# CofE Catalyzes the Addition of Two Glutamates to $F_{420}$-0 in $F_{420}$ Coenzyme Biosynthesis in *Methanococcus jannaschii*

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ABSTRACT: The protein product of the *Methanococcus jannaschii* MJ0768 gene has been expressed in *Escherichia coli*, purified to homogeneity, and shown to catalyze the GTP-dependent addition of two L-glutamates to the L-lactyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin ($F_{420}$-0) to form $F_{420}$-0-glutamyl-glutamate ($F_{420}$-2). Since the reaction is the fifth step in the biosynthesis of coenzyme $F_{420}$, the enzyme has been designated as CofE, the product of the *cofE* gene. Gel filtration chromatography indicates CofE is a dimer. The enzyme has no recognized sequence similarity to any previously characterized proteins. The enzyme has an absolute requirement for a divalent metal ion and a monovalent cation. Among the metal ions tested, a mixture of $Mn^{2+}$, $Mg^{2+}$, and $K^+$ is the most effective. CofE catalyzes amide bond formation with the cleavage of GTP to GDP and inorganic phosphate, likely involving the activation of the free carboxylate group of $F_{420}$-0 to give an acyl phosphate intermediate. Evidence for the occurrence of this intermediate is presented. A reaction mechanism for the enzyme is proposed and compared with other members of the ADP-forming amide bond ligase family.

The biosynthesis of amide bonds in living systems is an essential metabolic process. The largest portion of this biosynthesis is ribosomally mediated (*1*). Many amide bonds, however, are synthesized using nonribosomal mechanisms (*2*). The enzymes that catalyze these reactions are known to produce a wide variety of important natural products that range from bacterial cell walls to coenzymes (*2*−*4*). Coenzymes containing amide bonds include coenzyme $F_{420}$, glutathione, coenzyme B, folate, methanopterin, $B_{12}$, and methanofuran (*5*, *6*). The enzymes themselves possess common features, in that they all require three substrates: NTP, a carboxylate-containing compound, and a free primary amino group. A new amide bond is formed between the amino group and the carboxyl that is activated by ATP.

Typical enzymes that convert the carboxyl group to an activated acyl group for amide bond formation do so by coupling the condensation with the hydrolysis of ATP and exhibit two major cleavage patterns, ATP to ADP and phosphate or ATP to AMP and pyrophosphate. Tetrahydrofolate:L-glutamate $\gamma$-ligase (EC 6.3.2.17; folylpolyglutamate synthetases or FPGS[1]) (*7*) and UDP-*N*-acetylmuramoyl-L-alanine:glutamate ligase (EC 6.3.2.9; MurD) (*8*) are two members of the amide bond ligase (ADP-forming) superfamily. Although there is little sequence identity between them, these two enzymes are placed in the same superfamily because of the presence of a phosphate-binding loop and the same spatial arrangement of their secondary structural elements (*9*).

The redox-active coenzyme $F_{420}$-2 (*N*-(*N*-L-lactyl-$\gamma$-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate) was first isolated from *Methanobacterium* str. M.o.H. based on the oxidized cofactor's intense absorbance peak at 420 nm and its blue-green fluorescence (*10*). It is known to have a wide distribution among the archaea and a limited distribution among the high G + C Gram-positive bacteria (*11*). The structure of coenzyme $F_{420}$ from *Methanobacterium* ($F_{420}$-2) has been shown to contain FO, a phosphate, and a lactyl group ($F_{420}$-0), terminating with two L-glutamate groups $\gamma$-linked (Figure 1) (*12*). *Methanosarcina barkeri* has modified $F_{420}$ with four and five $\gamma$-linked glutamate groups ($F_{420}$-4 and $F_{420}$-5) (*13*). *Mycobacterium smegmatis* cells contain primarily five and six $\gamma$-glutamyl forms of $F_{420}$ (*14*), whereas the euryarchaeon *Methanococcus jannaschii* contains a new $F_{420}$ designated $\alpha$-$F_{420}$-3 with an $\alpha$-linked terminal glutamate (*15*).

The assembly of $F_{420}$-0 in *M. jannaschii* has been studied by biochemical methods (*5*, *16*). It has also been demonstrated by genetic methods that *fbiA* and *fbiB* genes are required for coenzyme $F_{420}$ biosynthesis in *Mycobacterium bovis* BCG (*17*). *M. bovis* mutants with an insertion in *fbiA* or *fbiB* gene made no $F_{420}$-5,6 but did make FO. Therefore, the products of these genes were expected to be involved in steps between FO and $F_{420}$-5,6 in that organism (*17*). Homologous genes were then identified in genome sequences of euryarchaea and high G + C Gram-positive bacteria (*17*). *M. jannaschii* gene at locus MJ0768 was found to be the homologous gene of *fbiB*. In this work, we cloned the MJ0768 gene, overexpressed the protein product of this gene

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[1] Abbreviations: FO, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; $F_{420}$, the *N*-(*N*-L-lactyl-$\gamma$-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin; $F_{420}$-0, $F_{420}$ with no glutamic acid; $F_{420}$-1, $F_{420}$ with one glutamic acid; $F_{420}$-2, $F_{420}$ with two glutamic acids; $F_{420}$-3, $F_{420}$ with three glutamic acids; HPLC, high performance liquid chromatography; FPGS, folylpolyglutamate synthetase.

