Characterization of the 2-Phospho-L-lactate Transferase Enzyme Involved in Coenzyme F_{420} Biosynthesis in *Methanococcus jannaschii*†

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ABSTRACT: The protein product of the *Methanococcus jannaschii* MJ1256 gene has been expressed in *Escherichia coli*, purified to homogeneity, and shown to be involved in coenzyme F_{420} biosynthesis. The protein catalyzes the transfer of the 2-phospholactate moiety from lactyl (2) diphospho-(5′)guanosine (LPPG) to 7,8-didemethyl-8-hydroxy-5-deazariboflavin (Fo) with the formation of the L-lactyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (F_{420}-0) and GMP. On the basis of the reaction catalyzed, the enzyme is named LPPG:Fo 2-phospho-L-lactate transferase. Since the reaction is the fourth step in the biosynthesis of coenzyme F_{420}, the enzyme has been designated as CofD, the product of the *cofD* gene. The transferase requires Mg^{2+} for activity, and the catalysis does not appear to proceed via a covalent intermediate. To a lesser extent CofD also catalyzes a number of additional reactions that include the formation of Fo-P, when the enzyme is incubated with Fo and GDP, GTP, pyrophosphate, or tripolyphosphate, and the hydrolysis of F_{420}-0 to Fo. All of these side reactions can be rationalized as occurring by a common mechanism. CofD has no recognized sequence similarity to any previously characterized enzyme.

Coenzyme F_{420}[1] (Figure 1) is a redox-active cofactor that is presently known to have only a limited distribution among the archaea and high G + C Gram-positive bacteria (*1*). Although playing a crucial role in methanoarchaeal metabolism (*2*), coenzyme F_{420} has also been found in various eubacteria such as *Streptomyces*, *Rhodococcus*, *Nocardioides*, *Mycobacterium*, and their relatives (*1*). The coenzyme, in fact, was first isolated as a cofactor involved in the biosynthesis of chlortetracycline in *Streptomyces aureofaciens* in 1960 (*3*). The coenzyme is currently known to be involved in the biosynthesis of a number of secondary metabolites (*4*), the degradation of nitroaromatics (*5*), and activation of nitroimidazofurans (*6*). *Mycobacterium* and *Nocardia* contain an F_{420}-dependent glucose-6-phosphate dehydrogenase (*7*, *8*), and an F_{420}-containing photolyase functions in DNA-repair mechanisms in a number of microorganisms (*9*).

The assembly of F_{420}-0 and its polyglutamate derivatives from 7,8-didemethyl-8-hydroxy-5-deazariboflavin (Fo), pyruvate, and glutamate requires at least six steps which are outlined in Figure 1 (*10*). The fourth step in this sequence of reactions is the reaction of lactyl (2) diphospho-(5′)guanosine (LPPG) with Fo to form F_{420}-0 (F_{420} with no glutamic acid) and GMP. The fact that the nonnucleotide portion of the LPPG is transferred to an acceptor places this reaction among a limited group of similar reactions found in biochemistry. One of these reactions catalyzed by cobalamin synthase (cobS) (*11*, *12*) is involved in adenosylcobalamin (AdoCbl) biosynthesis where adenosylcobalamin guanosine diphosphate (AdoCbi-GDP) reacts with the 2′-hydroxy group of α-ribazole to form AdoCbl and GMP. Another example of this type of reaction is the second step of the DNA ligase reaction where a pyrophosphate diester of an adenylylated DNA reacts with the 3′-hydroxyl from the same DNA molecule in the completion of the ligase reaction (*13*). Further examples occur in phospholipid biosynthesis, where CMP-activated phosphate monoesters react with a series of hydroxyl acceptors to generate the phosphodiester linkage present in phospholipids. These include reactions catalyzed by CDP-choline:1,2-diacylglycerol phosphocholine transferase and CDP ethanolamine:1,2-diacylglycerol phosphoethanolamine transferase (*14*, *15*). Analogously, phosphophatidylinositol synthase (*16*, *17*), glycerophosphate phosphatidyltransferase (*18*), and phosphotidylserine synthase in yeasts (*16*, *17*) or bacteria (*19*) catalyze the transfer of the 1,2-diacylglycerol phosphate portion of CDP-diacylglycerol to the respective acceptor. All of these enzymes have been grouped into a superfamily on the basis of their sequence similarities (*20*). To be added to this list is the recently

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[1] Abbreviations: 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-deazariboflavin; F_{420}-0, F_{420} with no glutamic acids; F_{420}-1, F_{420} with one glutamic acid; F_{420}-2, F_{420} containing two glutamic acids; F_{420}-3, F_{420} with three glutamic acids; F_{420}-4, F_{420} with four glutamic acids; H_4MPT, tetrahydromethanopterin; Fo-P, 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate (originally referred to as F^+); GC-MS, gas chromatography−mass spectrometry; L-P, 2-phospho-L-lactate; cL-P, cyclic 2-phospho-L-lactate; LPPG, lactyl (2) diphospho-(5′)guanosine; LPPA, lactyl (2) diphospho-(5′)adenosine; CH_3LPPG, guanosine(5′)-diphospho-L-lactic acid methyl ester; CH_3LPPA, adenosine(5′)-diphospho-L-lactic acid methyl ester; GPPG, guanosine(5′)diphospho-(5′)guanosine; APPA, adenosine(5′)diphospho-(5′)adenosine; TCA, trichloroacetic acid.

