Factor 390 Chromophores: Phosphodiester between AMP or GMP and Methanogen Factor 420†

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ABSTRACT: Two chromophores with absorbance maxima at 390 nm (factors 390) have been isolated from oxidized cells of *Methanobacterium thermoautotrophicum* ΔH. The isolation procedure included anion-exchange chromatography of the soluble cofactor pool followed by reverse-phase chromatography. The factor 390 species are novel derivatives of methanogen coenzyme factor 420 in which the 5-deazaflavin 8-hydroxy group is in a phosphodiester linkage to adenosine 5′-phosphate or guanosine 5′-phosphate. The structural assignments were based, in part, on the UV–visible and ^{1}H NMR spectra. In addition, the results from amino acid analysis, phosphate determination, ^{31}P NMR spectroscopy, and fast atom bombardment mass spectrometry were consistent with the proposed structures. Confirmation of the factor 390 structures was made following phosphodiesterase release of the nucleotide monophosphates from factor 420. The nucleotide monophosphates were identified as AMP and GMP by UV–visible spectra and based on elution position by using reverse-phase and anion-exchange high-performance liquid chromatography. The presence of AMP was further demonstrated by using adenylate-5′-phosphate kinase which induced a spectral shift during conversion of the sample to IMP. In addition, the presence of GMP was established by a specific enzymatic assay.

Methanogenic bacteria possess a large repertoire of novel coenzymes. Many of these compounds function in the energy-yielding pathways leading to methane production. Three unique, one-carbon carriers have been characterized and termed methano*furan* (Leigh et al., 1984), methano*pterin* (van Beelen et al., 1984), and coenzyme M (thioethanesulfonate) (Taylor & Wolfe, 1974). Methyl coenzyme M reductase, which catalyzes the last step in methane formation, contains the nickel tetrapyrrole coenzyme F_{430} (Ellefson et al., 1982; Pfaltz et al., 1982; Hausinger et al., 1984). This reaction also requires component B (Gunsalus & Wolfe, 1980), a coenzyme with, as yet, undefined role or structure. Coenzyme F_{420} (F_{420}),[1] a 5-deazaflavin derivative (Eirich et al., 1978; Jacobson & Walsh, 1984), serves as a low potential, cellular, two-electron redox carrier. Lastly, a cyclic glycerodiphosphate compound (Kanodia & Roberts, 1983; Seeley & Fahrney, 1983) serves an, as yet, unidentified role.

This paper extends our knowledge of the methanogen coenzyme ensemble to include two related compounds with absorbance maxima at 390 nm. These compounds, referred to here as F_{390}-A and F_{390}-G, can be isolated from cells that have been exposed to oxygen but are absent from anaerobic cells. Oxidative conditions lead to F_{420} disappearance in the cells (Schonheit et al., 1981) concomitant with F_{390} formation. Here, the two forms of F_{390} are demonstrated to be derivatives of F_{420} in which adenosine 5′-phosphate (F_{390}-A) and guanosine 5′-phosphate (F_{390}-G) are linked to the flavin via the F_{420} 8-hydroxy position. Substitution of the 8-hydroxyl group has not previously been observed in the biological chemistry of F_{420}. Thus, these results may suggest a potential new role for the 5-deazaflavins in methanogen biochemistry.

Experimental Procedures

Materials. DEAE-Sephadex was purchased from Pharmacia. HPLC-grade methanol and water were obtained from MCB reagents. Snake venom phosphodiesterase was from Worthington. Alkaline phosphatase, 5′-adenylic acid deaminase, guanylate-5′-phosphate kinase, pyruvate kinase, and lactate dehydrogenase were from Sigma. The 99.8% D_2O was from Aldrich.

Preparation of Crude Extract. Methanobacterium thermoautotrophicum ΔH was grown at 62 °C in low iron medium (10 μM) as described by Schonheit et al. (1981) using a 25-L New Brunswick fermenter. After an OD_{660} of 1 was reached, the cells were cooled to 30 °C and concentrated by using a Millipore Pellicon membrane filtration apparatus. The concentrated cells were pelleted at 10000*g* for 10 min, and the cell paste (60 g wet weight) was suspended in 300 mL of water. After incubation at 62 °C in an unstoppered bottle with stirring for 2 h, the cell suspension was passed twice through a French pressure cell at 18000 psi and centrifuged at 100000*g* for 60 min at 4 °C to yield oxidized crude extract.