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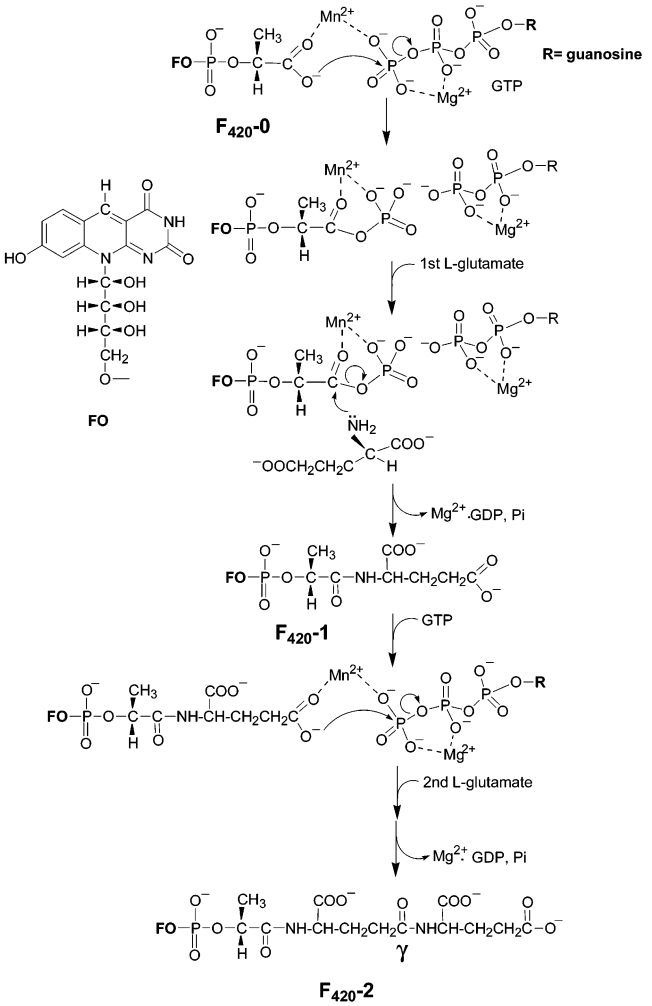


FIGURE 1: Reactions catalyzed by CofE.

in *E. coli*, purified the protein, and established that it catalyzes the fifth step in the biosynthesis of coenzyme $F_{420}$. On the basis of the position of the reaction in the $F_{420}$ biosynthesis pathway, we name this gene *cofE* and the enzyme CofE. We discovered that CofE adds two glutamate groups to $F_{420}$-0 to form $F_{420}$-2 with a $\gamma$-linkage between the two glutamates (Figure 1). CofE is the first member of this group of proteins to be characterized. The paralogous MJ1361 gene and homologous *Ms·cofE* gene in *M. smegmatis* were also cloned, and their respective proteins were expressed in *E. coli* and their activities examined. No activity was found for the MJ1361 paralog, but the Ms·CofE produced $F_{420}$-3 as the major product with lesser amount of $F_{420}$-4,5,6, consistent with it producing the polyglutamated $F_{420}$ compounds present in this organism.

## MATERIALS AND METHODS

*Materials.* All reagents were purchased from Sigma unless otherwise specified.

*Production of $F_{420}$ Compounds.* $F_{420}$-2 used was isolated from extracts of *Methanobacterium thermoautotrophicum* as previously described (*18*). It was analyzed by electrospray ionization mass spectrometry operated in negative ion mode. This technique produced an $(M-H)^-$ ion at 772.2 *m/z* corresponding to the expected molecular formula for $F_{420}$-2 of $C_{29}H_{36}O_{18}N_5P$. $F_{420}$ compounds containing no glutamyl residue ($F_{420}$-0) and one glutamyl residue ($F_{420}$-1) were generated by the treatment of $F_{420}$-2 with a solution of carboxypeptidase G from *Pseudomonas sp.* (5 units/100 $\mu$L, Sigma) and $\gamma$-glutamyl-transpeptidase IV from porcine kidney (50 units/500 $\mu$L, Sigma) in 50 mM TES/$Na^+$ and 10 mM $MgCl_2$ (pH 8.5) at 30 °C, respectively. At the completion of each incubation, proteins were precipitated by the addition of ethanol to 60% final concentration. After centrifugation, the supernatants were evaporated to dryness under a stream of nitrogen and dissolved in deionized water. Assignment and purity of the $F_{420}$ compounds were verified by high performance liquid chromatography (HPLC, see below). Concentrations of the solutions were determined by comparison of fluorescence intensities to $F_{420}$-0 with known concentration. The compounds were characterized as previously described (*16*).

$F_{420}$-0 was further purified to eliminate all traces of glutamate generated in the enzyme digestion, by passage the sample dissolved in 1% formic acid through a $C_{18}$ column (0.5 × 10 cm, 55−105 $\mu$m, Waters) equilibrated with 1% formic acid. The $F_{420}$-0 was then eluted with a step gradient of methanol in 1% formic acid. The bound $F_{420}$-0 was eluted from the column at 50% methanol. The elution was monitored by the $F_{420}$-0 fluorescence under the illumination of UV light.

*HPLC Method for the Analysis of $F_{420}$ Compounds.* Analysis of $F_{420}$ compounds were performed on a Shimadzu SCL-6B HPLC using a C-18 reversed-phase column (AXXI Chrom octyldecyl silane column, 5 $\mu$m, 4.6 mm × 25 cm) eluted with 15% methanol in 25 mM sodium acetate buffer (pH 6.0, 0.02% $NaN_3$) at a flow rate of 0.5 mL/min. The eluent was monitored by fluorescence (excitation wavelength 420 nm, emission wavelength 480 nm). Using this chromatographic condition, the $F_{420}$ derivatives showed the following retention times: $F_{420}$-2, 8.6 min; $F_{420}$-1, 13.2 min; $F_{420}$-0, 17.8 min; FO, 43.0 min.

*Nucleotide Product Identification.* The nucleotide products of the enzymatic reaction were analyzed by separation on a MonoQ HR anion-exchange column (0.5 × 5 cm, Amersham Biosciences) as described previously (*19*). Using the NaCl gradient, GMP was eluted at 26.1 min, GDP at 30.1 min, and GTP at 32.6 min. The elution of the guanosine nucleotides was monitored by UV absorbance at 254 nm.