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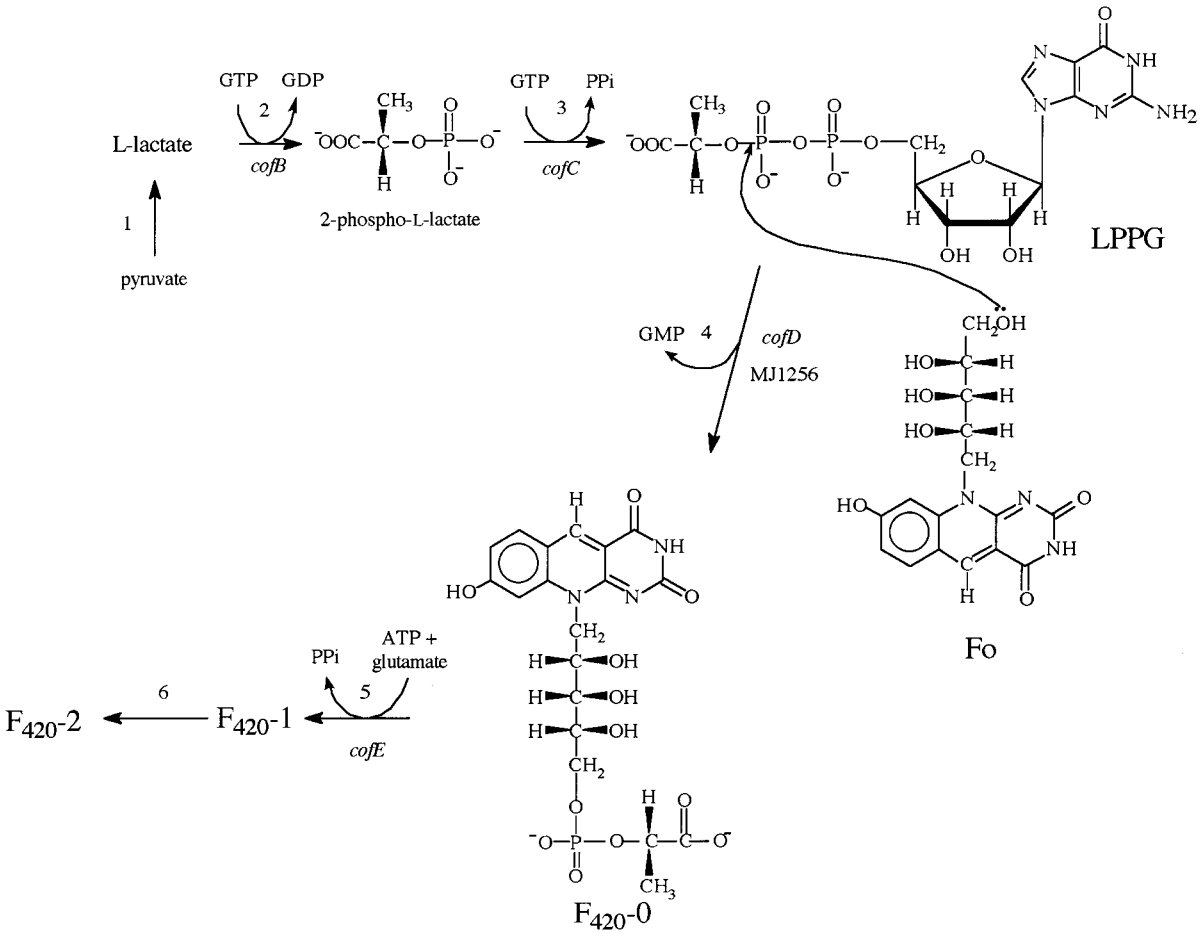

FIGURE 1: Pathway for coenzyme F_{420} biosynthesis.

characterized CDP-archaeol:serine archaeol-P transferase involved in the biosynthesis of archaeal phospholipids (*21*). In each of these reactions the nonnucleotide portion of a nucleotide pyrophosphate diester is transferred to a hydroxyl group in the substrate, as occurs in the reaction described here. In most nucleotide transferases the nucleotide portion of one substrate is transferred to the acceptor substrate. In some of these reactions the direct transfer of NMP occurs while others may involve an enzyme acyl-phosphate intermediate (*20*).

Recently, several genes in *Mycobacterium bovis* have been implicated in the biosynthesis of F_{420} (*22*). Among the identified genes is Rv3261, which has homology to the *Methanococcus jannaschii* gene MJ1256, as well as to genes from other archaea and bacteria known to contain F_{420}. In each case, these genes have been annotated as hypothetical proteins, and their alignments with other members of this group are shown in Figure 2. None of these genes have any recognized sequence similarity to any previously characterized enzyme.

We have now cloned the MJ1256 gene, overexpressed the protein product of this gene in *Escherichia coli*, purified the protein, and established that it catalyzes the fourth step in the biosynthesis of coenzyme F_{420}. On the basis of the position of the reaction in the F_{420} biosynthetic pathway, we name this gene *cofD* and the enzyme CofD. We have also discovered that the enzyme catalyzes a number of unexpected phosphoryl transferase reactions that can be rationalized on the basis of the proposed mechanism of this enzyme.

MATERIALS AND METHODS

7,8-Didemethyl-8-hydroxy-5-deazariboflavin (Fo) was prepared from 1-deoxy-1-[(3-hydroxyphenyl)amino]-D-ribitol and 6-chlorouracil as previously described (*23*). Guanosine(5′)-diphospho-L-lactate (LPPG) and adenosine(5′)-diphospho-L-lactate (LPPA) were synthesized as described previously (*10*). [8-^{3}H]Guanosine 5′-triphosphate tetrasodium salt (10 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO. Nucleotide pyrophosphatase type II from *Crotalus adamanteus* venom and β,γ-methyleneguanosine 5′-triphosphate were obtained from Sigma.

Production of F_{420} Intermediates. F_{420}'s containing either one (F_{420}-1) or no glutamyl residues (F_{420}-0) were generated by the treatment of F_{420} with a solution of γ-glutamyltranspeptidase type IV from porcine kidney (50 units/500 μL, Sigma) in 0.1 M TES/Na^+ and 10 mM $MgCl_2$ (pH 9.0) at room temperature. The F_{420} used in these incubations was isolated from extracts of *Methanobacterium thermoautotrophicum* as previously described for the isolation of methanopterin (*24*). For F_{420}-1 production, an incubation time of 3 h was used, while for the F_{420}-0, an incubation time of 2 days was used. At the completion of each incubation the proteins were precipitated by the addition of ethanol to 60%. After centrifugation (14000*g*) the supernatants were evaporated to dryness under a stream of nitrogen and dissolved in 200 μL of 10 mM NH_4OH. Concentrations of the solutions were measured at pH 8.0 using $\epsilon_{420} = 51500\ M^{-1}\ cm^{-1}$. Assignment and purity of samples (>95%) were verified by

