Biosynthesis of the Phosphodiester Bond in Coenzyme F_{420} in the Methanoarchaea†

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ABSTRACT: The biochemical route for the formation of the phosphodiester bond in coenzyme F_{420}, one of the methanogenic coenzymes, has been established in the methanoarchaea *Methanosarcina thermophila* and *Methanococcus jannaschii*. The first step in the formation of this portion of the F_{420} structure is the GTP-dependent phosphorylation of L-lactate to 2-phospho-L-lactate and GDP. The 2-phospho-L-lactate represents a new natural product that was chemically identified in *Methanobacterium thermoautotrophicum, M. thermophila*, and *Mc. jannaschii*. Incubation of cell extracts of both *M. thermophila* and *Mc. jannaschii* with [hydroxy-^{18}O, carboxyl-$^{18}O_2$]lactate and GTP produced 2-phospho-L-lactate with the same ^{18}O distribution as found in both the starting lactate and the lactate recovered from the incubation. These results indicate that the carboxyl oxygens are not involved in the phosphorylation reaction. Incubation of Sephadex G-25 purified cell extracts of *M. thermophila* or *Mc. jannaschii* with 7,8-didemethyl-8-hydroxy-5-deazariboflavin (Fo), 2-phospho-L-lactate, and GTP or ATP lead to the formation of F_{420}-0 (F_{420} with no glutamic acids). This transformation was shown to involve two steps: (i) the GTP- or ATP-dependent activation of 2-phospho-L-lactate to either lactyl(2)diphospho-(5′)guanosine (LPPG) or lactyl(2)diphospho-(5′)adenosine (LPPA) and (ii) the reaction of the resulting LPPG or LPPA with Fo to form F_{420}-0 with release of GMP or AMP. Attempts to identify LPPG or LPPA intermediates by incubation of cell extracts with L-[U-^{14}C]lactate, [U-^{14}C]2-phospho-L-lactate, or [8-^{3}H]GTP were not successful owing to the instability of these compounds toward hydrolysis. Synthetically prepared LPPG and LPPA had half-lives of 10 min at 50 °C (at pH 7.0) and decomposed into GMP or AMP and 2-phospho-L-lactate via cyclic 2-phospho-L-lactate. No evidence for the functioning of the cyclic 2-phospho-L-lactate in the in vitro biosynthesis could be demonstrated. Incubation of cell extracts of *M. thermophila* or *Mc. jannaschii* with either LPPG or LPPA and Fo generated F_{420}-0. In summary, this study demonstrates that the formation of the phosphodiester bond in coenzyme F_{420} follows a reaction scheme like that found in one of the steps of the DNA ligase reaction and in the biosynthesis of coenzyme B_{12} and phospholipids.

The idea of a connection between structures of nucleic acids and some of the coenzymes has long been recognized (*1*). This idea is based in part on the occurrence of β-ribosides in nucleic acids as well as in coenzymes such as NAD, FAD, ATP, etc. An equally important structural element of nucleic acids is the presence of phosphodiester bonds, which are known to be present in only three coenzymes: coenzyme F_{420}[1] (*2*), coenzyme B_{12} (*3*), and tetrahydromethanopterin (H_4MPT) (*4*) (Figure 1). The phosphodiester bonds in nucleic acids are formed by two different pathways. In the first route, which involves a step catalyzed by DNA and RNA polymerases, a 3′ secondary hydroxyl group of a ribose attacks the α-phosphate of a 5′ primary nucleotide triphosphate with subsequent displacement of PPi and formation of a phosphodiester bond (Figure 2, route I). In the second pathway, which involves a step like that catalyzed by DNA ligase, a 5′ primary monophosphate reacts with ATP or NAD to form an adenylylated adduct containing a pyrophosphate diester. The pyrophosphate diester subsequently reacts with the 3′ secondary hydroxyl group of ribose with displacement of AMP and formation of the phosphodiester bond (*5*, *6*) (Figure 2, route II).

The formation of the phosphodiester bond in tetrahydromethanopterin (H_4MPT) follows route I (*7*). Route II is used for the formation of the phosphodiester bond in coenzyme B_{12}, where adenosylcobinamide-P (AdoCbi-P) reacts with GTP to form adenosylcobinamide-GDP, which then reacts by displacement of GMP with the formation of adenosylcobinamin (*8*, *9*). A route mechanistically analogous to route II, but using CTP, is involved in the biosynthesis of the phosphodiester bond in phospholipids (*10*). In this paper, we show that the biosynthesis of the phosphodiester bond in coenzyme F_{420} is analogous to the latter process and

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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, guanosine(5′)-diphospho-L-lactate; LPPA, adenosine(5′)-diphospho-L-lactate; 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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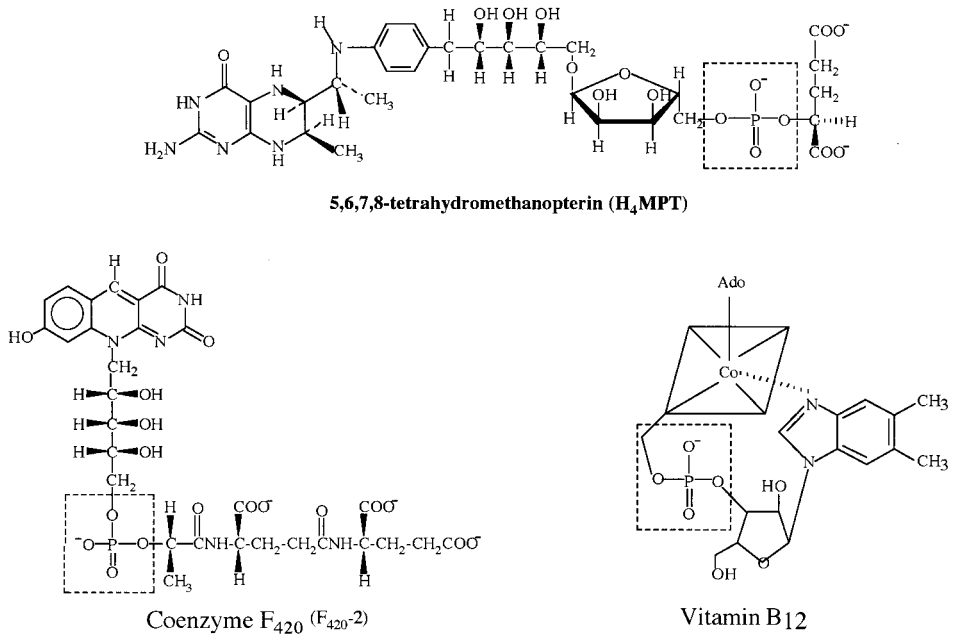

FIGURE 1: Chemical structures of coenzyme F_{420}, vitamin B_{12}, and tetrahydromethanopterin. The phosphodiester bonds present in each structure are indicated by the dotted frames.

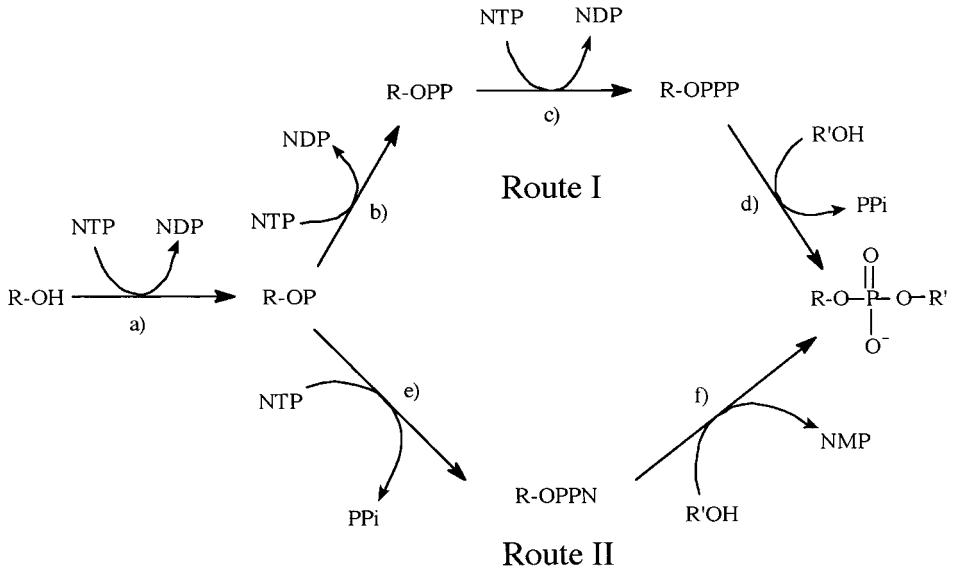

FIGURE 2: Possible biochemical pathways for the formation of phosphodiester bonds in coenzyme F_{420}. Route I resembles a reaction catalyzed by DNA and RNA polymerases using a nucleotide triphosphate for phosphodiester formation. The complete reaction sequence would require four enzymes catalyzing reactions a, b, c, and d. Enzymes involved in catalyzing these reactions would use mechanisms analogous to those found in the following enzymes: (a) kinases, (b) nucleoside monophosphate kinases, (c) nucleoside diphosphate kinases, (d) RNA polymerases. Route II follows reactions catalyzed by the DNA ligases and could use enzymes with mechanisms such as the following enzymes: (a) kinases, (e) nucleotidyl transferases (could also use NAD^+ for adenylylation), and (f) ligases.

involves the formation and utilization of lactyl(2)diphospho-(5′)guanosine (LPPG) or lactyl(2)diphospho-(5′)adenosine (LPPA) for the formation of F_{420}-0 (Figure 3).

MATERIALS AND METHODS

Chemicals. GTP, GDP, GMP, ATP, $POCl_3$, benzyl-L-lactate, diphenyl chlorophosphate, phenyl dichlorophosphate, guanosine 5-monophosphomorpholidate, adenosine 5-monophosphomorpholidate, D-ribose, 3-aminophenol, 6-chlorouracil, L-lactate, *Escherichia coli* type III alkaline phosphatase, and nucleotide pyrophosphatase type II from *Crotalus adamanteus* venom were obtained from Sigma-Aldrich Chemical Co. Dowex AG 50W-8X cation-exchange resin (100−200 mesh) was purchased from Bio Rad. [U-^{14}C]L-Lactate (150 mCi/mmol) and [8-^{3}H]guanosine 5′ triphosphate tetrasodium salt (10 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO. TLC plates (precoated silica gel 60 F-254) were obtained from E. Merck, Germany. Fo was prepared from D-ribose, 3-aminophenol, and 6-chlorouracil as previously described (*11*). All other reagents were obtained from standard sources.