*Cloning and Expression of the MJ0768, MJ1361, and Ms·cofE-Derived Proteins in E. coli.* The *Methanococcus jannaschii* genes at loci MJ0768 (Swiss-Prot accession number Q58178) and MJ1361 (Swiss-Prot accession number Q58756) were amplified by PCR from genomic DNA using oligonucleotide primers synthesized by Invitrogen. The MJ0768 gene was amplified using primers: Fwd, (5′-GGTGGTCATATGATTAAAGAAAAAAG-3′); and Rev, (5′-GATCGGATCCTTAATTTCTAAAAAC-3′). The MJ1361 gene was amplified using primers: Fwd, (5′-GGTCATATGAGAGCTTATCCC-3′); and Rev, (5′-GATCGGATCCTTATCTTTGAGATTCTGG-3′). *Ms·cofE*, the homologous gene of MJ0768 in *Mycobacterium smegmatis*, was amplified by PCR from *M. smegmatis* genomic DNA, which was extracted as previously described (*20*). The primers used for amplifications were: Fwd, (5′-GGTCATATGAGCGCCGCGGCGAACGCCGAG-3′); and Rev, (5′-GCTAAGCTTTCACTTGCGCACCAGCAGTTC-3′). PCR amplifications were performed as described previously (*21*) using a 50 °C

annealing temperature for MJ0768, 55 °C for MJ1361 and 65 °C for *Ms·cofE*. The primers introduced an *Nde*I and a *Hind*III site at the 5′- and 3′- end, respectively, for the amplified DNA from MJ0768 and *Ms·cofE*, and an *Nde*I and a *Bam*HI at the 5′- and 3′- end, respectively, for MJ1361. The amplified PCR products were purified by QIAQuick spin columns (Invitrogen) and then digested with restriction enzymes (*Nde*I and *Hind*III for MJ0768 and *Ms·cofE*; *Nde*I and a *Bam*HI for MJ1361) (Invitrogen). The MJ0768 and *Ms·cofE* genes were ligated into the compatible sites in plasmid pET19b (Novagen) by bacteriophage T4 DNA ligase (Invitrogen) to make the recombinant plasmids pMJ0768 and p*Ms·cofE*. The MJ1361gene was ligated into the compatible sites in plasmid pT7-7 (Novagen) to make the plasmid pMJ1361. DNA sequences were verified by dye-terminator sequencing at the University of Iowa DNA facility. The resulting plasmids pMJ0768 and pMJ1361 were transformed into *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene) cells. The plasmid p*Ms·cofE* was transformed into *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene) cells. Transformed cells were grown in Luria-Bertani medium (200 mL; Difco) supplemented with 100 $\mu$g/mL ampicillin at 37 °C with shaking until they reached an absorbance at 600 nm of 1.0. Recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional culture of 2 h, the cells were harvested by centrifugation (4000 × *g*, 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (12% T, 4% C acrylamide, using a Tris/glycine buffer system) analysis of total cellular proteins.

*Purification of the CofE.* Cell-free extracts were prepared by sonication of the *E. coli* cell pellets (400 mg, wet weight) suspended in 3 mL of buffer (20 mM Tris/HCl, pH 7.6) followed by centrifugation (14 000 × *g*, 15 min). The *E. coli* proteins were denatured by heating the soluble cell-free extract at 70 °C for 15 min, followed by centrifugation at 14 000 × *g* for 15 min to remove the insoluble material. Heat-soluble cell extract (2 mL) was applied to a Mono Q HR anion-exchange column (0.5 × 50 mm; Amersham Biosciences) equilibrated with buffer A (20 mM Tris/HCl, pH 7.5) (*19*). Bound protein was eluted with a 20 mL linear gradient from 0 to 1 M NaCl in buffer A at a flow rate of 0.5 mL/min. Elution of CofE was monitored by UV absorbance at 280 nm and activity. Active fractions were pooled and concentrated in a $M_r$ 10 000 cutoff Centricon YM-10 Centrifugal Filter Devices (Millipore). The protein was then frozen at −20 °C for future use.

Protein purity was evaluated by silver staining (Bio-Rad) of the SDS−PAGE separated proteins. Size of the denatured proteins was determined by comparison to low molecular weight protein standards (Bio-Rad). Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as the standard.

*Measurement of the Native Molecular Weight of CofE.* Size exclusion chromatography was performed on a Superose 12HR column (1 × 30 cm; Amersham Biosciences) equilibrated with a buffer containing 50 mM HEPES/NaOH, 0.15 M NaCl, 2 mM DTT, pH 7.2. Purified CofE in a volume of 160 $\mu$L was applied to the column and eluted at a flow rate of 0.4 mL/min. Protein standards were used to calibrate the sizing column as described previously (*19*).

*Measurement of CofE Activity.* In a standard assay, CofE (0.5−2 $\mu$g) was incubated with $F_{420}$-0 (0.4−2 $\mu$M) in a 50 $\mu$L reaction mixture containing 50 mM CHES/$Na^+$ buffer (pH 8.5), 0.2 M KCl, 10 mM L-glutamate, 5 mM GTP, 5 mM $MgCl_2$, and 5 mM $MnCl_2$ at 50 °C for 20 min. The reaction was initiated by adding $F_{420}$-0 and terminated by addition of EDTA to a final concentration of 10 mM. Quantitative analysis of the reaction products was conducted by HPLC as described above.

The nature of the linkage of the $F_{420}$ products in CofE standard assay was determined by incubation of the reaction product with peptidases of known specificity. The enzymes used were carboxypeptidase Y (specifically removes carboxyl terminal α-amino acids) and carboxypeptidase G (specifically cleaves terminal $\gamma$-glutamyl peptide bonds). After the completion of the CofE standard assay, the reaction mixture (2 $\mu$L) was treated with 5 $\mu$L of a solution of carboxypeptidase Y (baker's yeast (Sigma), 16 units/100 $\mu$L in 50 mM TES/$Na^+$, pH 7.5, 10 mM $MgCl_2$), or 5 $\mu$L of carboxypeptidase G (*Pseudomonas sp.*, (Sigma), 5 units/100 $\mu$L in 50 mM TES/$Na^+$, pH 7.5, 10 mM $MgCl_2$, Sigma) for 2 h at room temperature. The products were then determined on HPLC by their retention time and compared to the known $F_{420}$ compounds.