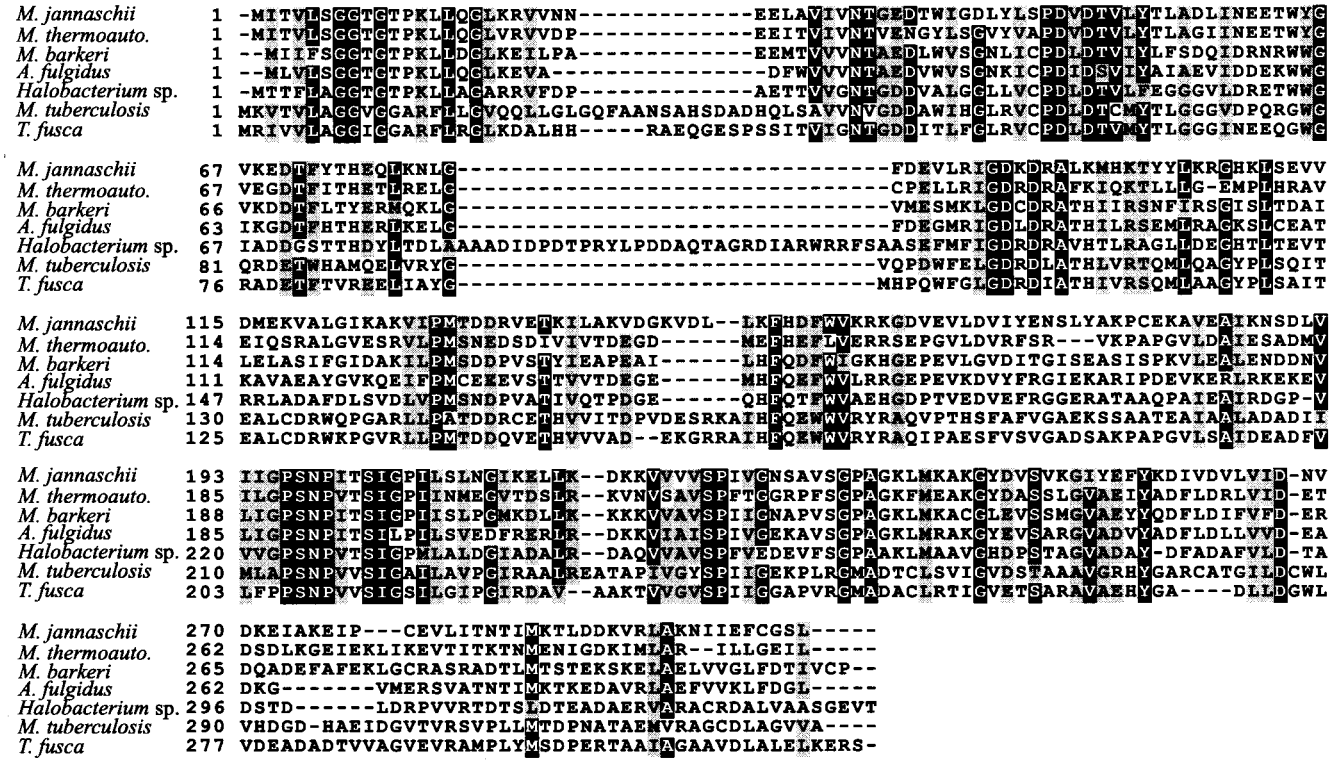

FIGURE 2: Alignment of *M. jannaschii* CofD with homologous proteins from archaea and bacteria that produce F_{420}. Protein sequences deposited in public databases have the following accession numbers: *M. jannaschii* (gb|AAB99260), *M. thermoautotrophicum* ΔH (gb|AAB85514), *Archaeoglobus fulgidus* VC-16 (gb|AAB90320), *Halobacterium* sp. NRC-1 (gb|AAG19743), and *Mycobacterium tuberculosis* H37Rv (emb|CAB07094). Sequences for *Methanosarcina barkeri* and *Thermobifida fusca* proteins were obtained from partial genome sequences (http://www.jgi.doe.gov/). The dark areas indicate invariant residues in all or all but one of the sequences. Shaded areas represent conservatively substituted residues.

HPLC and TLC using the solvent system acetonitrile−water−formic acid (88%) 40:10:5 v/v/v. Fo-P, the monophosphate ester of Fo, was obtained by acid hydrolysis of F_{420} as previously described (*25*).

HPLC Method for the Analysis of F_{420} Biosynthetic Intermediates. Analyses of Fo derivatives were performed on a Shimadzu SCL-6B HPLC using a C-18 reversed-phase column (AXXI Chrom ODS, 5 μm, 25 cm) eluted with a linear methanol gradient as previously described (*10*). The eluent was monitored by fluorescence (excitation wavelength 420 nm, emission wavelength 480 nm) and by absorbance at 280 nm. Using this HPLC method the different F_{420} derivatives showed the following retention times: F_{420}-4, 22.5 min; F_{420}-3, 23.2 min; F_{420}-2, 24.4 min; F_{420}-1, 25.8 min; F_{420}-0, 26.5 min; Fo-P, 27.8 min; Fo, 30.4.

Analysis and Separation of Reaction Intermediates on a Mono Q Column. Separations of the biochemical intermediates based on total charge were effected on a Mono Q HR 5/5 column (Pharmacia) attached to a BioLogic HR chromatographic system (Bio-Rad). The compounds were eluted using a linear sodium chloride gradient or a linear ammonium bicarbonate gradient as previously described (*10*). Using the NaCl gradient the following compounds eluted at the indicated times: lactate, 7.5 min; AMP, 10.4 min; GMP, 11.5 min; CH_3-LPPA, 11.6 min; CH_3-LPPG, 12.5 min; 2-phospholactate, 13 min; ADP, 18.8 min; GDP, 19.1 min; LPPA, 20.7 min; LPPG, 23.0 min; ATP, 24.5 min; GTP, 25.4 min; F_{420}-0, 32 min; F_{420}-1, 38 min; F_{420}-2 and higher F_{420} analogues, 42 min. Using the NH_4HCO_3 gradient the following compounds eluted at the indicated times: lactate, 8 min; AMP, 12.9 min; 2-phospholactate, 14 min; GMP, 15.0 min; ADP, 19.4 min; GDP, 21.2 min; LPPA, 22.0 min; ATP, 24.5 min; LPPG, 25.9 min; GTP, 26.8 min. Guanosine, adenosine, and F_{420}-containing materials were detected by absorbance recorded with an UV detector at 254 nm.

Cloning and Recombinant Expression of the MJ1256 Gene in E. coli. The MJ1256 gene (encoding protein gb|AAB99260.1) was amplified by PCR from *M. jannaschii* genomic DNA using oligodeoxynucleotide primers synthesized by Gibco BRL. The MJ1256-Fwd (5′-GGTGGTCATATGATATTTGTGATTAC-3′) primer introduced a *Nde*I restriction site at the 5′-end of the amplified DNA whereas MJ1256-Rev (5′-GATCGGATCCTTATAAAGACCCACAG-3′) introduced a *Bam*HI site at the 3′-end. PCR amplifications were performed as described previously using a 55 °C annealing temperature (*26*). PCR product DNA was purified using a QIAquick spin column (QIAGEN) and then digested with *Nde*I and *Bam*HI restriction enzymes (Gibco BRL). DNA fragments were ligated into compatible sites in plasmid pET17b (Novagen) using bacteriophage T4 DNA ligase (Gibco BRL). Recombinant plasmids were transformed into *E. coli* NovaBlue (Novagen) and *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene).

Transformed *E. coli* cells were grown in Luria−Bertani medium (Difco) (200 mL) supplemented with 100 mg/L ampicillin. Cultures were shaken at 37 °C until they reached an absorbance at 600 nm of 1.0. Recombinant protein production was then induced with 28 mM lactose. After an additional 4 h incubation with shaking at 37 °C the cells were harvested by centrifugation (4000*g*, 5 min) and frozen

at −20 °C. Induction of the desired protein was confirmed by SDS−PAGE analysis of total cellular protein.