Isolation of F_{390}-A and F_{390}-G. The protein fraction of oxidized crude extract was separated from the soluble cofactors by using Amicon pressure filtration with a PM10 membrane. The cofactor pool was chromatographed at 4 °C on a column (2.5 × 45 cm) of DEAE-Sephadex equilibrated in 25 mM Tris-HCl, pH 8.0, buffer. An 800-mL linear KCl gradient to 1 M KCl was used to develop the column, yielding an F_{390} pool at a conductivity of 35 mΩ^{-1}. Two F_{390} species were isolated by preparative reverse-phase chromatography using a 10-μm C-18 column (Alltech, 10 × 250 mm, equilibrated in 50 mM ammonium formate, pH 7.0, 2.0 mL/min). A 20-min linear gradient from 0.0% to 20% CH_3OH separated the F_{390} species. Rechromatography using a 40-min linear

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[1] Abbreviations: F_{390}-A and F_{390}-G, novel 5-deazaflavin derivatives of methanogenic bacteria; F_{420}, coenzyme F_{420}; F_0, riboflavin level coenzyme F_{420}; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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gradient from 5% to 20% CH_3OH yielded homogeneous F_{390}-A and F_{390}-G.

Phosphodiesterase Treatment of F_{390}'s. F_{390}-A and F_{390}-G were digested at 25 °C with snake venom phosphodiesterase (10 μg/mL). The extent of reaction was followed by monitoring changes at 400 nm. The reaction products were isolated by preparative reverse-phase HPLC as described above, except that elution conditions employed a 30-min linear gradient from 0.0% to 30% methanol.

Nucleotide Identification. Nucleotides were identified by their elution positions on reverse-phase (see above) and ion-exchange HPLC and comparison to authentic standards. A partisil 10 SAX column (Whatman, 4.6 × 250 mm, equilibrated in 50 mM phoshate buffer, pH 3.35, 0.67 mL/min) was used with detection at 254 nm for anion-exchange HPLC.

The presence of GMP was confirmed by the spectrophotometric method of Grassl (1974). The oxidation of NADH was followed in a coupled assay system involving guanosine-5′-phosphate kinase, pyruvate kinase, and lactate dehydrogenase.

Samples were tested for the presence of AMP with AMP deaminase (5 μg/mL) at 25 °C. The reaction was monitored at 265 nm, and the samples were scanned before and after digestion. Characteristic spectral changes in the AMP to IMP conversion demonstrated the presence of AMP.

Analytical Methods. UV–vis spectroscopy was performed by using a Perkin-Elmer Lambda 3 spectrophotometer equipped with a Perkin-Elmer 3600 data station. Fluorescence spectra were obtained on a Perkin-Elmer LS-3 fluorescence spectrophotometer. Bruker 250- and 270-MHz spectrometers were used to obtain pulse Fourier-transform ^1H and ^{31}P (109.3 MHz) NMR spectra. Organic phosphate determinations were performed by the methods of Ames (1966). Amino acid analysis was performed by using a Dionex D-500 instrument after a 24-h sample hydrolysis in 6 N HCl. The Du Pont Instruments HPLC used in this work was equipped with a series 8800 gradient controller, an 850 absorbance detector, and a Micromeritics 786 variable wavelength detector. Fast atom bombardment mass spectrometry was performed by using a Finnigan MAT 731 mass spectrometer equipped with an Ion Tech atom gun.

RESULTS

Isolation of F_{390}-A and F_{390}-G. Aerobic incubation of *M. thermoautotrophicum* ΔH at 62 °C led to a dramatic change in cell color from greenish brown to pink. Disrupted cells were centrifuged to remove the membrane fraction from the dark red-brown supernatant solution. Amicon filtration was used to separate the dark-red protein fraction from a yellow pool containing soluble cofactors.

As shown in Figure 1, DEAE-Sephadex chromatography of the cofactor pool separated three fractions having absorbance at 390 nm. Fraction A absorbs maximally at 430 nm and contains partially purified nickel tetrapyrrole F_{430} and its thermal degradation products (Hausinger et al., 1984; Diekert et al., 1981). Fraction C has maximal absorbance at 350 nm and was not further characterized. Fraction B possesses two, partially purified, F_{390} species. No coenzyme F_{420} was observed.

The two forms of F_{390} were separated from each other and from multiple contaminants by using HPLC as shown in Figure 2. The only other peak with significant absorbance at 390 nm absorbed maximally at 430 nm. The ratio of the two F_{390} peaks was variable in different preparations, but the F_{390} peak eluting first from the column (F_{390}-G) was consistently smaller than the second form (F_{390}-A). Rechromatography using different conditions was used to complete the purification. From 60 g of cells (wet weight) were obtained 0.5 μmol of F_{390}-G and 1.9 μmol of F_{390}-A.