*Substrate Specificity and Inhibitory Effects of Substrate Analogues.* Substrate specificity of CofE was investigated using different nucleotides, amino acids and amino acid analogues. Substrates tested were ATP, UTP, CTP, TTP, dGTP, $\beta,\gamma$-$CH_2$-GTP at 5 mM concentrations, and $\beta$-glutamate, D-glutamate, L-glutamine, $\gamma$-glutamyl-glutamate, L-aspartate, L-α-aminoadipic acid, L-homocysteic acid, DL-2-amino-4-phosphonobutyric acid, 2-carboxyethylphosphonic acid, and DL-2-amino-3-phosphonopropionic acid at 10 mM concentrations.

Inhibitory effects of various substrate analogues on CofE activity were also examined. The reactions included 5 mM GTP and other nucleotides, or 10 mM L-glutamate and 10 mM of the L-glutamate analogues.

*Heat Stability, pH Optimum, and Metal Effect on CofE Activity.* To test the heat stability of CofE, purified CofE was assayed at different temperatures from 25 to 90 °C or incubated for 15 min at those temperatures and then assayed at 50 °C. The effect of pH on CofE activity was studied by using 50 mM TES or CHES buffers in the pH range 6.0−10.0. DL-Dithiothreitol (DTT) (10 mM) was added to the standard assays to test its effect.

The effect of both monovalent and divalent cations on CofE activity was investigated. Different concentrations of KCl were used in the standard assay from 0 to 0.5 M. Other monovalent cations tested were 0.2 and 0.5 M concentrations of NaCl, 0.1 and 0.4 M concentrations of $NH_4Cl$, 0.2 M RbI, CsCl, and LiCl. Divalent metals used in the assay included 10 mM concentrations of $MgCl_2·6H_2O$ (Fisher), $MnCl_2·4H_2O$ (Fisher), $CoCl_2·6H_2O$ (J. T. Baker Inc.), $NiCl_2·6H_2O$, $CaCl_2·6H_2O$ (Fisher), and $ZnSO_4·7H_2O$.

*Kinetic Analysis of CofE.* For the determination of the kinetic parameters of CofE activity, initial reaction rates were measured in standard assays at various concentrations of $F_{420}$-0 (0−30 $\mu$M). Production of both $F_{420}$-1 and $F_{420}$-2 was monitored. A curve from the Michaelis-Menton equation was fit through the initial rate data by nonlinear regression, using the Sigma Plot 2000 program (SPSS Science).

Table 1: Purification of CofE from Recombinant *E. coli* Cell Paste (0.4 g)

| step | total protein,[a] mg | total activity, nmol/min | specific activity,[b] nmol min$^{-1}$ mg$^{-1}$ | yield, % |
|---|---|---|---|---|
| crude extract | 49 | 2.0 | 0.04 | 100 |
| heat treatment | 6.5 | 1.6 | 0.24 | 80 |
| mono Q | 2.0 | 1.2 | 0.60 | 60 |

[a] Protein concentration was measured using the Bradford protein assay (Bio-Rad). [b] Specific activity was measured with the standard assay. $F_{420}$-2 production was used to calculate the specific activity.

*Examination of MJ1361 and Ms·cofE Encoded Proteins.* Cell-free extracts were prepared as described for CofE. The soluble fraction of *E. coli* (MJ1361 expression) cell extracts were heated at 70 °C for 15 min followed by centrifugation at 14 000 × *g* for 15 min to remove the insoluble material. Heated-soluble cell extract was then used for the CofE standard assay. The soluble *E. coli* (*Ms·cofE* expression) cell extracts without heat treatment (10 $\mu$L) were used for the CofE standard assay, except that the reaction mixture was incubated at 37 °C, and both GTP and ATP were added in the assay. Both reactions were stopped by the addition of ethanol to a final concentration of 60%. After centrifugation, the supernatants were evaporated to dryness under a stream of nitrogen and dissolved in deionized water before the HPLC analysis of the reaction products.

*Phylogenetic Analysis of CofE Homologues* . Amino acid sequences similar to *M. jannaschii* CofE were aligned automatically by using the CLUSTALW program (Ver. 1.83) (*22*). From the alignment of 22 protein sequences, 214 positions were deemed to be confidently aligned. These were analyzed by protein maximum likelihood methods by using the PROML program (Ver. 3.6a2.1) (*23*) with the Jones, Taylor, and Thornton model of amino acid changes. Bootstrap proportions were calculated by using SEQBOOT, PROML, and CONSENSE programs (*23*) to create and evaluate 100 resampled alignments.

## RESULTS

*Purification of CofE.* The purification of the recombinantly expressed CofE is summarized in Table 1. We took advantage of the expected thermal stability of the CofE protein and purified the protein through a heat treatment to remove the bulk of the *E. coli* proteins. Following Mono Q ion-exchange chromatography, the resulting CofE protein showed a single band on the SDS−PAGE. When loaded on an analytical size-exclusion column, CofE eluted with an apparent molecular mass of 52 100 Da, compared to its predicted molecular mass of 27 150 Da, this elution profile suggests that the native CofE protein may form a dimer.

*CofE Catalyzed Reactions.* When incubated with $F_{420}$-0, L-glutamate, and GTP, CofE added two L-glutamates on $F_{420}$-0 to form $F_{420}$-2. The ion-exchange chromatography analysis indicated that the nucleotide product of the reaction was GDP. The product $F_{420}$-2 contains two glutamates with the terminal glutamate being bound through an amide bond to the $\gamma$-position of the first one (Figure 1). Analysis of the product formation throughout a 1 h incubation at a 2 $\mu$M concentration of $F_{420}$-0 showed a rapid production of $F_{420}$-1 followed by a decrease of $F_{420}$-1 which corresponded with the increase of $F_{420}$-2 (Figure 2). When increasing the concentration of $F_{420}$-0 to about 15 $\mu$M, the only product of CofE reaction is $F_{420}$-1, whereas at concentration below 3 $\mu$M, the major product is $F_{420}$-2 (data not shown). CofE is also able to add one L-glutamate on $F_{420}$-1 to form $F_{420}$-2.