Purification of CofD. Cell-free extracts were prepared by sonication of the *E. coli* cell pellets (300 mg wet weight) suspended in 3 mL of buffer (50 mM TES/Na^+, pH 7.0, 10 mM $MgCl_2$, and 20 mM mercaptoethanol) followed by centrifugation (14000*g*, 10 min). SDS−PAGE analysis of the intact cells, the pellet resulting from the above sonication, and the cell-free extract showed that the CofD protein represented >80% of the total cellular protein. Most of the overexpressed protein was in the insoluble pellet. A clear band for CofD, representing about 10% of the total soluble protein, was observed in the cell-free extract. The crude cell-free extracts were then heated for 30 min at 80 °C followed by centrifugation (14000*g*, 10 min) to remove the precipitated *E. coli* proteins. This heating resulted in only a slight drop in the total enzymatic activity. Portions of the supernatant diluted with 25 mM Tris-HCl, pH 7.0, buffer were loaded onto a Uno Q column (1.3 mL bed volume, Bio-Rad) and eluted with a linear gradient of 0−1 M NaCl in 20 mM Tris-HCl (pH 7.5). Elution of CofD was detected by activity, UV absorbance at 280 nm, and SDS−PAGE analysis. Active fractions showing a single band, as established by SDS−PAGE analysis, were pooled and used for the work reported here. The protein concentrations were determined using the Bio-Rad Protein Assay using BSA as standard. Solutions of CofD could be stored at 4 °C for 2 weeks without significant loss of activity.

Measurement of the Native Molecular Weight of CofD. A Superose 12 HR 10/30 column (Pharmacia), eluted with a buffer containing 50 mM TES/Na^+ and 150 mM NaCl (pH 7.0), was used to determine the molecular weight of CofD. Apoferritin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* were used as molecular weight markers. Purified CofD (150 μL, 0.8 mg/mL of protein) was applied to the column and eluted at a flow rate of 0.5 mL/min.

Measurement of the Enzymatic Activities of CofD. In a typical assay, CofD (1 μg) was preincubated with Fo in 25 μL of 50 mM TES/Na^+ buffer, pH 7.0, for 5 min at 37 °C. Because of the hydrolytic instability of the LPPG substrate it had to be prepared fresh at the start of each incubation (*10*). The reaction was initiated by adding LPPG, adjusting the volume to 50 μL, and incubating for 5 min at 37 °C unless otherwise indicated. The calculated final concentrations in the incubation mixture were 100 μM LPPG and 8 μM Fo. The reaction was stopped by addition of EDTA to a final concentration of 10 mM, and any precipitate present was removed by centrifugation (1 min, 14000*g*). The resulting solution could be quickly analyzed for the presence of products by TLC analysis [acetonitrile−water−formic acid (88%) 80/20/10 v/v/v] of a portion of the reaction mixture. Both Fo and F_{420}-0 were easily detected as blue-green fluorescent spots by exposing the TLC plates to UV light. With this solvent Fo had an R_f of 0.46 and F_{420}-0 an R_f of 0.33. Quantitative analysis of products was conducted by HPLC with a reverse-phased column. One unit of enzyme activity is equivalent to 1 μmol of F_{420}-0 produced per minute.

Incubations to study the other reactions catalyzed by CofD, including the hydrolysis of F_{420}-0, were conducted with 25 μg of enzyme and Fo (8 μM) or F_{420}-0 (8 μM) contained in 50 μL of the same volume of buffer but were incubated for 30 min at 50 °C. Substrates tested for their ability to phosphorylate Fo included GTP, GDP, cyclic trimetaphosphate, tripolyphosphate, and pyrophosphate and were each tested at a concentration of 10 mM.

Potential effectors added to the above-described incubation mixtures included 5 mM EDTA, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM $ZnCl_2$, 10 mM β,γ-methyleneguanosine 5′-triphosphate, and/or 0.1 M hydroxylamine.

For the determination of the kinetic parameters the apparent K_m for Fo was measured in the presence of 100 μM LPPG, and the apparent K_ms for LPPG and LPPA were determined in the presence of 34 μM Fo. Incubations with the enzyme and substrates were conducted at 30 °C to reduce errors resulting from the chemical hydrolysis of the LPPG and LPPA. LPPG had a measured half-life of 2 h at room temperature and 10 min at 50 °C.

Temperature Stability of CofD. The temperature stability of the CofD enzyme was established by incubating the enzyme at different temperatures for fixed periods of time and then measuring the remaining activity at 37 °C. The use of the 37 °C assay temperature was necessitated because of the increased stability of the LPPG and LPPA substrates at the lower temperatures. The samples heated above 100 °C were sealed in small glass tubes during the heating.

^{32}P-Labeling and Phosphoamino Acid Analysis. CofD (660 μg) was incubated with [γ-^{32}P]guanosine 5′-triphosphate (5.8 μCi), 23 μM GTP, and 2.3 μM $MgCl_2$ contained in 100 μL of 25 mM TES buffer, pH 7.0, for 30 min at 37 °C. The reaction was terminated by addition of SDS loading buffer and heating for 5 min at 95 °C. A SDS−PAGE separation was preformed on the sample and the resulting gel analyzed for ^{32}P using an electronic autoradiography system (Instant Imager from Packard). The radioactive protein band corresponding to the CofD protein was transferred to a PDVF membrane, cut into three equal pieces, and washed with H_2O, 1 M KOH, and 0.1 M HCl as previously described (*27*), and the remaining radioactivity was obtained by autoradiography. In a second experiment the SDS gel protein band was subjected to both tryptic digest and acid hydrolysis in an attempt to isolate ^{32}P-containing fragments. The presence of possible phosphoamino acids (phosphoserine, phosphothreonine, or phosphotyrosine) was determined by two-dimensional thin-layer chromatography (*27*).

[^{3}H]GMP Exchange Reaction. [8-^{3}H]GMP was prepared from [8-^{3}H]GTP by hydrolysis with nucleotide pyrophosphatase. The resulting [8-^{3}H]GMP was purified by preparative TLC (cellulose plates) using the solvent system composed of 1-butanol−acetone−acetic acid−ammonia (5%)−water (45:15:10:10:20 v/v/v/v/v). After elution of the [8-^{3}H]GMP from the plate with water, a radiochemical purity of >97% was confirmed by radiochromatography on the Mono Q HR 5/5 column (Pharmacia). The resulting [8-^{3}H]-GMP (80 μM) contained in 50 μL of 50 mM TES/Na^+, pH 7.0, buffer was incubated with CofD (2.5 μg) and LPPG (80 μM). After incubation for 1 h at 37 °C the proteins were removed with a centricon concentrator 10 (Amicon). The resulting filtrate was then chromatographed on a Mono Q column to separate the GMP from the LPPG. Fractions (0.5 mL) were collected, 1 mL of ScintiSafe (Fisher) was added, and the solutions were counted for radioactivity.