Bacterial Strains, Growth Conditions, and Preparation of Cell Extracts. *Methanosarcina thermophila* strain TM-1 was grown in the laboratory of Dr. James G. Ferry as previously described (*12*). Cell extracts of *M. thermophila* were prepared by French Press lysis (*13*) in buffer containing 25 mM Tris,

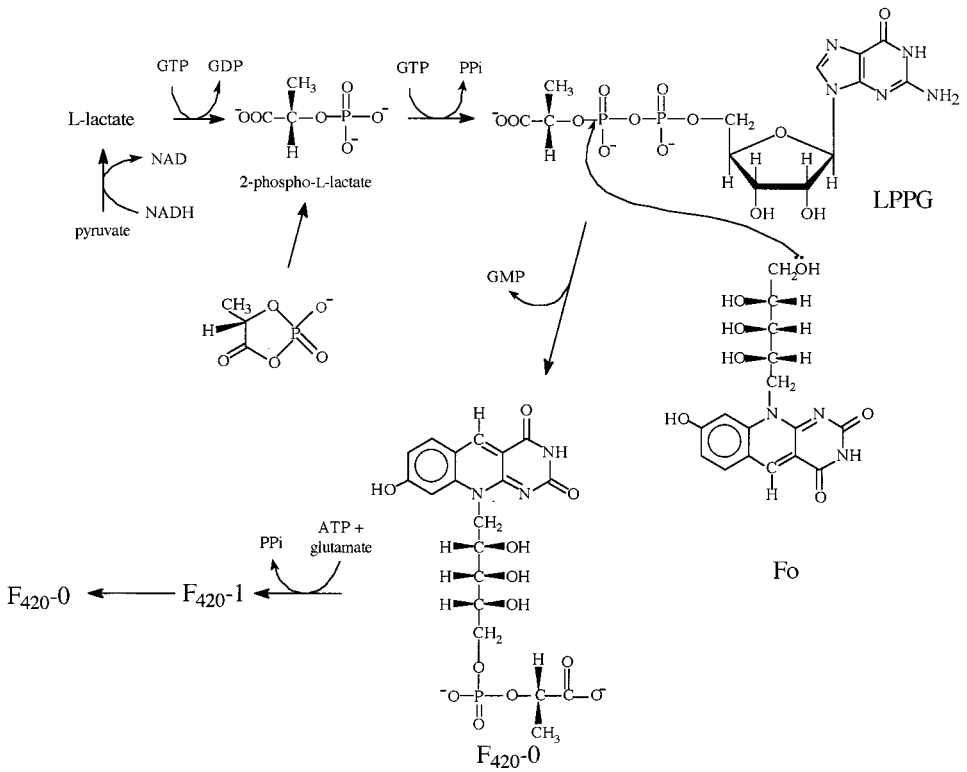

FIGURE 3: Proposed pathway for the formation of F_{420}-0 from lactate, Fo, and GTP.

pH 7.4, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 10 mM β-mercaptoethanol, and were stored frozen under argon at −78 °C until needed. Dr. Ralph S. Wolfe (Urbana, Illinois) kindly supplied frozen cells of *Methanobacterium thermoautotrophicum* strain ΔH, grown heterotrophically in the presence of yeast extract and tryptone. Dr. Biswarup Mukhopadhyay, Department of Microbiology, University of Illinois, Urbana (*14*), kindly supplied frozen cells of *Methanococcus jannaschii*. The cells were lysed by sonication under argon in buffer containing 50 mM TES, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, pH 7.0, and stored at −78 °C until used. The protein concentrations of cell extracts used typically ranged from 40 to 50 mg/mL.

Cell extracts (100 μL) of *Mc. jannaschii* or *M. thermophila* were passed through a Sephadex G-25 column (50−150 μm, 0.5 × 7 cm) equilibrated in buffer containing 50 mM TES/Na^+, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol at pH 7.0. The protein containing eluent (5−8 mg/mL protein) was frozen at −20 °C under argon until used.

Synthesis and Characterization of 2-Phospho-L-lactate. Benzyl L-lactate (0.54 g, 3 mmol) was dissolved in 4 mL of dry pyridine and reacted with diphenyl chlorophosphate (0.9 g, 3.36 mmol) at 0 °C with stirring. After the sample was mixed, the solution was allowed to warm to room temperature and stirred for 18 h. After addition of a few drops of water, the pyridine was removed from the solution by evaporation in a vacuum. The resulting colorless oil was dissolved in 5 mL of benzene, washed with 5 mL portions of water, 1 M HCl, and saturated $NaHCO_3$, and dried over Na_2SO_4 to give 1.3 g of chromatographically (TLC) pure oil. Mass spectral analysis ($M^+ = 412$ *m/z*) proved that the product was the desired diphenyl phosphate ester of benzyl lactate. The sample was dissolved in 30 mL of ethanol containing 0.13 g PtO_2 and hydrogenated at 25 psi for 1.5 h at room temperature. After removal of the Pt by filtration and evaporation of the ethanol in a vacuum, the final product was obtained as a colorless syrup, which was devoid of UV absorbance. Gas chromatography−mass spectrometry (GC-MS) analysis of the tritrimethylsilyl derivative showed a single peak with the same retention time and mass spectrum as 2-phospho-L-lactate, $M^+ = 386$ *m/z* having major fragment ions above 100 *m/z* at 133, 211, 269, 299, 342, and 371 *m/z*. The final sample was dissolved in water, titrated to a pH of 7.0 with NaOH, and diluted to a concentration of 0.1 M.

Synthesis, Purification, and Hydrolytic Stability of Cyclic 2-Phospho-L-lactate. Cyclic 2-phospho-L-lactate was synthesized in a direct reaction of L-lactic acid with phosphoryl oxychloride. Phosphoryl oxychloride (0.92 g, 6 mmol) was added to 0.51 mL of pyridine at 0 °C. This solution was added *carefully* to lactic acid (0.54 g, 6 mmol) dissolved in 0.51 mL of pyridine at 0 °C. The resulting white solid was stored at 3 °C for 24 h, then 1 h at room temperature, and was finally dissolved in 6 mL of water. After the solution was warmed to 100 °C for 1 min, the sample was adjusted to pH 7.0 with a saturated solution of LiOH at room temperature. After centrifugation to remove the lithium phosphate, 3 mL of the resulting clear and colorless solution was diluted to 60 mL with water and applied onto a DEAE-Sephadex (HCO_3^-, 1.5 × 8.5 cm) column. The sample was eluted with a linear gradient formed from 200 mL of water and 200 mL of 2 M NH_4HCO_3. Individual fractions (8 mL) were lyophilized, dissolved in ^2H_2O containing a fixed amount of [2,2,3,3-^{2}H] sodium 3-trimethylsilylpropionate. Both ^{1}H NMR and ^{31}P NMR were obtained on the fractions containing organic phosphate. On the basis of the ratios of the intensities of the methyl resonances of the eluted compounds with the methyl resonances of the TMS signal, the relative amount of material in each fraction was established. Three peaks were observed, one at fractions 8−9 for the phosphate, one at fractions 11−12 and one at fraction

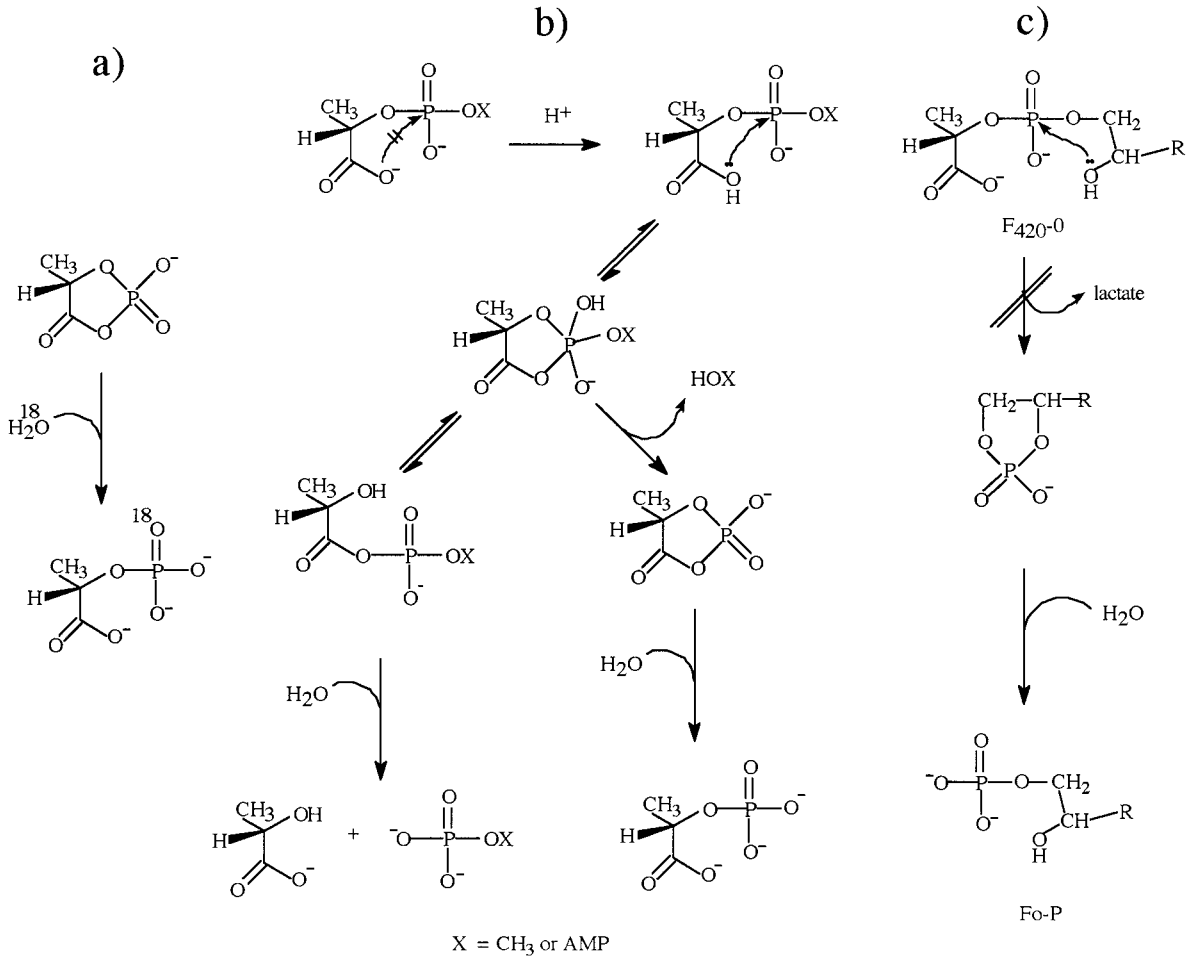

FIGURE 4: Chemical mechanisms for the hydrolysis of (a) cyclic 2-phospholactate in the presence of ^{18}O-labeled water, (b) phosphate diesters of phospholactate, and (c) and F_{420}-0.

