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Methanococcus jannaschii Coenzyme F_{420} Analogs Contain a Terminal α-Linked Glutamate

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Analyses of the F_{420}s present in *Methanococcus jannaschii* have shown that these cells contain a series of γ-glutamyl-linked F_{420}s capped with a single, terminal α-linked L-glutamate. The predominant form of F_{420} was designated as α-F_{420}-3 and represented 86% of the F_{420}s in these cells. Analyses of *Methanosarcina thermophila*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, and *Mycobacterium smegmatis* showed that they contained only γ-glutamyl-linked F_{420}s.

Coenzyme F_{420} (Fig. 1) is a name given to a group of redox active cofactors that are presently known to have only a limited distribution among the archaea and high G+C gram-positive bacteria (3). Although playing a crucial role in methanoarchaeal metabolism (4), coenzyme F_{420} has also been found in various eubacteria such as *Streptomyces*, *Rhodococcus*, *Nocardioides*, and *Mycobacterium* spp. and their relatives (3). The coenzyme, in fact, was first isolated in 1960 as a cofactor involved in the biosynthesis of chlortetracycline in *Streptomyces aureofaciens* (18). The coenzyme is presently known to be involved in the biosynthesis of a number of secondary metabolites (20), the degradation of nitroaromatics (5), and activation of nitroimidazofurans (23). *Mycobacterium* and *Nocardia* spp. contain an F_{420}-dependent glucose-6-phosphate dehydrogenase (21, 22), and an F_{420} containing photolyase functions in DNA repair mechanisms in a number of microorganisms (7).

The first F_{420} whose structure was completely characterized was the γ-F_{420}-2 [*N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin] isolated from *Methanobacterium thermoautotrophicum* (γ-F_{420}-2 in Fig. 1) (6). This F_{420} contained two glutamic acids, with the terminal glutamate being bound through an amide bond to the γ position of the other glutamate, as is found in most folates and glutathione. This structure will be designated here as γ-F_{420}-2 to indicate the γ attachment of this terminal glutamate to the core structure of F_{420}-1 (*N*-L-lactyl-γ-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin) (Fig. 1). Subsequent work, however, has revealed that most organisms contain a series of γ-polyglutamated F_{420} cofactors with up to a total of seven γ-linked glutamates (1, 10–12, 17, 19).

The assembly of F_{420}-0 and its polyglutamate derivatives from 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (Fig. 1), pyruvate, and glutamate requires at least six steps (14, 15). 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 glutamic acid is then added to the lactyl carboxyl group of F_{420}-0 (Fig. 1) to generate the F_{420}-1, which is followed by the repeated addition of single glutamates to generate the polyglutamate derivatives.

As part of our work to establish the genes and pathway involved in F_{420} biosynthesis in *Methanococcus jannaschii* (13–15) we examined the F_{420} species present in this euryarchaeon. These analyses established that the F_{420}s present in these cells are unique and consist of a series of γ-linked F_{420}s capped with a single terminal α-linked L-glutamate.

A cell extract of *M. jannaschii* was prepared by sonication of 4.67 g of frozen cells suspended in 10 ml of TES extraction buffer {50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-NaOH–10 mM $MgCl_2$ [pH 7.5]} under Ar for 5 min at 3°C. The resulting mixture was centrifuged under Ar (10 min at 27,000 × *g*) and stored frozen at −20°C until used. The protein concentration of the *M. jannaschii* extract was 38 mg/ml. Protein concentrations were measured using the BCA total protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. Similar procedures were used to prepare cell extracts of *Methanosarcina thermophila*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum* ΔH, and *Archaeoglobus fulgidus*, having protein concentrations of 38, 6.8, 37, and 20 mg/ml, respectively. *Mycobacterium smegmatis* cell pellet (104 mg) was extracted by heating for 10 min at 100°C with 0.5 ml of water. After the addition of 0.6 ml of methanol and after centrifugation (14,000 × *g* for 5 min) to remove insoluble material, the resulting clear yellow extract was separated, evaporated, and dissolved in water prior to high-performance liquid chromatography (HPLC) analysis.