Table 1: Purification of CofD from an Extract of *E. coli* Containing the Overexpressed Enzyme

	total protein (mg)	total units of activity (μmol/min)	specific activity (unit/mg)	purification fold	yield (%)
crude extract	69	1.3	0.019	1	100
heat treatment	8.4	0.87	0.10	5.3	65
Uno Q column	2.0	0.72	0.36	19	54

Table 2: Kinetic Parameters for CofD

substrate	K_M^{app} (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_M^{app} (M^{-1} s^{-1}) $\times$ 10^{-3}
Fo[a]	32 ± 5		
LPPG[b]	17 ± 2	1.4 ± 0.4	47.0
LPPA[b]	515 ± 50	0.1 ± 0.03	0.10

[a] LPPG concentration was 100 μM in the incubation. [b] Fo concentration was 34 μM in the incubation.

Table 3: Formation of GMP and F_{420}-0 from LPPG by CofD in the Presence of Fo

	products detected (μM)	
incubation mixture[a]	F_{420}-0	GMP
LPPG (no incubation)	nd[c]	64 ± 5[b]
LPPG (15 min incubation)	nd	80 ± 6
CofD + LPPG	nd	86 ± 7
CofD + LPPG + Fo + EDTA	nd	88 ± 6
CofD + LPPG + Fo	50 ± 5	104 ± 9

[a] LPPG (50 μM, which already contained 62 μM GMP as determined from the nonhydrolyzed LPPG methyl ester) was incubated as indicated with CofD (2.5 μg), 50 μM Fo, and 5 mM EDTA in 50 μL of 50 mM TES/Na^+, pH 7.0, for 15 min at 37 °C. Incubation mixtures not containing EDTA were stopped by addition of EDTA (5 mM) and immediately applied to the Mono Q column for analysis. [b] This GMP represents the GMP produced by the chemical hydrolysis of the LPPG. [c] nd = not detected.

RESULTS AND DISCUSSION

Purification of CofD. The purification of the recombinantly generated CofD is summarized in Table 1. Since *cofD* was derived from the hyperthermophile *M. jannaschii*, we took advantage of the expected thermal stability of the CofD enzyme to facilitate its separation from the *E. coli* host proteins by simply heating the extracts which denatured the *E. coli* proteins. Thus the first step used in the purification was a heat treatment (15 min at 80 °C) of the *E. coli* cell extract, followed by centrifugation to remove the bulk of the *E. coli* proteins. This mild heating was unlikely to cause deamidation reactions which have been found to proceed slowly or not at all in conformationally intact enzymes (*28*). The resulting heated extract was of sufficient purity that a single chromatographic step on an Uno Q column resulted in electrophoretically (SDS−PAGE) pure protein. The CofD protein showed one band on SDS−PAGE, which stained with an apparent molecular weight of 34400. This is in agreement with the predicted molecular weight value of 34600 from the gene sequence. The molecular weight measured from the gel filtration column of 66700 indicated that the native CofD enzyme consisted of a dimer.

Characterization of the Reactions Catalyzed by CofD. CofD readily catalyzed the condensation of LPPG or LPPA with Fo to form F_{420}-0, an established intermediate in coenzyme F_{420} biosynthesis. The other product of the reaction was the respective nucleotide monophosphate. The determination of the kinetic parameters for this enzymatic reaction is very difficult due to the simultaneous chemical breakdown of the LPPG and LPPA substrates. LPPG had a measured half-life of 2 h at room temperature and 10 min at 50 °C under the assay conditions. By running the reactions at 30 °C, we were able to measure apparent K_ms for Fo, LPPG, and LPPA (Table 2). The data clearly indicate that LPPG is the preferred substrate for the enzyme.

The exact quantitation of the moles of GMP produced by the enzyme was complicated because of the chemical degradation of the LPPG to GMP and 2-phospholactate during the incubation (Table 3). Even during the short incubation of LPPG at 37 °C followed by its analysis on the Mono Q column, a significant amount of the LPPG would hydrolyze to GMP. Addition of the CofD enzyme and Fo in the presence of the inhibitor EDTA did not significantly increase this rate of decomposition. But the addition of Fo resulted in complete conversion of Fo to F_{420}-0. From comparison of the conversion of Fo to F_{420}-0 and the increase in GMP, it is obvious that most of the LPPG is used for the catalytic process in the presence of Fo.

The CofD-catalyzed reaction also proceeded with LPPA instead of LPPG but to a much lesser extent (Table 2). CH_3LPPG did not function as a substrate for CofD, indicating that the free carboxyl group of the lactic acid may be involved in substrate binding.

If CofD is present in an incubation mixture in higher amounts, the formation of Fo-P from Fo using a series of molecules containing condensed polyphosphates such as GTP, GDP, PPP, and PP as substrates can be observed. Under the incubation conditions used, 10−15% of the Fo was converted into Fo-P with these substrates. CofD also catalyzed the hydrolysis of F_{420}-0 to Fo. Under the conditions described in the Materials and Methods section, 32% of F_{420}-0 was hydrolyzed to Fo in 30 min at 50 °C in the presence of 25 μg of the CofD enzyme. No Fo-P was observed as a product of this hydrolysis. The replacement of Fo with riboflavin in the CofD reaction did not lead to the formation of the riboflavin analogue of F_{420}-0, indicating that Fo substrate binding site of CofD is very specific for Fo.

From the kinetic parameters established for CofD, its substrate specificity, and distribution, it is clear that the enzyme catalyzes the fourth reaction in the biosynthesis of F_{420}. In this reaction, Fo reacts with LPPG with the formation of F_{420}-0 and GMP. The conversion of LPPG to F_{420}-0 was not inhibited by the product, GMP (240 μM), or by 10 mM GTP or GDP (data not shown).

Considering the instability of LPPG, it was considered that CofD might also catalyze the formation of LGGP from L-P and GTP where it would then be used directly for F_{420}-0. If this were occurring, it should be possible to observe the formation of F_{420}-0 from 2-phospho-L-lactate, GTP, and Fo incubated with CofD. This experiment was conducted, and no formation of F_{420}-0 was observed, indicating that another enzyme was responsible for the formation of LPPG. The enzyme catalyzing this reaction has now been identified as the product of the MJ1117 gene (unpublished results).