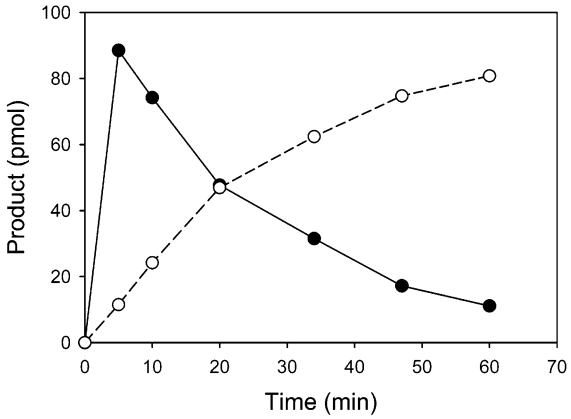


FIGURE 2: Product formation (●, $F_{420}$-1; ○, $F_{420}$-2) as a function of time from $F_{420}$-0 incubated with L-glutamate, GTP, and CofE. Standard assay conditions were used with 2 $\mu$M $F_{420}$-0 and 0.6 $\mu$g of CofE in a 50 $\mu$L volume. At each time point, samples (5 $\mu$L) were diluted into a 50 $\mu$L ice-cold 2 mM EDTA solution before HPLC analysis.

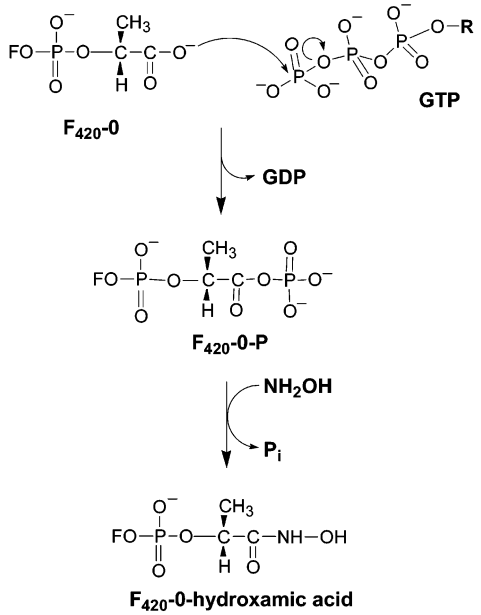


FIGURE 3: Proposed reaction of CofE incubated with $F_{420}$-0, GTP, and hydroxylamine.

An expected intermediate in the formation of $F_{420}$-1 would be the acyl phosphate of $F_{420}$-0. To increase the concentration of this compound and simultaneously convert it into a stable derivative, $F_{420}$-0 and GTP were incubated with CofE in the absence of glutamate and in the presence of 0.1 M hydroxylamine. Hydroxylamine is well-known to convert acyl phosphates into hydroxamic acids, as shown in Figure 3. Under these conditions, two new fluorescent compounds derived from the $F_{420}$-0 were detected by HPLC. The retention time for the first peak (11.2 min) was 2 min earlier than $F_{420}$-1 (13.2 min), and the second peak was eluted at 40 min, which was 3 min before FO (43.0 min). The retention times of these peaks would be that expected if the first peak was $F_{420}$-0-P, and the second was the hydroxamic acid derivative of $F_{420}$-0. This idea is based on the correlation between the

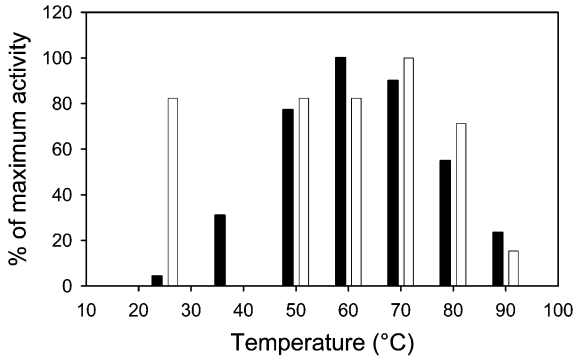


FIGURE 4: Heat stability of the CofE protein and activity assays at various temperatures. Solid bars: CofE activities measured at the indicated temperatures. Open bars: CofE activities measured at 50 °C after it was incubated at the indicated temperature for 15 min. Error range is less than 10% in these assays.

retention time and net charge of the $F_{420}$ derivatives, the more charge on the molecule, the earlier its elution time. Thus, $F_{420}$-0-P with two negative charges is eluted around $F_{420}$-1 (with two negative charges) and the hydroxamic acid derivative of $F_{420}$-0 around FO (both with no charge). In support of this peak assignment, we observed the disappearance of the first peak and the increase intensity of the second peak after another 30 min incubation. The proposed $F_{420}$-0-P intermediate was not seen in the standard CofE assay in the absence of L-glutamate; therefore, hydroxylamine may facilitate the dissociation of the acyl phosphate intermediate from the enzyme.

*Characterization of CofE Activity.* The activity of purified CofE was characterized under various conditions. Assays under different pH showed a maximum activity at pH 8.5 with 50 mM CHES/$Na^+$ buffer. No change of CofE activity was observed when it was assayed with the addition of 10 mM DTT to the reaction mixture. CofE is also a thermostable enzyme. When heated at 80 °C for 15 min, CofE retained over 70% of its activity, when assayed at 50 °C. The optimum temperature for assaying CofE activity was found to be 60 °C (Figure 4).

CofE absolutely requires a monovalent cation for activity, and the greatest extent of activation is achieved by $K^+$, with maximum stimulation occurring at 0.2 M KCl. $NH_4^+$ stimulates activity to a lesser extent, whereas $Na^+$ and $Li^+$ have no effect on CofE activity. In the presence of either $Rb^+$ or $Cs^+$ at 0.2 M concentration, the only product of reaction was $F_{420}$-1.