14. A known sample of 2-phospholactate eluted at fraction 14. The ^{1}H NMR of fractions 11−12 and 14 each showed 2-phospholactate as a major compound ^{1}H NMR: 1.42, 3H, d, $J_{3\rightarrow2} = 7.0$ Hz; 4.50, 1H dq, $J_{2\rightarrow3} = 7.0$ Hz, $J_{2\rightarrow P} = 9.0$ Hz. COSY (^{31}P × ^{1}H) showed the −0.5 ppm phosphorus signal (phosphate set at 0 ppm) coupled to the C-2 hydrogen at 4.5. Fractions 11 and 12 also showed another doublet at 1.44 ppm ($J_{3\rightarrow2} = 7.0$ Hz) which was assigned to the methyl group of cyclic 2-phospho-L-lactate. Although the chemical shift of the 1.42 methyl resonance of the 2-phospholactate was found to be slightly different from sample to sample due to small pH changes, the 1.44 peak did not change, indicating that no ionizable hydrogen was associated with this molecule in the pH range of the samples (pH 7−9). ^{31}P NMR of fractions 11−12 and 14 showed several peaks, which were assigned as follows: 2-phospho-L-lactate, −0.5 ppm; cyclic 2-phospho-L-lactate, −2.4 ppm; and trimetacyclic phosphate, −22 ppm.

Confirmation that the fraction 12 contained cyclic 2-phospholactate was obtained by mixing a portion of the dried fraction 12 with $H_2{}^{18}O$ water (95% ^{18}O) and heating at 100 °C for 15 min. After dilution with water and passing of the solution through a Dowex AG 50W-8X column in the pyridinium form, the sample was dried and converted to the TMS derivative as described above. GC-MS analysis of the 2-phospholactate showed that the molecule had incorporated a single ^{18}O to an extent of 30%. Since the label was incorporated only in the 299 *m*/*z* fragment ion that contains only the phosphate, this means that cleavage of the O−P bond of the carboxylic acid phosphoanhydride bond had occurred as shown in scheme a of Figure 4. The extent of label incorporation was similar to the amount of cyclic 2-phospho-L-lactate present in the sample. Control incubations with 2-phospho-L-lactate showed no incorporation of ^{18}O.

Chemical Synthesis and Hydrolysis of the Methyl Phosphate Ester of 2-Phospho-L-lactate. Benzyl L-lactate (0.32 g, 2 mmol) was dissolved in 2.5 mL of methylene chloride to which was added, with stirring, phenyl dichlorophosphate (0.3 mL, 2 mmol) followed by triethylamine (0.28 mL, 2 mmol). After 12 h at room temperature, 0.1 mL of methanol was added to the stirred solution followed by an additional 0.28 mL of triethylamine. A thick precipitate of triethylamine HCl was formed in the reaction. After an additional 6 h at room temperature, the reaction mixture was extracted sequentially with 5-mL portions of 1 M HCl, saturated $NaHCO_3$, and dried with Na_2SO_4 to produce 0.56 g of yellow oil. TLC analysis (benzene/methyl acetate, 8:2 vol/vol) showed the material consisted of only two major UV absorbing spots of about equal intensities with R_f values of 0.38 and 0.29. Preparative TLC purification of these two major products in the same solvent system gave chromatographically pure compounds each of which gave the same UV and mass spectral data: $M^+ = 350$ *m*/*z*, $M^+ - OC_6H_5 = 257$ *m*/*z*, $M^+ - CO_2Bz = 215$ *m*/*z*, and other major ions at 91, 189, 171. ^{1}H NMR (top TLC spot) (δ) 7.35 (5H, m,

benzyl); 7.2 (5H, m, phenyl); 5.225 (1H, d, $J = 12.15$ cps, methylene hydrogen); 5.165 (1H, d, $J = 12.15$ Hz, other methylene hydrogen); 5.03 (1H, dq, $J_{2\rightarrow3} = 7.0$, $J_{H\rightarrow P} = 8.24$, H-2), 3.85 (3H, d, $J_{H\rightarrow P} = 11.54$ Hz, CH_3); and 1.5 (3H, dd, $J_{3\rightarrow2} = 7.0$, $J_{H\rightarrow P} = 0.82$, H-3). ^{1}H NMR (bottom TLC spot) (δ) 7.35 (5H, m, benzyl); 7.2 (5H, m, phenyl); 5.18 (2H, m, methylene hydrogens); 5.04 (1H, dq, $J_{2\rightarrow3} = 6.9$, $J_{H\rightarrow P} = 8.65$, H-2), 3.48(3H, d, $J_{H\rightarrow P} = 11.5$ Hz, CH_3); and 1.61-(3H, dd, $J_{3\rightarrow2} = 6.8$, H-3). On the basis of these data, the two isomers represent the two diastereomers of the methyl phosphate ester of 2-phospho-L-lactic acid benzyl ester. Since we did not expect any significant difference in the rates of hydrolysis of these two diastereomers, we used the mixture of the two compounds to conduct the hydrolysis experiments.

Hydrogenation, at atmospheric pressure, of an equal mixture of the two diastereomers in ethanol with Pt for 1 h followed by GC-MS analysis of the TMS derivatives of the products gave one peak corresponding to $(TMS)_2$ derivative of methyl phosphate ester of 2-phospho-L-lactate, $M^+ = 328$ *m/z*, $M^+ - 15 = 313$ *m/z*, with other major ions at 169, 211, 147, and 284. The product from the hydrogenation was then treated under different hydrolytic conditions, and the products were assayed by GC-MS of the TMS derivatives. Incubation of the methyl phosphate ester of 2-phospho-L-lactate in water for 2 h at room temperature produced no hydrolysis. Hydrolysis in 0.17 M HCl at 100 °C for 10 min produced complete hydrolysis to 2-phospho-L-lactate. Hydrolysis in pyridine buffer (pH 4) at 100 °C for 10 min produced 50% hydrolysis to 2-phospho-L-lactate. In addition, a significant amount of lactate and methyl phosphate was also observed. Finally, hydrolysis in triethylamine buffer (pH 7.0) at 100 °C for 10 min produced no hydrolysis to 2-phospho-L-lactate.

Synthesis of DL-[Hydroxy-^{18}O, Carboxyl-$^{18}O_2$]Lactic Acid. 2-Bromopropionic acid (90 μL, 1 mmol) was mixed with 100 μL of $H_2{}^{18}O$ water (95 atom % ^{18}O) and diisopropylamine (280 μL, 2 mmol) and heated for 3 h at 100 °C. The resulting homogeneous solution was cooled to room temperature whereupon it crystallized to a solid mass. After solution in 2 mL of water, the solution was passed through a Dowex AG 50W-8X/H^+ column to remove the diisopropylamine, and the resulting acidic solution was titrated with solid $ZnCO_3$ to a pH of 6.5. Upon concentration and addition of ethanol, crystals of zinc lactate were collected and dried in air to give 78 mg of white solid. This corresponded to a 50% yield based on the weight of this material and the amount of 2-bromopropionic acid starting material. A solution of ^{18}O-lactic acid was prepared by passing a solution of the material dissolved in water through a Dowex 50W-8X/H^+ column to remove the zinc. Titration showed two equivalents of lactate per mole of zinc. A portion of this sample was converted into the $(TMS)_2$ derivative and analyzed by GC-MS to measure the extent and position of the labeled oxygens that were incorporated into the molecule. The $M^+ - 15$ ion at 219 *m/z*, which contains all of the oxygens in the lactate, had the following atom % distribution of ^{18}O: 45% no ^{18}O, 46% $^{18}O_1$, 8.9% $^{18}O_2$, and 1.1% $^{18}O_3$, clearly showing that enriched ^{18}O had been incorporated into all three of the oxygen positions present in the molecule. The $M^+ -$ COOTMS fragment ion at *m/z* 117, which contained only the C-2 hydroxyl oxygen, was found to contain 43 atom % ^{18}O. From the difference between the ^{18}O distributions of the whole molecule measured from the molecular ion and that measured from the *m/z* fragment ion at *m/z* 117, each of the carboxylic acid oxygens had 12 atom % ^{18}O. The calculation involved solving for the values of *a*, *b*, *c*, and *d* in the expression $(a + b)(c + d)^2$, where $a =$ mole fraction of molecules with no ^{18}O, and $b =$ mole fraction of molecules with one ^{18}O at C-2, and the values of *c* and *d* represent the mole fraction of molecules with no ^{18}O or one ^{18}O at each C-1 oxygen. Because the labeling of the carboxyl oxygens was less than that in the C-2 hydroxyl group, complete exchange of the carboxyl oxygens must not have occurred during the synthesis.

Synthesis of [U-^{14}C]2-Phospho-L-lactate. Sodium [U-^{14}C]-L-lactate (6.7 nmol) was mixed with 20 μL of pyridine and 2 μL of $POCl_3$. After storage of the sample overnight at 3 °C, 0.5 mL of water was added and the pH of the solution was adjusted to pH 7−8 with saturated LiOH. After 45 min at room temperature, the sample was applied to a DEAE-Sephadex column and eluted with the NH_4HCO_3 gradient as described above. Three major radioactive peaks were observed. One major peak was unreacted lactate eluting maximally at fraction 4. The two other peaks, eluting maximally at fractions 11 and 14, and each accounting for about 10% of the radioactivity, were assigned as [U-^{14}C]-cyclic 2-phospho-L-lactate and [U-^{14}C]2-phospho-L-lactate. This assignment of peaks was based on the elution positions of the known compound on the DEAE Sephadex column and enzymatic and chemical analysis of the products by TLC. Thus, the compound found in fraction 11 when heated with water produced the compound found in fraction 14. This compound as well as the compound found in fraction 14 when treated with alkaline phosphatase both produced lactate. TLC analysis of fraction 14 showed the same R_f as for 2-phospho-L-lactate.

