomes that have no genes for de novo riboflavin synthesis, such as "*Pyrococcus abyssi*" GE5, *Pyrococcus horikoshii* OT3, and *Thermoplasma acidophilum* DSM 1728, provides a clear interpretation of FMN and FAD biogenesis via a salvage pathway from exogenous riboflavin.

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ADDENDUM

While this work was under review, Ammelburg et al. published data showing that the MJ0056 gene product is a riboflavin kinase and proposed an evolutionary lineage of MJ0056 to other riboflavin kinases (2).

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