Metal Ion Dependency of the CofD Reaction. Nucleotide-dependent reactions are very often dependent on divalent metal ions. The isolated CofD enzyme was not activated by

Table 4: Effect of Divalent Metal Ions and EDTA on the Activity of the CofD Enzyme

	specific activity (μmol min^{-1} mg^{-1})	
experiment[a]		EDTA + excess M^{2+}
no additions	0.30	
EDTA	0.009	
Mg^{2+}	0.30	0.36
Mn^{2+}	0.009	0.026
Zn^{2+}	0.009	0.009

[a] The incubations were conducted as described in the text. No additional Me^{2+} ions or EDTA were added to the first incubation mixture. The concentration of Mg^{2+}, Mn^{2+}, and Zn^{2+} was 10 mM and that of EDTA was 5 mM. EDTA was added to the preincubation mixture, and the metal ions were added together with LPPG.

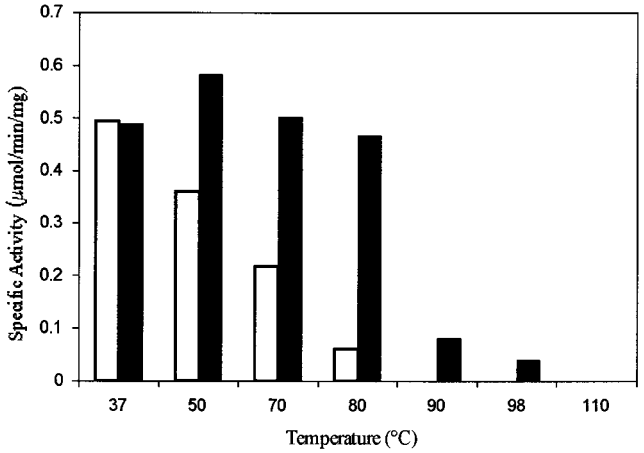

FIGURE 3: Heat stability of the CofD enzyme and activity assays at elevated temperatures. Solid bars: CofD activities measured at 37 °C after CofD was exposed to the indicated temperature for 30 min. Open bars: CofD activity measured at the indicated temperatures. (For details, see Materials and Methods.)

the addition of 0−4 mM $MgCl_2$. However, the activity was completely eliminated by including EDTA in the incubation mixtures. This inactivation by EDTA could be completely reversed by the addition of excess Mg^{2+} but not by the addition of Zn^{2+} or Mn^{2+} (Table 4).

Thermostability of CofD. Since the LPPG substrate is labile, one must distinguish between the thermostability of the CofD enzyme and the LPPG substrate. The apparent CofD activity is highest when measured at 37 °C (Figure 3). At 50 °C and higher temperatures the activity drops significantly. This temperature profile reflects only the increased instability of the LPPG substrate at elevated temperatures, because CofD can be heated for 24 h at 80 °C without significant loss of activity as expected for an enzyme from an hyperthermophilic organism. Heating at 98 °C for 30 min, however, begins to decrease its activity, and heating at 110 °C for 30 min does not leave any residual activity.

Search for Catalytically Important Residues. As can be seen in Figure 2, CofD-related proteins have several conserved amino acid residues that could be involved in the formation of a covalent intermediate. These include six conserved aspartic acids and three conserved serines. To test the possible involvement of an enzyme-bound phospholactate acyl-phosphate intermediate involving one of these aspartic acids, we conducted the enzymatic reaction in the presence of 0.1 M hydroxylamine and observed no effect on the enzymatic reaction. Had an acyl-phosphate intermediate been involved in the reaction, it should have reacted with the hydroxylamine, resulting in inactivation of the enzyme by the formation of the hydroxamic acid. One site-directed mutant (S121A) was generated and shown to have the same specific activity as the wild type (data not shown).

Search for Covalent Intermediates. Considering that CofD was able to form Fo-P when incubated with Fo and GTP, it was considered that a covalent phosphoryl intermediate arising from the γ-phosphate of the GTP could be involved in the reaction. Thus a sample of CofD was incubated with [γ-^{32}P]GTP, and the sample was separated by SDS−PAGE electrophoresis and analyzed for the presence of covalently bound phosphate. A trace amount of label (<0.003%) was found associated with the CofD protein band that could not be washed out with water. Washing with 0.1 M HCl and 1 M KOH reduced this remaining radioactivity by 42% and 54%, respectively. Since phosphoserine in proteins is known to be resistant to acid but labile in base (*29*), these data are not consistent with the presence of a phosphoserine. A control experiment where BSA was incubated with [γ-^{32}P]GTP showed an identical pattern of labeling, indicating that the observed CofD labeling was nonspecific. Tryptic digest and acid hydrolysis of the protein failed to produce any phosphoserine.

Finally, the incubation of the enzyme with [^{3}H]GMP and LPPG failed to produce [^{3}H]LPPG, indicating that no back-reaction was occurring. In total, these experiments are consistent with their being no covalent intermediate in the reaction catalyzed by CofD.

Similarities of CofD to Other Proteins. As can be seen in Figure 2, proteins with sequences homologous to CofD are found in the genomes of all organisms currently known to produce coenzyme F_{420}. These include not only the methanogenic archaea like *M. jannaschii* and *M. thermoautotrophicum* but also nonmethanogenic archaea such as *Halobacteria* and *Archaeoglobus*, as well as some eubacteria, such as *Streptomyces*, *Mycobacterium*, *Nocardia*, and *Thermobifida*. CofD-related proteins also have homologues in what are currently considered to be non-F_{420}-containing microorganisms. One conserved motif present in the C-terminus of the proteins from the F_{420} producers is PSNPXXSI. This motif is absent among the proteins of the expected non-F_{420} producers. The lack of this motif may indicate that the enzymes of the nonproducers catalyze a reaction with a similar mechanism, which is not involved in F_{420} biosynthetic reaction.

The only recognizable motif common to all of these proteins is the short N-terminal sequence ^{9}GTGTPK^{14}, which is in some ways similar to the GXG(RS)$(X)_4$PK motif found in the recently established pyrophosphorylase superfamily (*30*−*32*). This suggests that the LPPG might be bound to the N-terminal end of the protein.