Synthesis and Characterization of Guanosine(5′)diphospho-L-lactate and Adenosine(5′)diphospho-L-lactate. The steps used in the synthesis of adenosine(5′)diphospho-L-lactate are shown in Figure 5. Methyl (*S*)−(−)-lactate (0.208 g, 2 mmol) was dissolved in 1.3 mL of pyridine with stirring at room temperature, and then diphenyl chlorophosphate (0.56 g, 2.09 mmol) was added. After reaction of the sample overnight at room temperature, 50 μL of water were added, and the pyridine was evaporated with a stream nitrogen gas. The resulting sample was dissolved in 2 mL of benzene and washed sequentially with 2 mL portions of water, 1 M HCl, water, and saturated $NaHCO_3$. The benzene solution was dried with Na_2SO_4, and the benzene was evaporated with a stream nitrogen gas to give a chromatographically pure colorless oil of the methyl ester of 2-diphenylphospho-L-lactate: $M^+ = 336$ *m/z* with important fragment ions at $M^+ - COOCH_3 = 277$ *m/z*, and $M^+ - OC_6H_5 = 243$ *m/z*. ^{1}H NMR (500 MHz) ($DCCl_3$): δ 7.16−7.36 (10H, m, phenyl), 5.096 (1H, dq, $J_{H-2\rightarrow P} = 8.5$ Hz; $J_{H-2\rightarrow H-3} = 6.8$ Hz, H-2), 3.70 (3H, s, OCH_3), 1.52 (3H, d, $J_{H-3\rightarrow H-2} = 6.8$ Hz). Hydrogenation of the sample dissolved in ethanol (100 mg/mL, 10 mg of PtO_2) for 1 h with 30 psi hydrogen, followed by neutralization with triethylamine, gave the desired triethylamine salt of 2-phospho-L-lactic acid methyl ester: ^{1}H NMR (500 MHz) ($DCCl_3$): δ 10.28 (1H, bs, HN), 4.80 (1H, dq, $J_{H-2\rightarrow P} = 8.9$ Hz; $J_{H-2\rightarrow H-3} = 6.8$ Hz, H-2), 3.71 (3H, s, OCH_3), 3.12 (6H, m, CH_2N), 1.495 (3H, d, $J_{H-3\rightarrow H-2} = 6.8$ Hz) 1.318 (8H, t, $C\underline{H}_3CH_2N$). GC-MS of the diTMS

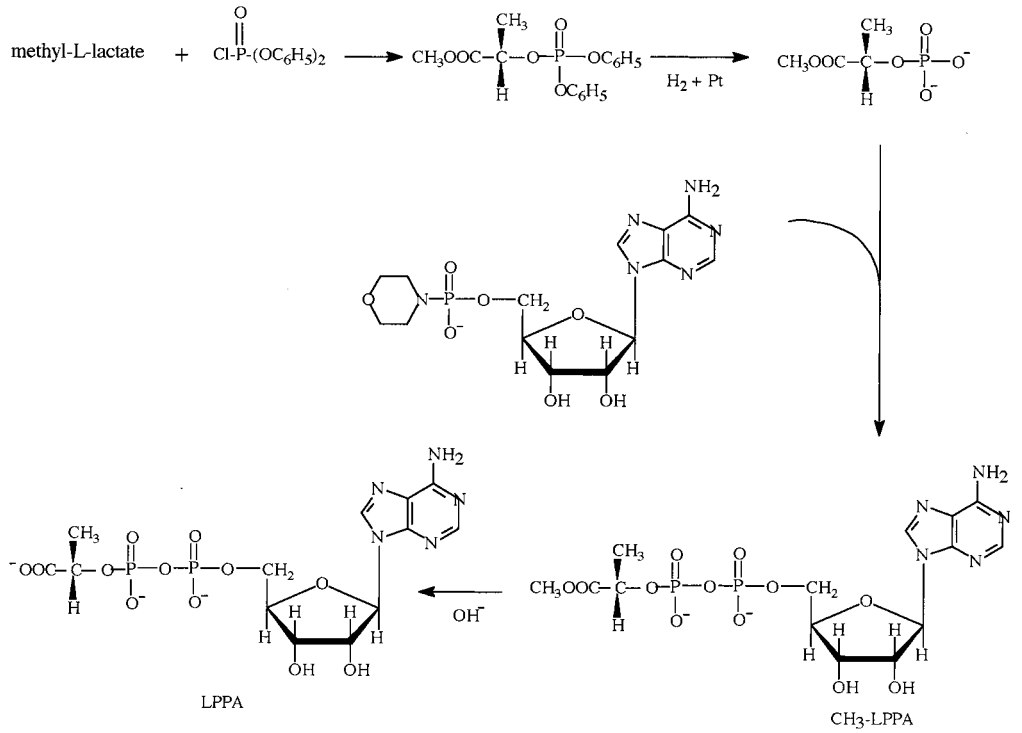

FIGURE 5: Scheme for the chemical synthesis of LPPA.

derivative of the product gave essentially one GC peak having the desired mass spectra: *m/z* $M^+ = 328$ *m/z* with important fragment ions at $M^+ - CH_3 = 313$ *m/z* and $M^+ - COOCH_3 = 269$ *m/z*. This triethylamine salt of 2-phospho-L-lactic acid methyl ester was coupled with 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine of adenosine- or guanosine 5-monophosphomorpholidate in the presence of 1*H*-tetrazole using reactions conditions similar to those described for the preparation of guanosine(5′)diphospho-α-L-fucose (*15*). The preparation of the adenosine derivative will be described, but the preparation and analysis of the guanosine derivative are identical. Thus, the triethylamine salt of 2-phospho-L-lactic acid methyl ester (23 mg, 92 μmol) containing two-thirds parts of triethylamine·HCl by ^{1}H NMR was coevaporated with dry pyridine (3 × 200 μL). The final sample was dissolved in 0.6 mL of pyridine, and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium adenosine 5-monophosphomorpholidate (44 mg, 62 μmol) was added. The suspension was stirred for 5 min until solution. 1*H*-Tetrazole (7 mg, 101 μmol) was added, and the solution was placed at 37 °C for 24 h. At the end of this period, 0.4 mL of ^2H_2O was added, and the ^{31}P NMR was obtained. Resonances from four compounds were observed. Relative to phosphoric acid, which was added to the sample as internal standard and assigned a value of 0.00, the phosphorus chemical shifts of these compounds were recorded: −0.04, AMP; −0.8, 2-phospho-L-lactic acid methyl ester; −10.4, APPA; and two doublets centered at −10.64 and −11.6 ($J_{P\rightarrow P} = 18.6$ Hz). On the basis of the intensities of the adenosine 5-monophosphomorpholidate derived resonances, 13% of the compound were hydrolyzed to AMP, 18% were converted into APPA, and 68% were converted into CH_3LPPA. A portion of the sample was purified on the Mono Q column HR 5/5 column (Pharmacia) (H_2O−NaCl gradient), lyophilized, and after exchange of the water with ^2H_2O the ^{1}H NMR of CH_3LPPA was obtained. ^{1}H NMR (δ) 8.62 (1H, s, aden, H-2); 8.27 (1H, s, aden, H-8); 6.15 (1H, d, $J = 6.0$ Hz, H-1); ∼4.71 (H-2, under the water peak); 4.51 (1H, t, $J_{H-3\rightarrow H-2} = 4.95$, $J_{H-3\rightarrow H-4} = 3.51$, H-3); 4.44 (1H, dt, $J_{H-2\rightarrow H-3} = 7.0$, $J_{H-2\rightarrow P} = 8.24$, lactate H-2), 4.37 (1H, m, H-4); 4.0 (2H, dd, $J_{H-5\rightarrow P} = 4.12$ Hz, $J_{H-5\rightarrow H-4} = 3.5$, H-5); 3.59 (3H, s, CH_3O); and 1.395 (3H, d, $J_{H-3\rightarrow H-2} = 7.0$, lactate CH_3).

CH_3LPPA was hydrolyzed in 0.1 M NaOH for 15 min at room temperature and neutralized with HCl. The final product was immediately analyzed on a Mono Q column (see below) or used as a substrate for incubations with cell extracts. The concentrations of LPPA and LPPG were determined using a known amount of AMP or GMP as standards. The stability of LPPA or LPPG toward acids was tested by exposing the compounds to 0.1 M HCl or 3% TCA for 15 min at room temperature. After neutralization, the samples were analyzed on the Mono Q column. To study the temperature stability, both compounds were incubated for certain specific times at 50 °C or room temperature and immediately applied onto the Mono Q column for analysis.

Isolation and Identification of Lactate and 2-Phospho-L-lactate in Mb. thermoautotrophicum and Mc. jannaschii. Mb. thermoautotrophicum cell paste (10.5 g) was suspended in a mixture of water and methanol (1:1 vol %:vol %) and heated in a closed vial at 100 °C for 10 min. After cooling to room temperature and centrifugation (14000*g*, 10 min) of the sample, the resulting clear supernatant was separated from the pellet, which was resuspended in 10 mL of 50% methanol and extracted in the same manner as in the first extraction. The combined supernatants were then evaporated to about 5 mL with a stream of nitrogen gas to remove the methanol and diluted to 20 mL with water for purification by ion exchange chromatography. The resulting sample was then applied to a DEAE-sephadex (HCO_3^-, 1.5 × 8.5 cm) column, which was eluted with a linear gradient formed from 200 mL of water and 200 mL of 2 M NH_4HCO_3. Fraction volumes were 8 mL. Fractions containing lactic acid and

2-phospho-L-lactate had been previously identified by the separation of known compounds under the same conditions. The presence of lactate in the fractions was determined by TLC analysis and the presence of 2-phospholactate by organic phosphate analysis. The NH_4HCO_3 was removed from fractions containing lactic acid and 2-phospho-L-lactate by evaporation under a stream of nitrogen gas, while the samples were maintained at 100 °C. This process had to be completed three times to totally remove the NH_4HCO_3. The final samples were then dissolved in water and passed through a Dowex-50W-8X H^+ column (2 × 5 mm) to remove the cations. After evaporation of the water, the samples were converted into the trimethylsilyl derivatives by reaction for 1 h at 40 °C with 20 μL of a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine (1/3/9 vol/vol/vol). Quantitation of lactate and 2-phospholactate was based on peak areas as compared with known amounts of standards.

Production of Fo-P and the Deglutamated F_{420}−0 Intermediate. F_{420}-2 samples (isolated from *Mb. thermoautotrophicum* described above) were incubated with a solution of γ-glutamyltranspeptidase type IV from porcine kidney (50 units/500 μL, Sigma) in 0.1 M TES/Na^+, 10 mM $MgCl_2$ (pH 9.0) at room temperature for 2 days (*16*). Shorter incubation times lead to F_{420}-1 preparations. The reaction was terminated by the addition of ethanol to 60% (vol/vol). After centrifugation of the sample, the supernatant was evaporated under a stream of nitrogen gas and redissolved in 10 mM NH_4OH. Assignment and purity of samples were proved by HPLC and TLC (acetonitrile/H_2O/HCOOH, 80:20:2, vol, vol, vol). The thermal stability of F_{420}-0 was investigated in a neutral solution that was heated for various times at 100 °C. The resulting samples were analyzed for decomposition products by HPLC. Fo-P was obtained by acid hydrolysis of F_{420}-2 (*2*).