F_{420} present in the cell extracts produced by sonication was isolated by precipitation of the proteins from the cell extracts (50 μl) with the addition of 80 μl of methanol, followed by centrifugation (14,000 × *g* for 5 min). After separation from the pellet, the clear liquid was diluted to 1 ml with water and 20-μl portions were analyzed by HPLC. Analyses were performed on a Shimadzu SCL-6B HPLC using a C-18 reversed phase column (AXXI-Chrom octyldecyl silane column; 5-μm particle size; 4.6 mm [internal diameter] by 25 cm) eluted isocratically with 15% methanol in 25 mM sodium acetate (pH 6.5) buffer at a flow rate of 0.5 ml per min. The eluent was monitored by fluorescence (excitation wavelength, 420 nm;

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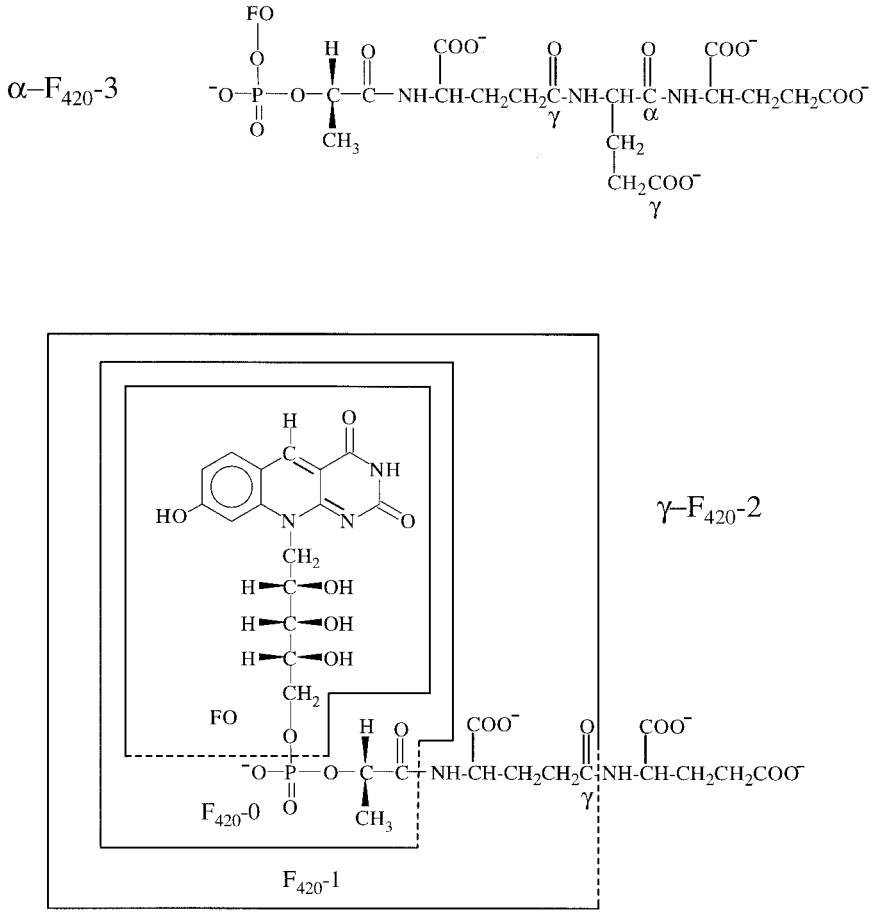

FIG. 1. The chemical structures of the F_{420} coenzyme analogs and their biosynthetic precursors. FO is defined in the text and is the fluorescent chromophore in F_{420}.

emission wavelength, 480 nm) and by absorbance at 280 nm. By use of this HPLC method, the different F_{420} derivatives showed the following retention times: γ-F_{420}-7, 4.88 min; γ-F_{420}-6, 5.04 min; γ-F_{420}-5, 5.84 min; γ-F_{420}-4, 5.99 min; α-F_{420}-5, 6.05 min; α-F_{420}-4, 6.51 min; γ-F_{420}-3, 6.95 min; α-F_{420}-3, 7.41 min; γ-F_{420}-2, 9.07 min; F_{420}-1, 13.0 min; F_{420}-0, 26.5 min; FO-P, 27.8 min; and FO, 30.4 min. The γ-F_{420}s were identified by coinjection with previously characterized γ-F_{420} samples from *Methanosarcina barkeri* and *Mycobacterium smegmatis* (1). Those peaks not corresponding with these known samples were the α-F_{420}s and were identified with regard to the number of glutamates by their elution positions relative to the known samples and by the observed difference between the elution times of F_{420}s that differed by one glutamate.