Proposed Reaction Mechanism for CofD. On the basis of analogy with other nucleotidyl and phosphoryl transferase reactions, one can envision two general mechanisms on how the CofD reaction could proceed. The first would involve the direct displacement of the GMP from LPPG by the hydroxyl group of the Fo. The second would involve a double displacement mechanism with a protein-bound 2-phospho-L-lactate serving as an intermediate. In chemical terms the direct displacement mechanism would be analogous to that catalyzed by the DNA polymerase superfamily of enzymes

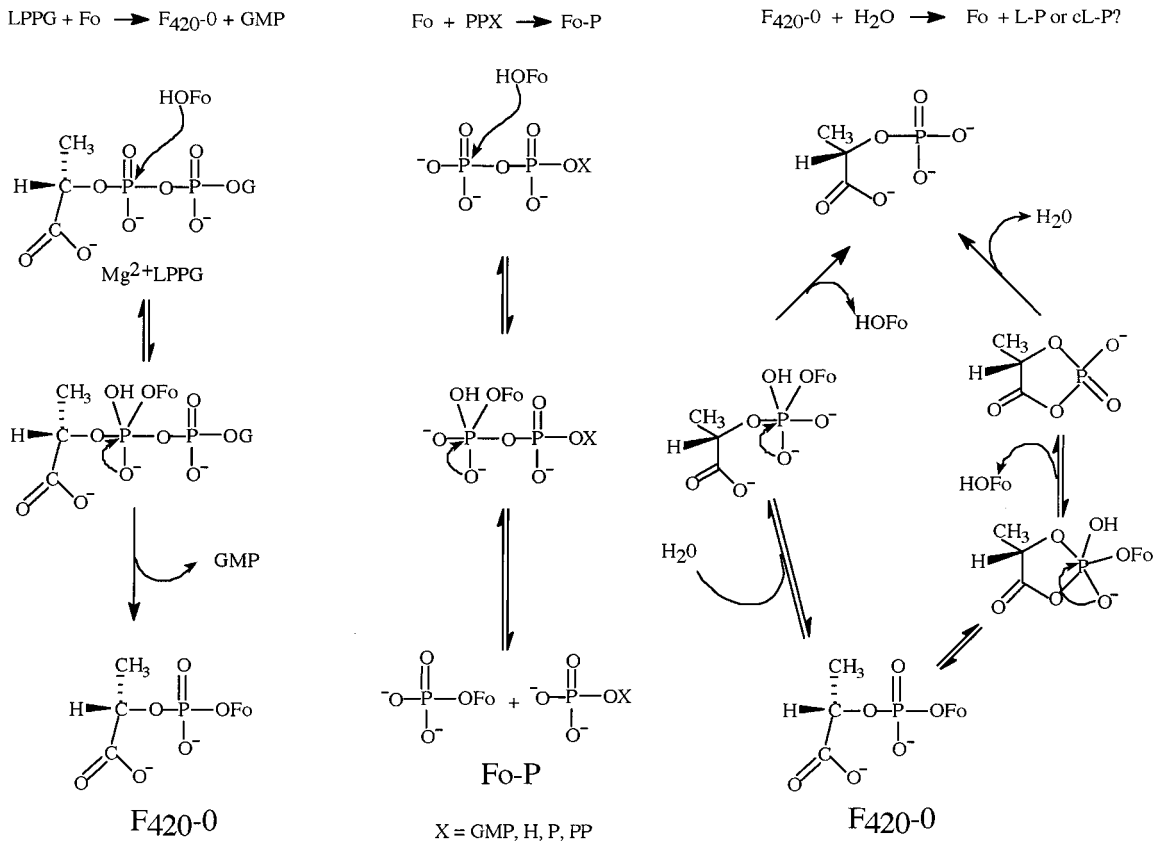

FIGURE 4: Reactions catalyzed by CofD.

(*33*). In these reactions a nucleophilic hydroxyl group attacks the α-phosphate of a NTP with direct displacement of pyrophosphate and the formation of a phosphodiester. In many, and perhaps all, of these enzymes, the reactions are mediated by the so-called two metal ion mechanism (*34*, *35*). In this mechanism one metal ion coordinates and facilitates the formation of the attaching anion, and the second metal coordinates the phosphate of the leaving monophosphate ester.

Whether this two metal ion mechanism is a required feature of all nucleotidyl transferases is not clear since many of the enzymes have been crystallized in the absence of metals (*36*) and thus it is not possible to establish that two metals are always involved. It is well established that divalent metal ion(s) are essential for all of the enzymes which proceed via direct displacement mechanisms. The enzymes only provide a scaffold to hold the metals in the correct orientation to be able to stabilize the pentavalent transition state at the α-phosphate. No enzymatic group is covalently attached to a phosphate during the course of the reaction. In the currently known examples of this type of reaction, pyrophosphate is the leaving group, whereas in our example, GMP would be the leaving group. Thus the reaction is most analogous to the phospholipid synthases, where the non-nucleotide portion of the pyrophosphate diester is transferred to the substrate. Phospholipid synthases are integral membrane proteins; thus little is known about their biochemical properties and reaction mechanisms, but divalent metal ions are still essential for their catalytic activity. Accordingly, only limited data are available for another mechanistically similar enzyme, cobalamin synthase (CobS), which catalyzes the condensation of adenosylcobalamin guanosine diphosphate

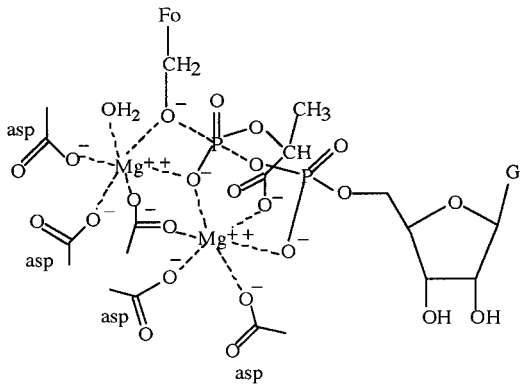

FIGURE 5: Possible structure of a two metal ion mechanism for the reaction between LPPG and Fo.

(AdoCbi-GDP) with α-ribazole to form adenosylcobalamin (AdoCbl) (*12*). The extent of involvement of metals in this reaction is at present unknown. Although we have not established the exact nature and number of the metal ions involved in the CofD enzyme, we have established that Mg^{2+} is required and that Mg^{2+} is not replaceable by Zn^{2+} or Mn^{2+}. The possible binding of the lactate carboxylate of the LPPG substrate with the Mg^{2+} may result in a different type of substrate binding to the Mg^{2+} than is found in these other Mg^{2+}-requiring enzymes. Considering the two conserved DXD motifs in CofD (Figure 2) which are highly conserved in the CofD among the F_{420}-producing microorganisms and their possible involvement as likely binding sites for two Mg^{2+} ions, we propose a possible transition state for CofD as shown in Figure 5. The same DXD motif is found in DNA polymerases III (*37*), while it is missing in the non-F_{420}-producing CofD homologues.

The other possible mechanism for CofD would involve an enzyme-bound covalent L-P intermediate in the reaction. On the basis of analogy with other phosphoryl enzymes this covalent intermediate could involve histidine (*38*, *39*), lysine (*13*, *40*, *41*), aspartic acid (*42*), or serine (*43*) residues of the protein. On the basis of the identity of the conserved amino acids in the CofD-related proteins, one of the three serines or, less likely, one of the six conserved aspartic acids could function as the covalent acceptor in the enzyme. Attempts to label the enzyme with γ-^{32}P-labeled GTP gave no evidence for the presence of a covalent intermediate. Although the experiments indicated that some small labeling of the enzyme had occurred, the same extent of labeling was observed in control experiments with BSA. In neither case did acid hydrolysis produce any phosphoserine or phosphothreonine. Furthermore, site-directed mutagenesis of serine S212A in the enzyme had no effect on the enzymatic activity (unpublished results).