HPLC Methods for the Analysis of F_{420} Biosynthetic Intermediates. HPLC analysis was performed on a Shimadzu SCL-6B using a C-18 reversed phase column (AXXI Chrom ODS, 5 micron, 25 cm) preceded by a guard column (RP-18 NEWGUARD, 7 μm, 1.5 cm) (*17*). A sodium acetate/methanol linear gradient was used for separation at a flow rate of 0.5 mL/min. For the first 5 min, the sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) contained 5% methanol. Then the methanol concentration was raised linearly to 80% over the next 40 min and kept for 5 min at this methanol concentration. The eluent was monitored for fluorescence at an emission wavelength of 480 nm (excitation wavelength 420 nm) and for absorbance at 280 nm. The 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; and Fo, 30.4 min under these conditions.

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 on a BioLogic HR chromatographic system (BioRad) using a linear sodium chloride gradient (pH 7.0) or an ammonium bicarbonate gradient (pH 9.2). Comparison of changes in the elution positions of the compounds between these two buffers systems allowed for the determination of the presence of a guanosine residue in the substrate because of its pK_a of 9. For the NaCl gradient, the following buffers were used: buffer A was 25 mM TES (adjusted to pH 7.0 with sodium hydroxide), and buffer B contained additionally 1.5 M NaCl. After injection of the sample onto the Mono Q column, the NaCl concentration was increased linearly from 0 to 60% buffer B over 60 min at a flow rate of 1 mL/min. 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. For the separation of CH_3LPPA on the Mono Q column for ^{1}H NMR analysis, the TES in buffer A and B was omitted (H_2O−NaCl gradient) to circumvent interferences from protons of the buffer.

For the NH_4HCO_3 gradient, buffer A was water and buffer B was 1 M NH_4HCO_3 (pH 9.0). The NH_4HCO_3 concentration was raised linearly from 0 to 100% buffer B over 60 min at a flow rate of 1 mL/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- and adenosine-containing materials were detected by absorbance recorded with an UV detector at 254 nm. Lactate was detected by TLC analysis of the fractions after spraying with molybdenum spray reagent (20 g NH_4-MoO_4 dissolved in 25 mL H_2SO_4 and diluted with 400 mL of water) and heating a few minutes at 150 °C. The cyclic 2-phospholactate, LPL, and 2-phospholactate were detected by organic phosphate analysis (*18*). In each elution, fractions were generally collected at 1-min intervals.

TLC Analysis of Radioactive Products. Fractions of the Mono Q column containing radioactive compounds from incubations were evaporated to dryness under a stream of nitrogen gas at room temperature to remove the NH_4HCO_3. After dissolving in water, the liquid was applied onto a 5 × 10 cm silica gel TLC plate which was developed in the solvent system acetonitrile/water/formic acid (88%) (40:10:5 v/v/v). The support of the TLC plate was removed from eight equal areas, ground to a powder, mixed with 1 mL of ScintiSafe (Fisher) scintillation fluid, and counted for radioactivity. In this solvent system, the following compounds had the following R_f values: GTP, 0.00; GDP, 0.00; GMP, 0.02; 2-phospholactate, 0.37; guanosine, 0.49; and lactate, 0.68.

To further confirm the presence of radioactivity labeled 2-phospholactate, the appropriate fractions were mixed with carrier 2-phospho-L-lactate, converted into their trimethyl ester derivatives, and separated by TLC. Briefly, the methyl ester was prepared by passing the sample containing the 2-phospholactate through a small column of Dowex AG 50W-8X H^+ (2 × 5 mm) to remove the cations. After evaporation of the solvent, the residue was dissolved in methanol (50 μL) and treated with diazomethane in diethyl ether until yellow. TLC analysis of the recovered oil was carried out with methyl acetate as the solvent system in which the trimethyl ester of 2-phospholactate had an R_f of 0.46.

Incubation of Cell Extracts with Precursors and Processing of Incubated Samples for Analyses. Cell extracts of *M. thermophila* (25 μL) or cell extracts of Sephadex-purified *Mc. jannaschii* (10−90 μL) were mixed with the indicated

amounts of anaerobic solutions of the substrates and incubated at 50 °C for 0.5−2 h in a total volume of 50 to 150 μL. Typically, all incubation mixtures contained 5−10 μM Fo and one or more of the following: 0.2−2 mM GTP, ATP or other nucleotides, 0.4−10 mM 2-phospho-L-lactate, 10−25 mM L-lactate, and/or 50−60 μM LPPG or LPPA. The proteins were precipitated by addition of 60 vol % EtOH. After centrifugation of the sample, the liquid was separated from the pellet, and the EtOH was removed from the aqueous solution under a stream of nitrogen. Final solutions were stored in 10 mM NH_4OH and portions were analyzed by HPLC.

Incubations with the radioactive substrates, [U-^{14}C]L-lactate and [8-^{3}H]GTP, were performed with *Mc. jannaschii* extracts (50 μL) in the presence of carrier L-lactate (0.07 mM) or GTP (0.05 mM) to give the same specific activities (78 000 cpm/nmol of substrate). The incubations with radioactive substrates were conducted for 30 min at 50 °C and then processed in either of two different manners to remove the bulk of the proteins for subsequent analyses. In the first procedure, the proteins were precipitated at 0 °C by the addition of TCA to a final concentration of 3% (vol %). Following centrifugation (16000*g* × 10 min) to remove the proteins, the pellet was extracted with an equal volume of 3% TCA at 0 °C, and the combined supernatants were extracted three times with 1-mL portions of ethyl ether to remove the TCA. This extraction also removed two-thirds of the lactate. In the second procedure, which avoided the acidic treatment with the TCA, the proteins were precipitated by the addition of ethanol to a final concentration 60 vol %. The precipitated proteins were removed by centrifugation (16000*g* × 10 min), and the pellet was washed with 100 μL of EtOH/H_2O (1:1). The supernatants were combined, and the ethanol was removed by evaporating under a stream of nitrogen.

Nucleotide-containing compounds present in incubation mixtures were removed by passing the incubated extracts through a small carbon column (Norite/Celite 1:1 wt/wt, 0.3 × 0.3 cm) that had been extensively washed with water and extraction buffer. This column quantitatively absorbed nucleotides from known solutions of GTP, GDP, and GMP by removing all of the A_{260} absorbance.

Incubation of Cell Extracts of M. thermophila and Mc. jannnaschii with DL-[Hydroxy-^{18}O, Carboxyl-$^{18}O_2$]Lactate and GTP. To 100 μL portions of a cell extract of *Mc. jannaschii* is added 10 μL of a 0.3 M solution of the ^{18}O-labeled DL-lactate and 10 μL of a 0.1 M solution of either GTP or ATP. In these incubations, the final concentrations of the lactate was 25 mM and that for the GTP or ATP was 8 mM. *M. thermophila* extracts (50 μL) were incubated with 60 mM ^{18}O-labeled lactate with or without 10 mM GTP. After incubation under argon for 2 h at 50 °C, an equal volume of ethanol was added and the samples were heated for 5 min at 80 °C and centrifuged to remove the protein pellet. After washing the pellet with 60 μL of 50% ethanol, the combined supernatants were evaporated to dryness, and, after forming a solution in 100 μL of water, were passed through a (0.3 × 0.5 mm) Dowex 50W−X8 H^+ column and evaporated to dryness. GC-MS analyses were performed on the TMS derivative. Quantitation was based on the intensities of the 190 *m*/*z* for the lactate and the *m*/*z* 371 for the phospholactate.

Treatment of Samples with Alkaline Phosphatase and Nucleotide Pyrophosphatase. Alkaline phosphatase was used to cleave GTP to guanosine in samples from incubations with [8-^{3}H]GTP to discover minor intermediates. This was accomplished by an equal volume of 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ to the samples followed by alkaline phosphatase (1 μL, 0.2 units). Treatment of samples with nucleotide pyrophosphatase was performed in 50 mM TES, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, pH 7.0 buffer. After incubation for 1 h at room temperature, the samples were processed in the same manner as the untreated ones.

GC-MS Analyses. GC-MS was performed using a VG-70-70EHF gas chromatography−mass spectrometer operating at 70 eV and equipped with a HP-5 column (0.32 mm by 30 m) programmed from 95 to 280 °C at 10 °C per min. The trimethylsilyl derivatives were produced by reacting samples with a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine (1/3/9 vol/vol/vol). After separation of the NH_4Cl precipitate by centrifugation, the resulting clear solutions were directly injected onto the column. Under these chromatographic conditions, the following compounds as their trimethylsilyl derivatives had the following retention times (min) and mass spectral data [molecular weight, base peak, the two-five most abundant ions with masses over 100 *m*/*z* listed in order of decreasing intensities]: ditrimethylsilyl derivative of lactate, (3.58) [$M^+ = 234$, 147, 117, 190, 133, 219 ($M^+ - 15$)]; tritrimethylsilyl derivative of phosphate, (6.56) [$M^+ = 314$, 299 ($M^+ - 15$), 314, 133, 207]; tritrimethylsilyl derivative of 2-phospholactate, (10.7) [$M^+ = 386$, 299, 371 ($M^+ - 15$), 342, 211, 269, 315].

RESULTS

Identification and Quantitation of Possible Intermediates in F_{420} Biosynthesis in Methanogens. Extracts of *Mb. thermoautotrophicum* cells were separated on a DEAE Sephadex column, and fractions were analyzed for possible intermediates involved in the formation of the phosphodiester bond in coenzyme F_{420}. On the basis of the amounts of intermediates recovered from the *Mb. thermoautotrophicum* extracts, lactate was calculated to be present in these cells at a concentration of about 600 μM, and 2-phospholactate was present at a concentration of about 17 μM. These data were obtained from intensities of the peaks from GC-MS analyses of the TMS derivatives of lactate and phospholactate recovered from the appropriate fractions as compared to known samples. HPLC analysis of Fo-P containing fractions demonstrated that the Fo-P concentration was less than 0.01 μM. (These calculations were based on the assumption that 70% of the wet weight of the *Mb. thermoautotrophicum* cells was water). To establish that the lack of detection of Fo-P was not the result of phosphatase cleavage of the Fo-P during extraction and sample work up, we conducted the extraction of the *Mb. thermoautotrophicum* cells in the presence of added Fo-P. In these experiments, the added Fo-P was recovered with no cleavage to Fo, indicating that no hydrolysis of this substrate occurred.