Divalent cation requirement was examined by assaying CofE with different divalent metals. No activity was detected in reactions without added metals. Maximum CofE activity was observed with the addition of 10 mM $MnCl_2$. Reactions containing 10 mM either $MgCl_2$ or $CoCl_2$ produced 40% of the $F_{420}$-2 of that was produced with $MnCl_2$. Under the same assay conditions, in the presence of $NiCl_2$, all the $F_{420}$-0 was converted to $F_{420}$-1, but no $F_{420}$-2 was observed, whereas only 26% and 8% $F_{420}$-0 were converted to $F_{420}$-1 with $ZnSO_4$ and $CaCl_2$, respectively. The nature of the metal requirement of the CofE was further investigated in experiments in which the $MgCl_2$ concentration was fixed to 0.5, 2, and 5 mM in the standard reaction mixture, while the concentration of $MnCl_2$ was varied from 0 to 5 mM. Activation experiments under various concentrations of $MgCl_2$ and $MnCl_2$ showed that the higher $Mg^{2+}$ concentration used, the lower $Mn^{2+}$ concentration needed to achieve the maximum activity. The combination of 5 mM $MgCl_2$ and 2−5 mM of $MnCl_2$ supported the highest activity. The combination of 5 mM $MgCl_2$ and 5 mM $MnCl_2$ was chosen in the standard CofE activity assay.

Table 2: Nucleotide Analogs as Substrates and Inhibitors for CofE

| nucleotide, 5 mM | relative activity[a] (as substrate), % | relative activity[b] (as inhibitor), % |
|---|---|---|
| GTP | 100 | 100 |
| ATP | *c* | 70 |
| dGTP | 25 | 90 |
| UTP | 66 | 89 |
| CTP | 0 | 107 |
| dTTP | 0 | 98 |
| $\beta$-$\gamma$-$CH_2$-GTP | 0 | 44 |
| GDP | 0 | 33 |
| GMP | 0 | 100 |
| Pi | 0 | 100 |

[a] Activities measured with individual nucleotide are expressed as the percentage of activity for $F_{420}$-2 formation compared to that obtained with 5 mM GTP. [b] Relative activities are expressed as the percentage of activity compared to that obtained with 10 mM GTP only. Analogues were added at a concentration of 5 mM to the reaction mixture containing 5 mM GTP in the CofE standard assay. [c] Reaction with ATP only produces 100% $F_{420}$-1.

*Substrate Specificity of CofE.* A number of amino acids and derivatives were tested for their ability to be used as the substrate for CofE compared to L-glutamate. CofE incubated with 10 mM $\beta$-glutamate, D-glutamate, $\gamma$-glutamyl-glutamate, DL-2-amino-3-phosphonopropionic acid, 2-carboxyethylphosphonic acid, or L-α-aminoadipic acid produced no $F_{420}$-1, $F_{420}$-2 or other $F_{420}$ analogues. $F_{420}$ analogues were formed and detected by HPLC (% conversion, retention time) when CofE was incubated with GTP and 10 mM of the following compound: L-aspartate (20%, 12.0 min), L-glutamine (10%, 12.2 min), L-homocysteic acid (100%, 10.1 min), or DL-amino-4-phosphono-butyric acid (57%, 9.8 min). The analogues are most likely products that had one molecule added to the carboxyl end of the $F_{420}$-0 through their amino group. When these analogues were tested for their inhibitory effects on CofE activity of L-glutamate at the same concentration, no significant inhibitions were observed.

The effectiveness of various nucleotides as substrates for CofE was tested at 5 mM concentration (Table 2). CofE exhibited maximum activity with GTP, compared with UTP (66%) and dGTP (25%). With ATP, only $F_{420}$-1 was observed as the product. CTP and TTP supported no activity. The inhibition study also indicated that ATP, dGTP, UTP, CTP, and TTP had little or no effect on CofE activity. $\beta$-$\gamma$-$CH_2$-GTP, the phosphonate derivative of GTP, was not a substrate for the enzyme but was an effective inhibitor. GDP was also an inhibitor of the activity, but not GMP and $P_i$.

Besides $F_{420}$-0, CofE also adds one L-glutamate on $F_{420}$-1 to form $F_{420}$-2. It cannot use $F_{420}$-2 as substrate to add more glutamates. Initial rates for CofE using $F_{420}$-0 or $F_{420}$-1 as substrate to produce $F_{420}$-2 were measured at various substrate concentrations. Both sets of data were fit to the Michaelis-Menton first-order rate equation. At 50 °C, apparent kinetic parameters of CofE using $F_{420}$-0 as substrate were $K_m = 1.0 \pm 0.15$ $\mu$M, $V_{max} = 2.4 \pm 0.09$ nmol $min^{-1}$ $mg^{-1}$. The rate of $F_{420}$-1 formation was about 10-fold higher that of the $F_{420}$-2 formation when $F_{420}$-0 concentration was 0.5 $\mu$M in the assays. Using $F_{420}$-1 as substrate, the

parameters were $K_m = 0.21 \pm 0.05$ $\mu$M, $V_{max} = 0.96 \pm 0.07$ nmol min$^{-1}$ mg$^{-1}$.

*Activities of MJ1361 and Ms·cofE-Encoded Proteins.* In *M. jannaschii*, CofE catalyzes the addition of two glutamates to $F_{420}$-0 with a $\gamma$-linkage between them. A paralogous gene of *cofE* in this organism is MJ1361. We tested its gene product, and no activity was observed when it was incubated with $F_{420}$-0 in the standard assay of CofE. The structures of $F_{420}$ coenzymes in *Mycobacterium* species have also been examined, and apparently *Mycobacterium smegmatis* has $F_{420}$ derivatives with five and six $\gamma$-linked glutamate groups attached (*14*, *15*). We expressed the *cofE* homologous gene from *M. smegmatis*, namely, *Ms·cofE*, in *E. coli*. Incubation of the cell crude extract with $F_{420}$-0, L-glutamate, KCl, ATP, and GTP led to the production of $F_{420}$-3 as the major product and a small amount of $F_{420}$-4,5,6 detected by HPLC by comparing their retention time to the known $F_{420}$-5,6 extracted from *M. smegmatis* (data not shown). This result confirmed that another member of this family catalyzes the same reaction adding multiple $\gamma$-linked glutamates to form polyglutamate $F_{420}$ derivatives.