HPLC analysis of the F_{420}s from *M. jannaschii* showed that they consisted of four major fluorescent peaks (percent of total fluorescence): γ-F_{420}-2 (2.9%), α-F_{420}-3 (85.9%), α-F_{420}-4 (10.1%), and α-F_{420}-5 (1.5%) (Table 1). The measured total amount of F_{420} in this organism (>2.0 μmol/g [dry weight]) was among the highest reported for any organism (16). Except for the γ-F_{420}-2 peak, each peak had chromatographic retention times different from that of the γ-linked F_{420} present in any of the other archaea tested, which included *Methanosarcina thermophila*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, and *Mycobacterium smegmatis*. These organisms were found to have the distributions of γ-F_{420} (percent of total fluorescence) shown in Table 1. No F_{420}-1 was detected in any sample.

To establish the nature of the linkages in the samples of F_{420}, they were incubated with peptidases of known specificity and the products were measured by HPLC. The enzymes used

TABLE 1. Distribution and types of F_{420} in the assayed cells

F_{420}	Distribution of F_{420} type (% of total F_{420}s) in:				
	Methanococcus jannaschii	*Methanosarcina thermophila*	*Methanosarcina barkeri*	*Archaeoglobus fulgidus*	*Mycobacterium smegmatis*
γ-F_{420}-2	2.9	2.4	31	0.3	Trace
γ-F_{420}-3		19	6.8	0.5	
α-F_{420}-3	85.9				
γ-F_{420}-4		62	32	89[a]	
α-F_{420}-4	10.1				
γ-F_{420}-5		16	25		67
α-F_{420}-5	1.5				
γ-F_{420}-6		1.0	4.0	10[b]	31
γ-F_{420}-7		Trace	0.7		7.2

[a] The sum of γ-F_{420}-4 and -5 was 89%.
[b] The sum of γ-F_{420}-6 and -7 was 10%.

were carboxypeptidase Y (a peptidyl-L-amino acid hydrolase that specifically removes carboxyl-terminal α-amino acids) and glutamyltranspeptidase and carboxypeptidase G (both γ-glutamyl hydrolases that specifically cleave terminal γ-linked glutamyl peptide bonds). Thus, treatment of 2 μl of *M. jannaschii* cell extract with 10 μl of a solution of carboxypeptidase Y from baker's yeast (Sigma) (4.35 U in 50 mM TES-Na^+–10 mM $MgCl_2$ [pH 7.5]) for 1 h at room temperature converted the sample to a mixture of γ-F_{420}-2, γ-F_{420}-3, and γ-F_{420}-4 based on their HPLC retention times. The abundances of F_{420}s in the resulting mixture matched the ratios of the starting F_{420}s. Treatment of the extracts containing the F_{420} analogs from the other organisms in the same manner had no effect on their HPLC profiles. Treatment of all of the cell extracts with glutamyltranspeptidase type IV from porcine kidney (Sigma) as previously described (15) had no effect on the *M. jannaschii* α-F_{420}s but degraded all of the other γ-F_{420}s, including the γ-F_{420}-2 present in *M. jannaschii*, to F_{420}-1. Treatment of 2 μl of *M. jannaschii* cell extract with 10 μl of carboxypeptidase G from *Pseudomonas* spp. (Sigma) (50 U per ml of 50 mM TES-Na^+–10 mM $MgCl_2$ [pH 7.5]) for 2 h at room temperature had no effect on the major *M. jannaschii* α-F_{420}s but did hydrolyze the γ-F_{420}-2 present in *M. jannaschii* to F_{420}-1. A similar treatment of the F_{420} samples from the other organisms degraded all of them to F_{420}-0.