Incubation of Cell Extracts of M. thermophila and Mc. jannaschii with DL-[Hydroxy-^{18}O, Carboxyl-$^{18}O_2$]Lactate and GTP. A cell extract of *M. thermophila* (50 μL) incubated

Table 1: Formation of F_{420-0} from Fo and L-Lactate or 2-Phospho-L-lactate by Cell Extracts of Methanogens[a]

substrate	nucleotide	pmol F_{420}-0 (per h per mg of protein) *M. thermophila*	*Mc. jannaschii*
1. lactate		3	nd[b]
2. lactate	ATP	4	1
3. lactate	GTP	16	71
4. 2-phospho-L-lactate		54	190
5. 2-phospho-L-lactate	ATP	240	1500
6. 2-phospho-L-lactate	GTP	170	1200

[a] Incubation conditions: For the experiments with L-lactate, *M. thermophila* extracts (50 μL) and Sephadex-purified *Mc. jannaschii* extracts (90 μL) were incubated with 20 mM L-lactate, 7 mM GTP or ATP, 5 μM Fo. For the 2-phospho-L-lactate experiments, Sephadex-purified *M. thermophila* extract (50 μL) was incubated with 6 μM Fo, 7 mM GTP or ATP, and 10 mM 2-phospho-L-lactate for 30 min at 50 °C. No F_{420}-0 (less than 0.017 pmol) was detected in incubations unless lactate or 2-phospholactate was added to the incubation. Incubations of the crude *M. thermophila* extract with 2-phospho-L-lactate did not result in the formation of F_{420}-0. After incubation for 2 h at 50 °C, the samples were precipitated with EtOH, processed, and the F_{420}-0 formation analyzed by HPLC. [b] nd, not detected.

with 60 mM ^{18}O-labeled DL-lactate and 10 mM GTP converted 20% of the lactate to 2-phospho-L-lactate, and no product was detected with ATP. The same experiment conducted with an extract of *Mc. jannaschii* was found to convert 6% of the lactate to 2-phospholactate in the presence of GTP and 1% in the presence of ATP. In both experiments, the 2-phospholactate had the same ^{18}O distribution as found in both the starting lactate and the lactate recovered from the incubation.

F_{420}-0 Formation in Extracts of Methanoarchaea Extracts. Incubation of cell extracts of *M. thermophila* with Fo and L-lactic acid produced an increase in F_{420}-0 over that observed in extracts with no additions (Table 1, experiment 1). The addition of GTP, but not ATP, to the incubation mixture produced an additional increase in F_{420}-0 over that formed with incubation of only lactate (Table 1, experiments 2 and 3). This result indicated that GTP alone could affect the incorporation of Fo and lactate into F_{420}-0. A Sephadex-purified *M. thermophila* cell extract incubated with 2-phospho-L-lactate gave a large increase in the amount of F_{420}-0 produced, which was further stimulated by the addition of GTP or ATP to the incubation mixture (Table 1, experiments 4−6).

To expand the above observations in another methanoarchaea, similar experiments were conducted with extracts of *Mc. jannaschii*. Because of the large amounts of different F_{420} polyglutamates in these cells (unpublished results) that interfered with the HPLC analyses of the F_{420}-0 produced, these incubations were conducted with Sephadex G-25 purified extracts that contained greatly reduced amounts of the F_{420}-polyglutamate coenzymes. As can be seen in Table 1, the production of F_{420}-0 in such an extract of *Mc. jannaschii* closely followed that observed in *M. thermophila* except that more F_{420}-0 was generally produced. Again, as was observed with the *M. thermophila* extract, there is no explicit nucleotide dependency for this reaction, whereas the lactate kinase reaction is dependent on GTP. Incubations without the addition of nucleotides, but in the presence of 2-phospholactate also, produced small amounts of F_{420}-0, suggesting that the necessary nucleotide component(s) required for the observed synthesis was present in the extract in a bound form (experiment 4).

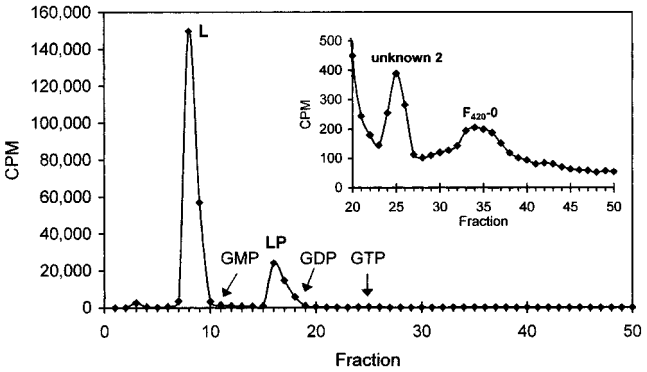

FIGURE 6: Mono Q elution profile (sodium chloride gradient) from the [U-^{14}C]L-lactate incubation. The sample was generated by an extract of *Mc. jannaschii* (25 μL) incubated with [U-^{14}C]L-lactate (0.3 mM), carrier L-lactate (1 mM), and GTP (1 mM) for 30 min at 50 °C followed by TCA precipitation. The arrows with GMP, GDP, and GTP indicate the elution positions of these compounds based on their UV absorbance.

Characterization of Biosynthetic Intermediates Involved in F_{420}-0 Production from Fo, 2-Phospho-L-lactate, and GTP. To characterize the nature of possible intermediates involved in the coupling of Fo with 2-phospho-L-lactate, extracts of *Mc. jannaschii* were incubated with radioactive precursors and analyzed for the production such intermediates. After the reaction mixtures were stopped and the proteins were precipitated with either TCA or ethanol, the resulting soluble compounds were separated on a Mono Q column using the two different solvent systems. Comparisons of the elution positions of the resulting radioactive peaks with those of known compounds were used to establish the total charges on the intermediates and thus give insight into the possible structures of the observed intermediate(s). Further chemical and enzymatic cleavages as well as HPLC analysis of each of the labeled peaks was followed by TLC and/or Mono Q analysis to establish the identity of the resulting radioactive compound. Three groups of such experiments were conducted and included incubations with [U-^{14}C]L-lactate and GTP, [U-^{14}C]2-phospho-L-lactate and GTP, and 2-phospho-L-lactate and [8-^{3}H]GTP.

Incubation of Mc. jannaschii Extracts with [U-^{14}C]Lactate and GTP. Cell extracts of *Mc. jannaschii* were incubated with [U-^{14}C]L-lactate and GTP, the proteins were precipitated, and the resulting soluble compounds were separated on a Mono Q column with the NaCl gradient elution. Samples precipitated with either TCA or ethanol produced similar profiles containing up to four clearly defined radioactive peaks (Figure 6). The elution profile for the TCA precipitated samples showed sharper peaks than for the ethanol precipitated samples. Radioactive peaks will be referred to by the fraction number containing the maximum radioactivity. The first peak eluted at the same fraction position 8 as a known sample of lactate, and all of the radioactivity in this peak could be accounted for as lactate based on radio-TLC analysis of the material. In addition, the radioactivity was found to be volatile as is observed for lactate.

The second peak eluted maximally at fraction 16, the same position as a known sample of 2-phospho-L-lactate which contains three negative charges. The third peak eluted

maximally at fraction 25, the same position as GTP which has four negative charges. The fourth peak eluted as a broad peak from fractions 33−36 where F_{420}-0 eluted. The amount of radioactivity present in the third and fourth peaks were, respectively, 11 and 14% of that found in the 2-phospholactate. TLC analysis of the compounds contained in fraction 16 showed that 21% of the radioactivity was present as 2-phospholactate. This was confirmed by its quantitative cleavage to lactate with alkaline phosphatase. The remaining radioactivity in fraction 16 remained at the origin of the TLC plate. The chemical structure of this material was not established and will be referred to as unknown 1. The third peak, fraction 25, was called unknown compound 2. This compound was retained by a carbon column and after passage through a Dowex 50W-8X H^+ column had a TLC R_f just above the origin. The chemical structure of this material was likewise not established.

The last broad fourth peak, corresponding to the elution time of F_{420}-0, was shown to consist of only two major radioactive compounds by radio-HPLC analysis. Each peak was a compound derived from Fo based on the detection of the Fo chromophore fluorescence. The later eluting HPLC peak coeluted with F_{420}-0 and was assigned to this structure. The earlier eluting HPLC peak was tentatively identified as an activated form of F_{420}-0 possibly used in the addition of the glutamic acids, based on its elution position from both the Mono Q column and the reversed phase HPLC column and that it contained F_{420} fluorescence.

Close examination of the radioactive compound(s) contained in fractions 10 and 11, which was a shoulder on the large lactate peak and at the same elution position as cyclic 2-phospholactate, confirmed the presence of cyclic 2-phospholactate in these fractions. This was accomplished by hydrolysis of the material in the fractions to 2-phospholactate, mixing the hydrolyzed material with carrier 2-phospholactate and analysis of the resulting products by TLC. Analysis of the samples both as free 2-phospholactate and as the trimethyl ester derivative demonstrated the presence of radiolabeled 2-phospholactate.

Incubation of Mc. jannaschii Extracts with [U-^{14}C]2-Phospho-L-lactate and GTP. Incubation of *Mc. jannaschii* extract with a biochemically generated sample of [U-^{14}C]2-phospho-L-lactate (purified on the Mono Q column with the NH_4HCO_3 gradient), Fo, and GTP followed by phosphatase treatment and separation on the Mono Q with the NH_4HCO_3 gradient showed the presence of six radiolabeled peaks (Figure 7). These peaks, eluting maximally in the indicated fraction numbers, were assigned as follows: lactate, 8; cyclic 2-phophalactate, 11; 2-phospholactate, 16; unknown compound 1, 21; unknown compound 2, 29, and F_{420}-0, 54. The radioactivity contained in each peak based on the percent of the 2-phospholactate peak was lactate, 88%; cyclic 2-phospholactate, 1.6%; 2-phospholactate, 100%; unknown compound 1, 4%; unknown compound 2, 0.6%, and F_{420}-0, 3.8%. Radioactive unknowns 1 and 2 when incubated with *Mc. jannaschii* extracts along with Fo and GTP failed to label any F_{420}-containing compounds.