*Phylogenetic Analysis of CofE Homologues* . The evolutionary history of genes homologous to *cofE* was analyzed using a phylogeny inferred from an alignment of 22 protein sequences (Figure 5). The phylogeny resolves two gene lineages: orthologs of the *M. jannaschii cofE* gene are found in microorganisms that produce $F_{420}$ (*14*), whereas paralogs of *cofE* (such as the gene at locus MJ1361 in *M. jannaschii*) must have a different, yet to be identified function. This phylogeny is consistent with the *cofE* gene being vertically inherited in the $F_{420}$-producing archaea, with a single lateral transfer event giving rise to the bacterial homologues . These bacterial genes are found not only in high % G + C Gram-positive bacteria that produce $F_{420}$, but also in the pathogen *Streptococcus pyogenes* and the anoxygenic phototroph *Chloroflexus aurantiacus*. These two bacteria are not known to produce $F_{420}$, and their genomes are missing other genes known to be required for $F_{420}$ biosynthesis. Homologous genes were identified in the genomes of several *Chlamydia* spp., intracellular parasites that are unlikely to produce $F_{420}$. Finally, no homologues were identified in genome sequences of cyanobacteria that produce FO (unpublished experiments), implying that these organisms do not produce the polyglutamyl forms of $F_{420}$ described here.

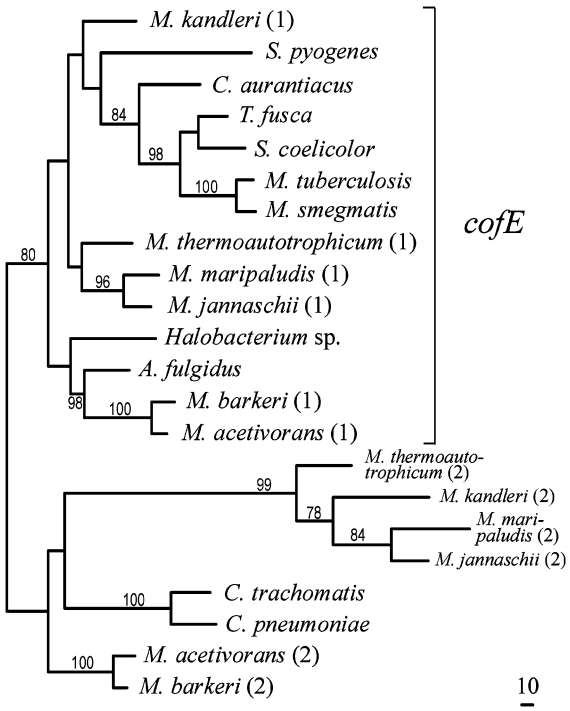


FIGURE 5: Phylogeny of *cofE* homologues inferred by the protein maximum likelihood method. Sequences from microorganisms that produce coenzyme $F_{420}$ included those from *Methanopyrus kandleri* (GenBank accession numbers 19887484 and 19887314) *Thermobifida fusca* (23018836), *Streptomyces coelicolor* (7649501), *Mycobacterium tuberculosis* (15610398), *Mycobacterium smegmatis* (http://www.tigr.org), *Methanobacterium thermoautotrophicum* (15679037 and 15679664), *Methanococcus maripaludis* (http://www.genome.washington.edu/UWGC/methanococus/), *Methanococcus jannaschii* (1499588 (MJ0768, *cofE*) and 1592005 (MJ1361)), *Halobacterium* sp. (15791326), *Archaeoglobus fulgidus* (11499837), *Methanosarcina barkeri* (23052365 and 23051094), and *Methanosarcina acetivorans* (19913891 and 19917566). Homologues from microorganisms that are not known to produce $F_{420}$ include those from *Streptococcus pyogenes* (NP 268810.1), *Chloroflexus aurantiacus* (22972964), *Chlamydia trachomatis* (3329056), and *Chlamydia pneumoniae* (4377067). Paralogs are distinguished by numbers in parentheses after the species' names. The tree is rooted by using the methanogen paralogs as an outgroup. Bootstrap percentages are indicated for branches supported by a plurality of bootstrap replicates. (Bar = 10 amino acid replacements per 100 positions.)

## DISCUSSION

CofE has no recognized sequence similarity to any previously characterized enzyme. It is the first enzyme studied among its homologous proteins from the $F_{420}$-containing organisms (Figure 5). The enzyme catalyzes the addition of two glutamates to $F_{420}$-0 to form $F_{420}$-2 in *M. jannaschii*. The homologous protein of CofE in *M. smegmatis* was able to add up to five glutamates to $F_{420}$-0. These results establish that other members of this family can catalyze the addition of multiple $\gamma$-linked glutamates to form polyglutamated $F_{420}$ derivatives.

The activities of the CofE protein of *M. jannaschii* are similar to those folylpolyglutamate synthetases from *E. coli* (*24*), *Corynebacterium sp.* (*25*) and rat liver (*26*). Despite the fact that there is no amino acid sequence similarity among these enzymes, they all catalyze the addition of multiple $\gamma$-linked L-glutamates to their substrate and there is an absolute requirement for $K^+$, a divalent cation, and nucleotide triphosphate. However, a number of differences do exist. Specific activity of the purified CofE is lower than that of the *E. coli* and the *Corynebacterium sp.* enzymes, but close to the eukaryotic FPGSs (*26*, *27*). A critical concern about the observed low specific activity would be if it is high enough to account for the amount of $F_{420}$ produced by the cells. Considering that the cells contain about 0.6 nmol of $F_{420}$ per mg protein (*15*) and have a doubling time of about 1 h (*28*), it would be enough to generate that amount of $F_{420}$ if 0.5% of the cell protein was this enzyme.