Our concern about the involvement of a covalent intermediate in the reaction was generated to help to explain the side reactions that were found to be catalyzed by CofD (Figure 4). The formation of Fo-P as a result of the incubation of the enzyme with Fo and several different phosphate donors, such as pyrophosphate, GTP, GDP, and triphosphate, could be explained by the formation of a phosphorylated enzyme intermediate which then transfers the phosphate group to Fo. The specific protein residue that would be phosphorylated would be the same as that to which the L-P would have been bound. Since evidence for such a covalent intermediate cannot be found, we must assume that the terminal phosphate of each of these compounds binds at the same site as the 2-phospho-L-lactate phosphate in the LPPG substrate. This phosphate would then be bound in the correct position for transfer to Fo to produce Fo-P. As with LPPG, each of these substrates can bring a single Mg^{2+} to the active site upon binding to the enzyme which contains the other Mg^{2+} ion. The hydrolysis of F_{420}-0 can be explained by the substitution of water for phosphate in the reaction.

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REFERENCES

1. Daniels, L. (1993) in *The Biochemistry of the Archaea (Archaeabacteria)* (Kates, M., Kushner, D. J., and Matheson, A. T., Eds.) pp 41−112, Elsevier, Amsterdam.
2. DiMarco, A. A., Bobik, T. A., and Wolfe, R. S. (1990) *Annu. Rev. Biochem. 59*, 355−394.
3. McCormick, J. R. D., and Morton, G. O. (1982) *J. Am. Chem. Soc. 104*, 4014−4015.
4. Peschke, U., Schmidt, H., Zhang, H. Z., and Piepersberg, W. (1995) *Mol. Microbiol. 16*, 1137−1156.
5. Ebert, S., Rieger, P. G., and Knackmuss, H. J. (1999) *J. Bacteriol. 181*, 2669−2674.
6. Stover, C. K., Warrener, P., VanDevanter, D. R., Sherman, D. R., Arain, T. M., Langhorne, M. H., Anderson, S. W., Towell, J. A., Yuan, Y., McMurray, D. N., Kreiswirth, B. N., Barry, C. E., and Baker, W. R. (2000) *Nature 405*, 962−966.
7. Purwantini, E., Gillis, T. P., and Daniels, L. (1997) *FEMS Microbiol. Lett. 146*, 129−134.
8. Purwantini, E., and Daniels, L. (1996) *J. Bacteriol. 178*, 2861−2866.
9. Eker, A. P., Kooiman, P., Hessels, J. K., and Yasui, A. (1990) *J. Biol. Chem. 265*, 8009−8015.
10. Graupner, M., and White, R. H. (2001) *Biochemistry 40*, 10859−10872.
11. O'Toole, G. A., Rondon, M. R., and Escalante-Semerena, J. C. (1993) *J. Bacteriol. 175*, 3317−3326.
12. Maggio-Hall, L. A., and Escalante-Semerena, J. C. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 11798−11803.
13. Doherty, A. J., Ashford, S. R., Subramanya, H. S., and Wigley, D. B. (1996) *J. Biol. Chem. 271*, 11083−11089.
14. McMaster, C. R., and Bell, R. M. (1997) *Biochim. Biophys. Acta 1348*, 117−123.
15. McMaster, C. R., and Bell, R. M. (1997) *Biochim. Biophys. Acta 1348*, 100−110.
16. Yamashita, S., and Nikawa, J. (1997) *Biochim. Biophys. Acta 1348*, 228−235.
17. Nikawa, J., and Yamashita, S. (1997) *Biochim. Biophys. Acta 1348*, 173−178.
18. Minskoff, S. A., and Greenberg, M. L. (1997) *Biochim. Biophys. Acta 1348*, 187−191.
19. Matsumoto, K. (1997) *Biochim. Biophys. Acta 1348*, 214−227.
20. Koonin, E. V. (1996) *Trends Biochem. Sci. 21*, 242−243.
21. Morii, H., Nishihara, M., and Koga, Y. (2000) *J. Biol. Chem. 275*, 36568−36574.
22. Chio, K. P., Bair, T. B., Bae, Y. M., and Daniels, L. (2001) *J. Bacteriol.* (in press).
23. Ashton, W. T., and Brown, R. D. (1980) *J. Heterocycl. Chem. 17*, 1709−1712.
24. White, R. H. (1990) *Biochemistry 29*, 5397−5404.
25. Eirich, L. D., Vogels, G. D., and Wolfe, R. S. (1978) *Biochemistry 17*, 4583−4593.
26. Graupner, M., Xu, H., and White, R. H. (2000) *J. Bacteriol. 182*, 3688−3692.
27. Duclos, B., Marcandier, S., and Cozzone, A. J. (1991) *Methods Enzymol. 201*, 10−21.
28. Daniel, M., Dines, M., and Petach, H. (1996) *Biochem. J. 317*, 1−11.
29. Buss, J. E., and Stull, J. T. (1983) *Methods Enzymol. 99*, 7−14.
30. Vaara, M. (1992) *FEMS Microbiol. Lett. 76*, 249−254.
31. Brown, K., Pompeo, F., Dixon, S., Mengin-Lecreulx, D., Cambillau, C., and Bourne, Y. (1999) *EMBO J. 18*, 4096−4107.
32. Olsen, L. R., and Roderick, S. L. (2001) *Biochemistry 40*, 1913−1921.
33. Aravind, L., and Koonin, E. V. (1999) *Nucleic Acids Res. 27*, 1609−1618.
34. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) *Biochemistry 36*, 11205−11215.
35. Steitz, T. A. (1999) *J. Biol. Chem. 274*, 17395−17398.
36. Weber, C. H., Park, Y. S., Sanker, S., Kent, C., and Ludwig, M. L. (1999) *Struct. Folding Des. 7*, 1113−1124.
37. Pritchard, A. E., and McHenry, C. S. (1998) *J. Mol. Biol. 285*, 1067−1080.
38. Thomas, M. G., and Escalante-Semerena, J. C. (2000) *J. Bacteriol. 182*, 4227−4233.
39. O'Toole, G. A., and Escalante-Semerena, J. C. (1995) *J. Biol. Chem. 270*, 23560−23569.
40. Atencia, E. A., Montes, M., Gunther Sillero, M. A., and Sillero, A. (2000) *Eur. J. Biochem. 267*, 1707−1714.
41. Tomkinson, A. E., Totty, N. F., Ginsburg, M., and Lindahl, T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 400−404.
42. Degani, C., and Boyer, P. D. (1973) *J. Biol. Chem. 248*, 8222−8226.
43. Schwartz, J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 871−878.

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