Incubation of Mc. jannaschii Extracts with [8-^{3}H]GTP and 2-Phospho-L-lactate. Incubation of Sephadex G-25 purified cell extracts of *Mc. jannaschii* with [8-^{3}H]GTP and 2-phospholactate followed by separation of the incubation mixture on the Mono Q column with a NH_4HCO_3 gradient produced

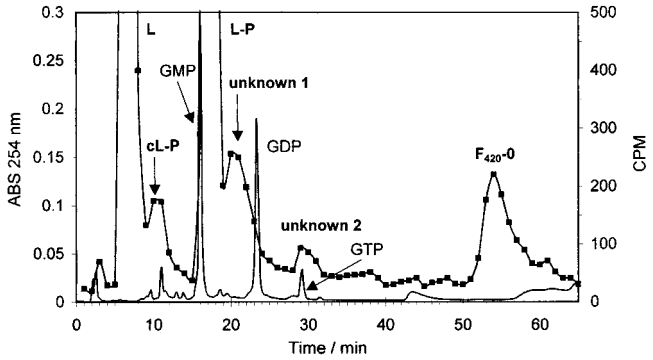

FIGURE 7: Mono Q elution profile (ammonium bicarbonate gradient) of radiolabeled compounds from an incubation for 30 min at 50 °C of *Mc. jannaschii* extracts (20 μL) with [U-^{14}C]2-phospholactate (0.05 mM), carrier 2-phospholactate (0.6 mM), and GTP (8 mM) after EtOH precipitation. -■- is cpm and − is absorbance.

four major radioactive peaks eluting maximally at fractions 4, 16, 23, and 27. These corresponded to the elution positions of guanosine, GMP, GDP, and GTP. To discover hidden minor compounds, the incubation was repeated and the sample was then treated with alkaline phosphatase prior to separation to remove the nucleotides. Separation of such a phosphatase treated sample produced, aside from the large peak at fraction 4 for guanosine, peaks at fractions 15, 21, 23, 27, and 29 (shoulder). The peaks 15, 21, and 27 were GMP, GDP, and GTP that were not completely hydrolyzed by the phosphatase. This was confirmed by their hydrolysis to guanosine in a second treatment with the alkaline phosphatase. Peak 23 and peak 29, each representing only 0.1% of the labeled GTP, were stable over time and were resistant to alkaline phosphatase and nucleotide pyrophosphatase. Thus, these unknown peaks could not be assigned to be intermediates in F_{420} biosynthesis. Although these peaks eluted from the Mono Q column with either gradient at about the same time as the unknown compounds 1 and 2, it was not possible to establish the identity of these compounds because of the low cpm detected.

Trapping of Nucleotide Containing Compounds by Chromography on a Carbon Column. Mc. jannaschii extracts were incubated with 2-phospho-L-lactate and GTP for 30 min and immediately passed through a carbon column. The flow-through was reincubated with *Mc. jannaschii* extract, Fo, with and without GTP. In each case, the formation of F_{420}-0 was found to still be dependent on the addition of GTP, indicating that any nucleotide containing activated 2-phospholactate was trapped on the carbon column.

Characterization of Guanosine(5′)diphospho-L-lactic Acid and Adenosine(5′)diphospho-L-lactic Acid. Repeated attempts to prepare these compounds by condensing 2-phospho-L-lactate with GMP or AMP using established procedures (*15*, *19*−*21*) were not successful. It was rationalized that the reason for this failure was the nucleophilic displacement of the NMP by the free carboxylate anion of the lactic acid. As shown in Figure 5, the synthesis was successfully accomplished by using the methyl ester of 2-phospho-L-lactate to yield the stable methyl esters of LPPA and LPPG. The final compounds were obtained by base hydrolysis of the methyl esters. Both compounds proved to be unstable, showing a half-life of 2 h at pH 7 at room temperature and a half-life of only 10 min at 50 °C. Neither LPPA nor LPPG

Table 2: Formation of F_{420}−0 from Fo and LPPG or LPPA by Cell Extracts of *Mc. jannaschii* and *M. thermophila*[a]

substrate	nucleotide	pmol F_{420-0} (per h per mg of protein) *M. thermophila*	*Mc. jannaschii*
LPPG		440	6300
LPPG	GTP	1200	7100
LPPA		180	210
LPPA	ATP	20	190

[a] Incubation conditions: Sephadex-purified cell extracts of *Mc. jannaschii* (30 μL) or *M. thermophila* (40 μL) were incubated with 4 μM Fo, 52 μM LPPG or LPPA, and as indicated 2 mM GTP or ATP for 30 min at 50 °C in a total volume of 80 μL. After incubation, the samples were precipitated with EtOH, processed, and analyzed for F_{420}-0 formation by HPLC. No F_{420}-0 (less than 0.017 pmol) was detected in incubations unless lactate or 2-phospholactate was added to the incubation.

could be recovered from acidic solutions such as 0.1 M HCl or 3% TCA, whereas exposure to 60% EtOH did not increase the amount of hydrolysis of the compounds. In contrast, the side product from the synthesis, guanosine(5′)diphospho(5′)-guanosine (GPPG), could be easily recovered after acidic treatments and is quite stable.

Incubation of Mc. jannaschii Extracts with LPPG and LPPA. Sephadex-purified extracts of *Mc. jannaschii* and *M. thermophila* were incubated with Fo and LPPG or LPPA (Table 2). Both extracts showed higher activities with LPPG than with LPPA. Addition of GTP increased the amount of F_{420}-0, while the addition of ATP led to a decrease in F_{420}-0 formation.

Characterization of Cyclic 2-Phospho-L-lactate. Separated reaction products from the synthesis of 2-phospholactate from L-lactate with $POCl_3$ showed an additional peak that would lead to the formation of F_{420}-0 when incubated with GTP and a cell extract of *M. thermophila*. To determine the structure of the compound, we undertook an extensive analysis of the products of this synthesis. ^{31}P NMR spectra of the crude reaction products showed several resonances that could be assigned to P_i, 2-phospholactate, PP_i, cyclic 2-phospholactate, cyclic trimetaphosphate, and likely the 4,5 and six-membered cyclic polyphosphates. No compound was found to contain P−P coupling eliminating compounds with nonsymmetrical P−P bonds. The resonances for P_i, 2-phospholactate, PP_i, and cyclic trimetaphosphate were confirmed by observing the increase in the assigned signal by the addition of the known compounds. Heating the sample for 100 °C for 30 min converted the suspected cyclic 2-phospholactate signal at −2.4 ppm to the 2-phospholactate signal at −0.5.

To establish the chemical structure of this material and its possible involvement in F_{420}-0 biosynthesis, the crude reaction products were separated on both a DEAE-Sephadex and the Mono Q columns, and the fractions were assayed for their ability to form F_{420}-0 in the presence of GTP. One major peak of activity was observed eluting from the DEAE Sephadex column at a position corresponding to a molecule containing one negative charge. On both columns, the compound eluted before the 2-phospho-L-lactate peak. On the basis of the position of elution, diphospholactate and triphospholactate could be eliminated as the compounds responsible for the increased activity, since these compounds would have 3 and 4 negative charges, respectively. GC-MS analysis of the TMS derivatives of the compounds contained in this fraction showed the presence of 2-phospho-L-lactate, lactyllactate, and Pi as major compounds. Since 2-phospho-L-lactate eluted after this compound in both separation methods, it must be assumed that the 2-phospho-L-lactate contained in the early fraction resulted from the decomposition of a compound that could produce 2-phospho-L-lactate. Only the cyclic 2-phospho-L-lactate is consistent with these data.

DISCUSSION

Identification and Quantification of Intermediates in Coenzyme F_{420} Biosynthesis in the Methanogens. The most likely substrates for F_{420} biosynthesis are Fo and L-lactate, one of which has to be phosphorylated and then coupled to the other partner to form the final product F_{420}-0. Considering the currently established routes for the formation of phosphodiester bonds (Figure 2), a pathway analogous to one of these routes may function in F_{420} biosynthesis. We would therefore expect to find either Fo-P or 2-phospho-L-lactate in cell extracts of the methanogens. It has been reported by others that cell extracts of *Mb. thermoautotrophicum* do not contain detectable amounts of Fo-P and that Fo appears to serve as the biosynthetic intermediate in the formation F_{420} (*22*, *23*). Fo, but not Fo-P, is also known to be produced by and secreted into the medium by growing cells of *Mb. thermoautotrophicum* (*24*, *25*). Our analysis of the *Mb. thermoautotrophicum* extract failed to show the presence of Fo-P. Because of its fluorescence, very low amounts of Fo-P can be detected, allowing us to establish that Fo-P could not have been present in these cells at concentrations higher that 10 nM. To address the concern that our inability to detect Fo-P in the cell extracts was a result of its cleavage by a phosphatase, we extracted cells in the presence of added Fo-P. These experiments resulted in the full recovery of Fo-P, demonstrating that no hydrolysis of the Fo-P was occurring with the extraction procedures used. The absence of Fo-P in *Mb. thermoautotrophicum* cells is in accordance with the lack of formation of Fo-P by incubations of extracts of *M. thermophila* and *Mc. jannaschii* with Fo and GTP or ATP. It is also consistent with the observation that cell extracts of *M. thermophilia* incubated with Fo-P, ATP, GTP, and lactate failed to produce any F_{420}-0 or Fo. From these results, it can be concluded that Fo-P is not an intermediate in the formation of the phosphodiester bond in coenzyme F_{420}.

No information is currently available on the occurrence of lactate and 2-phospholactate in the methanogens. GC-MS analyses of *Mb. thermoautotrophicum* extracts confirms the presence of both lactate (0.6 mM) and 2-phospho-L-lactate (17 μM) in these cells. Although several chemical syntheses for 2-phospho-L-lactate (*26*−*28*) as well as its enzymatic formation from L-lactate and ATP by pyruvate kinase (*29*) have been described, this is the first report of the natural occurrence of this compound. Previous interest in 2-phospho-L-lactate has been concerned with its functioning either as an alternate substrate or inhibitor of PEP-utilizing enzymes (*28*, *30*), phosphoglycerate mutase (*27*), or phosphoenolpyruvate carboxylase (*31*, *32*).