CofE uses GTP more efficiently than ATP, whereas the FPGSs prefer ATP to GTP. Although $Mg^{2+}$ supports the maximum activity for the FPGSs, there are differences in the ability to use $Mn^{2+}$ (*24*, *25*). CofE, on the other hand, is likely to use both $Mn^{2+}$ and $Mg^{2+}$ for its activity, as data showed that adding the two metals together at nonsaturated concentrations for CofE assay gave 5−10-fold higher activity

than with any individual metal. The *E. coli* FPGS is only capable of diglutamate synthesis with its preferred form of folate substrate (*24*), whereas the *Corynebacterium* FPGS not only adds a single glutamate to the monoglutamate substrate effectively, but also produces up to tetraglutamate folate derivatives. CofE can use both $F_{420}$-0 and $F_{420}$-1 as substrates, adding two or one L-glutamate, respectively, to form $F_{420}$-2. No conditions were found for the addition of more glutamates. The affinities for $F_{420}$-0 and $F_{420}$-1 are close to those of FPGSs for folate derivatives.

Acyl phosphate intermediates have been detected with FPGS isolated from *E. coli*, hog liver and rat liver (*29*, *30*). It was also shown that ADP is the only nucleotide product formed in the reaction catalyzed by FPGS from *Corynebacterium sp.* (*25*) or from bacteria and mammalian cells (*31*). All these results indicated that the folate substrate is phosphorylated first and then reacts with the amino group of L-glutamate to form the amide bond and release the phosphate. For the CofE catalyzed reaction, we also observed that GDP is the only nucleotide product formed. Taken together with results from the hydroxylamine experiment (Figure 3), it is likely that the CofE reaction also involves the activation of the free carboxylate group of $F_{420}$-0 or $F_{420}$-1 by GTP, forming an acyl-phosphate intermediate, followed by nucleophilic attack by the amino group of the incoming glutamate to form the respective glutamated $F_{420}$ compounds.

The order of the substrate binding with FPGS from *Corynebacterium sp*. has been investigated (*32*, *33*). Initial velocity, product inhibition, and competitive inhibition studies were consistent with the mechanism of MgATP binding first to the enzyme, tetrahydrofolate or other folate anologs second, and glutamate last. However, recent X-ray crystallographic data suggested that although the binding order of the first two substrates (ATP and folate) can be random for FPGS, folate may effectively be the first substrate to bind and causes a conformational change in FPGS that is essential for the initiation of catalysis (*7*, *34*, *35*). Whether $F_{420}$-0 or GTP binds first to CofE is unknown; however, there may be a similar requirement for the conformational change at the active site, so that the third substrate L-glutamate can bind. Since a second L-glutamate has to be added to $F_{420}$-1 as well, more adjustment of the active site might be needed. We propose that this might occur by an expansion of the protein structure so that the FO moiety of $F_{420}$-1 and the carboxyl group of the glutamate are still bound at the same positions as the corresponding groups of $F_{420}$-0. For the CofE homologous protein in *M. smegmatis*, further expansion may be needed for the addition of up to five glutamate residues to $F_{420}$-0. Similar conformational changes at the active site can be expected for the FPGSs, especially for some mammalian enzymes that can synthesize derivatives with between six and nine glutamate residues (*27*, *34*).

Both $F_{420}$-1 and $F_{420}$-2 were detected over a 1 h reaction time course, and at the end of the reaction, most $F_{420}$-1 was converted to $F_{420}$-2 (Figure 2), indicating that glutamate was added stepwise and the produced $F_{420}$-1 must be released by the enzyme and then rebind to it. The high concentration of $F_{420}$-0 seems to impact CofE's ability to react with the second glutamate. Similar results were reported with the mammalian FPGSs that the lengths of the products are inversely dependent on the initial substrate concentration (*26*, *27*). In addition, when CofE was incubated with $Rb^+$ or $Cs^+$ instead of $K^+$ or ATP instead of GTP, $F_{420}$-1 was the only product of the reaction. The binding of these compounds may cause CofE unable to make the conformational changes required for the addition of the second L-glutamate.

Some kinases have been reported to require two divalent cations for their full activities, such as the pyruvate kinase (*36*) and phosphoenolpyruvate carboxykinase (*37*, *38*). For both enzymes, one $Mg^{2+}$ coordinates with the nucleotide, whereas the second cation, usually a transition metal, has a separate distinct binding site (*39*), similar to the metal sites shown in Figure 1. Recent crystal structures of FPGS (*34*) and MurD (*40*) both revealed two divalent metal ions bound at the active site. The first $Mg^{2+}$ binding site is a classical $Mg^{2+}$ site common to many ATP- and GTP-binding proteins (*41*). The second $Mg^{2+}$ or $Mn^{2+}$ site has been proposed to be involved in mediating the nucleophilic attack by the L-glutamate substrate (*34*, *40*). In addition, $Mn^{2+}$ was suggested to be a better candidate for the second metal binding site, and the reaction geometry is similar to that observed in the phosphoenolpyruvate carboxykinase active site (*38*, *40*). For CofE, apparently $Mn^{2+}$ activates the activity better than $Mg^{2+}$ and other divalent cations. The combination of $Mn^{2+}$ and $Mg^{2+}$ activates CofE most effectively. Therefore, $Mn^{2+}$ here likely plays a role in stabilizing the acyl phosphate intermediate and the successive transition state of the enzyme. Figure 1 shows the possible metal binding sites at the active site, in which $Mn^{2+}$ and $Mg^{2+}$ can be coordinated through the conserved aspartate or glutamate in CofE and its homologous proteins.

In summary, this work describes the enzyme catalyzing amide bond formation between the $F_{420}$-0 and L-glutamate in coenzyme $F_{420}$ biosynthesis. We identified the gene in *M. jannaschii*, *cofE*, responsible for this reaction and established the functions of the encoded protein, CofE. It is the first protein among its homologous proteins to be characterized and may be the first member of a new protein family. Although having no sequence similarity to the well studied folylpolyglutamate synthetase, CofE catalyzes the addition of glutamates to $F_{420}$-0 through a manner strikingly similar to that enzyme. We expect that future studies of the enzyme's structure will help to illustrate CofE's complicated mechanism and its relationship to the folylpolyglutamate synthetase if any.

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