For the purification of the F_{420} analogs from *M. jannaschii*, cell extracts were separated on a MonoQ HR 5/5 column on a BioLogic HR chromatographic system (Bio-Rad) using a linear sodium chloride gradient. Buffer A was 25 mM Tris–HCl [pH 7.5] buffer, and buffer B was the same but containing 1 M NaCl. The total flow was 0.5 ml per min for 40 min. In this system, the F_{420} eluted at 0.6 M NaCl and was identified by its fluorescence intensity (excitation, 420 nm; emission, 480 nm). The F_{420}-containing fractions were combined and concentrated and applied to a C-18 column (0.5 by 10 cm; 55–105 μm; Waters) equilibrated with water. The F_{420} was eluted with 10% methanol in water. The F_{420}-containing fractions were combined and concentrated, formic acid was added to a concentration of 1%, and the sample was applied to a C-18 column (0.5 by 10 cm; 55–105 μm; Waters) equilibrated with 1% formic acid. The F_{420}, which was tightly bound to the column, was then eluted with a step gradient of 1% formic acid–methanol. The F_{420} was eluted around 50% methanol. The F_{420}, which has no color at this pH, was observed via its fluorescence under a UV light. The final material was then purified by preparative thin-layer chromatography using the solvent system acetonitrile-water-formic acid (80:20:10 [vol/vol/vol]) with a R_f of 0.22. This material was subjected to acid hydrolysis (6 M HCl; 110°C; 12 h), and the resulting amino acids were converted into their *N*-trifluoroacetyl methyl ester derivatives for gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analysis as previously described (25) showed only the presence of the glutamate derivative. GC-MS analysis of the sample using an Alpha Dex 120 fused chiral silica capillary column (30 m by 0.25 mm by 0.25-μm film thickness; Supelco, Bellefonte, Pa.) showed that it was composed of only L-glutamic acid.

In total, these data are consistent with the conclusion that the F_{420}s in *M. jannaschii*, consisting of γ-F_{420}-2, γ-F_{420}-3, and γ-F_{420}-4, each have a single α-linked terminal glutamate, as shown in Fig. 1. These structures will be referred to as α-F_{420}. The cells also contain a small amount of γ-F_{420}-2, which could serve as the biosynthetic precursor to the other structures.

Although no example of α-linked glutamates in F_{420} has been described in any organism, the occurrence of α-linked glutamates in folates in *E. coli* (8) and an α-glutamylmethanopterin, sarcinapterin, in *Methanosarcina* and some *Methanococcus* species has been described (24). In the case of the folates, it has been established that a bifunctional dihydrofolate synthetase-folylpolyglutamate synthetase (FolC) adds the first glutamate to pteroic acid and then up to three additional γ-linked glutamates. A second enzyme then extends the folylpolyglutamate chain via the addition of α-linked glutamates (9). The enzyme adding these α-linked glutamates was purified and shown to differ from FolC, but the gene for its generation was not identified. It is very likely that the same sequence of events is occurring in *M. jannaschii*. In *M. jannaschii*, there could be one enzyme, which would add the glutamate to F_{420}-0 to generate the series of γ-linked glutamate F_{420} analogs. This process would be analogous to that with the bifunctional FolC. The resulting γ-linked glutamate F_{420} analogs would then serve as substrates for the addition of the α-linked glutamate. In each case, the addition of the glutamates would likely proceed via acylphosphate intermediates, generated from a nucleotide triphosphate, as is known to occur with FolC (2). The major difference however, between the biosynthesis of the α-linked glutamates in folates and the *M. jannaschii* F_{420}s is that only one α-linked glutamate would be added.

Since *M. jannaschii* contains α-linked glutamate F_{420} analogs, many enzymes in these cells which are known to use the γ-linked glutamate F_{420} analogs in other bacteria and archaea must have adapted in *M. jannaschii* to use the α-linked analogs. This list of enzymes for *M. jannaschii* in which F_{420} is essential would include hydrogenase, formate dehydrogenase, methylene-tetrahydromethanopterin dehydrogenase, alcohol dehydrogenase, methylene-tetrahydromethanopterin reductase, and F_{420}-dependent $NADP^+$ oxidoreductase. Questions still to be answered are how and why this specific change in coenzyme structure specifically occurred in *M. jannaschii* and what enzymes are involved.

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