Demonstration of the Phosphorylation of Lactate. The presence of 2-phospho-L-lactate in the methanogens indicates that the first step in the formation of phosphodiester bond

in coenzyme F_{420} is the phosphorylation of L-lactate to 2-phospho-L-lactate. No obvious gene is present in the genome of *Mc. jannaschii* to encode a lactate kinase, but an enzyme annotated as pyruvate kinase could possibly do this reaction. Considering the presence of ADP-kinases in the archaea, it is also possible that an ADP/GDP kinase could function in the phosphorylation. The phosphorylation of lactate was confirmed by the demonstration that a cell extract of either *M. thermophila* or *Mc. jannaschii* incubated with [hydroxyl-^{18}O, carboxyl-$^{18}O_2$]lactic acid and GTP produced 2-phospholactate with the same atom % distribution as the labeled lactate. In the *M. thermophila* incubations, the conversion of 20% of the lactate to 2-phospholactate was accomplished with GTP, but no product was measured with ATP. Incubations of the *Mc. jannaschii* extracts with GTP and lactate gave a 6% conversion of lactate to 2-phospholactate and 1% conversion with ATP. These results not only confirmed the formation of 2-phospholactate but also established that the amount of lactate and 2-phospholactate in the cell extracts was much lower than that present in the incubated samples because there was no dilution of the isotopically labeled compounds. In addition, because the carboxylic acid oxygens still contained the same labeling pattern as the original lactate, no reaction involving exchange or loss of the carboxyl oxygens was involved in the phosphorylation. This eliminated 1-phospholactate from being an intermediate in the formation of 2-phospho-L-lactate, because hydrolysis of this acyl phosphate would have resulted in the loss of one of the labeled carboxyl oxygens (*33*).

It is assumed that the lactate and the 2-phospholactate present in the *Mb. thermoautotrophicum* cells have the same L-stereochemistry as the L-lactate found in F_{420} (*2*) as well as its γ-glutamyl derivatives (*16*). The origin of this lactate in the methanogens is not clear. One obvious possibility would be the reduction of pyruvate by a lactate dehydrogenase. The genomes of the methanogens do not show an annotation for a lactate dehydrogenase, but we have established that the reduction of pyruvate to L-lactate can be carried out by one of the malate dehydrogenases (the MJ1425 gene product) present in *Mc. jannaschii* (*34*).

All these results are consistent that in the case of F_{420} biosynthesis the R group in Figure 2 is L-lactate and not Fo. Incubations of cell extracts of *M. thermophila* and *Mc. jannaschii* with Fo, 2-phospho-L-lactate, and GTP readily lead to the formation of F_{420}-0 (Table 1). Next we have to consider whether routes I or II in Figure 2 were involved in the subsequent reaction to form the activated intermediate that allows for the coupling of 2-phospho-L-lactate to Fo. The possible compounds are either LPPG or LPPA, if using route II, or the di- and triphosphates of lactate, if following route I.

Intermediates Involved in the Formation of F_{420}-0 from Fo and 2-Phospholactate. To establish if a nucleotide-containing intermediate such as LPPG or LPPPG was involved in formation of F_{420}-0, cell extracts of *Mc. jannaschii* were incubated with 2-phospho-L-lactate and GTP. Then any nucleotide-containing intermediate was removed by passing the samples through an activated carbon column. The resulting solutions have been tested for their ability to form F_{420}-0 when incubated with a *Mc. jannaschii* extract and Fo. These incubations repeatedly failed to show any production of F_{420}-0, indicating that if intermediates were formed, they were completely retained by the carbon as would be expected for nucleotide containing compounds. These results also demonstrated that no intermediates such as diphospholactate or triphospholactate were involved in the biosynthesis because they should have passed through the carbon column and thus allow F_{420}-0 formation without further addition of nucleotides.

To obtain additional information on the chemical nature of the intermediates involved in the formation of F_{420}-0, *Mc. jannaschii* extracts were incubated in separate experiments with the radioactively labeled substrates [U-^{14}C]L-lactate, [U-^{14}C]2-phospho-L-lactate and [8-^{3}H]GTP and separated on the Mono Q column. We identified the radioactively labeled anionic compounds, 2-phospholactate, cyclic-2-phospholactate, and F_{420}-0 in these experiments as well as found two unknown compounds 1 and 2. Since the two unknown compounds were found not to function as precursors for F_{420}-0 synthesis, it must be concluded that lactate and GTP are metabolized to additional compounds by *Mc. jannaschii* extracts which were not related to F_{420} biosynthesis.

Finally, incubations of extracts of *Mc. jannaschii* and *M. thermophila* with LPPG or LPPA and Fo resulted in F_{420}-0 formation. This result is of interest for two reasons: first, it is consistent with 2-phospholactate and not Fo-P as the precursor for F_{420}-0, and second, it proves that LPPG and LPPA are substrates for the enzyme that ligates Fo with 2-phospholactate. At least two steps are involved in the conversion of Fo and 2-phopholactate to F_{420}-0. The first step is the nucleotidyl transfer reaction, and the second is the ligation of Fo with the activated 2-phospholactate with release of a mononucleotide. This sequence of reactions resembles route II in Figure 2, analogous to the one found in DNA ligation reactions, in coenzyme B_{12} biosynthesis, and in phospholipid biosynthesis. In general, nucleotidyl transferases are rather specific enzymes concerning their nucleotide preference. In coenzyme B_{12} biosynthesis it is GTP, in phospholipid biosynthesis it is CTP, and in the DNA-ligase reactions it is ATP. The nucleotidyl transferase reaction in *Mc. jannaschii and M. thermophila* extracts was performed with GTP and ATP to about equal extents, whereas for the ligase reaction unequivocally LPPG was preferred by both extracts. The determination of the real nucleotide preference requires the availability of the isolated enzymes.

Chemical Stability of the Proposed F_{420}-0 Intermediates. The instability of phosphodiesters, containing a nucleophilic group like the carboxyl group in alpha position to the phosphodiester bond, is well-documented for their ability for decomposition. Well-studied examples of the hydrolysis of such compounds include the base-catalyzed hydrolysis of RNA, NDP sugar derivatives (*35*, *36*), FAD (*37*), coenzyme A (*38*), phosphate ester derivatives of phosphoenolpyruvate (*39*−*41*), and phenyl phosphate esters of phospholactate (*42*). In the case of FAD and RNA, the nucleophilic attack is committed by the 2-hydroxy group of the ribose, whereas in the case of 2-phospholactate and PEP phosphate esters the nucleophilic center is the carboxylate anion. The proposed F_{420}-0 intermediate in coenzyme F_{420} biosynthesis has both the alpha-hydroxyl group of the Fo moiety and the carboxylate anion from the lactate to serve as possible nucleophilic groups for its decomposition. However, since both RNA and

FAD are relatively stable at neutral pH we would not expect the involvement of the alpha-hydroxyl group of F_{420}-0 to be significant for its decomposition. The effect of the carboxyl group is of more concern since model compounds such as the phosphophenyl ester of 2-phospholactate are known to undergo hydrolysis 10^7 times faster than the phosphodiester diphenyl phosphate (*42*). In fact, as we could show, F_{420}-0 is a relatively stable compound in slightly basic solutions and can be heated for 30 min at 100 °C without significant degradation to Fo (Figure 4, reaction scheme c).

In contrast to F_{420}-0, the hydrolysis of the pyrophosphoester bond is expected to be more enhanced in LPPG and LPPA with GMP and AMP being very good leaving groups. To test this concern, we have prepared the methyl phosphate ester of 2-phospholactate and measured its stability and the products generated as a function of pH. As expected, the compound is found to be very unstable in dilute acid but to be quite stable in neutral or basic solutions. We have found both lactate and methyl phosphate as products of the hydrolysis, indicating that a cyclic intermediate is involved in the hydrolysis as shown in Figure 4. On the basis of this limited data, we conclude that the stability of the compound at neutral or basic pH originates from the simple electrostatic repulsion between the charged anionic carboxyl group and the charged anionic phosphate group. Under acidic conditions, the charge of the carboxyl group is removed and the cyclic pentavalent phosphate intermediate can be formed. This cyclic intermediate can decompose to either cyclic 2-phospholactate or to 1-methylphospholactate which hydrolyzes to methyl phosphate and lactate. This multiple mode of cleavage, for similar compounds, has been described earlier (*41*). We then examined the stability of LPPA and LPPG. Both compounds are quite unstable in water having a half-life of about 2 h at room temperature and only 10 min at 50 °C. LPPG and LPPA decompose into 2-phospholactate or cyclic 2-phospholactate and GMP or AMP. The mechanism of the decomposition is directly dependent on the nucleophilic attack of the carboxyl group of lactate, since the methyl esters of LPPG or LPPA are significantly more stable.

Involvement of Cyclic 2-Phospholactate in F_{420}-0 Biosynthesis. The repeated appearance of the formation of cyclic 2-phospholactate in the incubation mixtures required its consideration as a possible intermediate in the generation of the phosphodiester bond in F_{420}. Very few examples of structures containing a cyclic phosphoric acid-carboxylic anhydride have been described. For the most part, they have been proposed as intermediates in the hydrolysis of phosphate esters with an alpha carboxylate group. The literature appears to have only two examples which includes cyclic derivatives of PEP (*39*−*41*) and 2-phospholactate (*42*, *43*). There was no indication of its biochemical contribution for the phosphodiester bond formation from incubation experiments, since the cyclic 2-phospholactate led, only in the presence of GTP, to the generation of F_{420}-0. This can be explained by its hydrolysis to 2-phospholactate.

We propose that the cyclic 2-phospholactate produced in these incubations originates from the intramolecular chemical cyclization of the LPPG or LPPA intermediate as shown in Figure 4, reaction scheme b. As expected, the cyclic 2-phospholactate is unstable toward hydrolysis and is finally converted to 2-phospholactate which can then be recycled back into F_{420}-0 in the presence of GTP. Since the incorporation of cyclic 2-phospho-L-lactate containing fractions into F_{420}-0 always requires the addition of GTP or ATP to the incubation mixture we conclude that it represents only a side product of the LPPG metabolism.

Pathway for the Formation of F_{420}-0. In summary, this work describes the biosynthetic pathway for the formation of the phosphodiester bond in coenzyme F_{420}. From our study of the methanogenic extracts, we propose that F_{420}-0 is an intermediate in F_{420} biosynthesis. F_{420}-0 is then generated from Fo and L-lactate by three enzymes: The first reaction is the GTP-dependent phosphorylation of L-lactate to 2-phospho-L-lactate catalyzed by a lactate kinase. The next step is the nucleotide-dependent activation of 2-phospho-L-lactate to LPPG or LPPA. The nucleotide preference for this step is clearly defined in the cell extracts of *Mc. jannaschii* and *M. thermophila*. The last step is the coupling of the activated 2-phospho-L-lactate onto Fo under release of GMP or AMP. Thus formation and use of LPPG in the coupling of 2-phospho-L-lactate with Fo proceeds in an analogous manner to that found for B_{12} biosynthesis (*8*, *9*).

Further experiments to reveal the mechanism of the phosphodiester bond formation will primarily involve the identification of the enzymes/genes to support our findings.

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