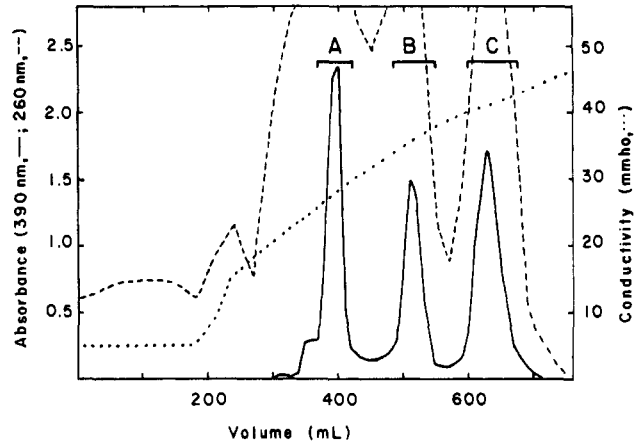


FIGURE 1: DEAE-Sephadex chromatography of soluble cofactors. The protein-free cofactor pool from oxidized *Methanobacterium thermoautotrophicum* was chromatographed on a column of DEAE-Sephadex by using conditions described in the text. Absorbance was monitered at 390 (—) and 260 nm (---).

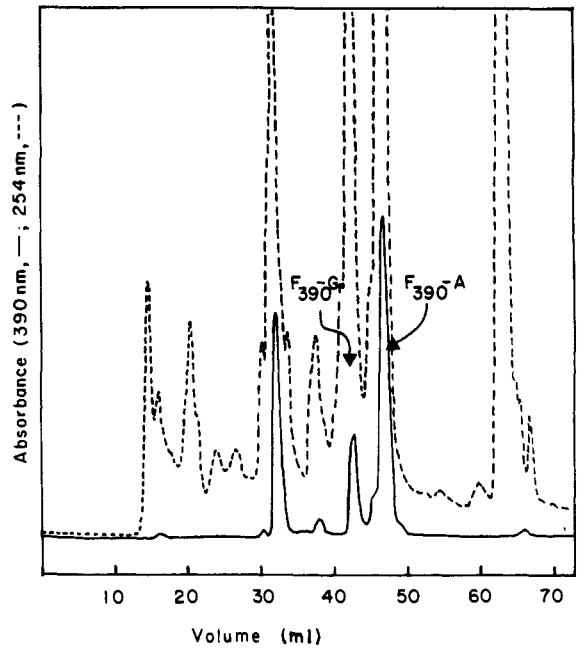


FIGURE 2: Reverse-phase HPLC purification of F_{390}'s. Pool B from Figure 1 was chromatographed by using preparative reverse-phase HPLC as described in the text. The absorbance was monitered at 390 (—) and 254 nm (---).

UV–Visible Spectroscopy. The spectrum of each F_{390} is shown in Figure 3. The spectra of the two species are very similar, but differences are apparent in the region of 255–270 nm. The general characteristics of the two spectra are very similar to the spectrum of 5-deazariboflavin (7,8-dimethyl) (O'Brien et al., 1970), also shown in Figure 3 for comparison. Chemical reduction using $NaBH_4$ led to a reduced F_{390} species with an absorbance maximum at 325 nm, analogous to the reduced 5-deazariboflavin spectrum (Spencer et al., 1976). Further, like other 5-deazaflavins, the reduced molecule was very stable to oxygen and required several days to fully oxidize (Hersh & Walsh, 1980). Little change in the absorbance spectrum was observed over a range of pH from 3.5 to 11.

Fluorescence Spectroscopy. The F_{390} species exhibited very little fluorescence at neutral pH; however, intense fluorescence was observed as the pH was decreased. The uncorrected

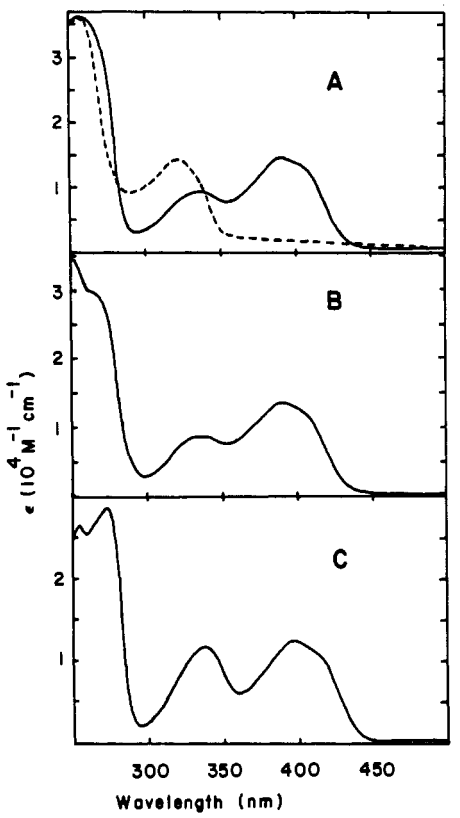


FIGURE 3: UV–visible spectra of F_{390}-A, F_{390}-G, and 5-deazariboflavin. Samples of (A) F_{390}-A as isolated (—) and after $NaBH_4$ reduction (---), (B) F_{390}-G, and (C) 5-deazariboflavin were scanned at pH 8.0. The extinction coefficients for F_{390}-A and F_{390}-G were determined by using phosphodiesterase treatment as described in the text.

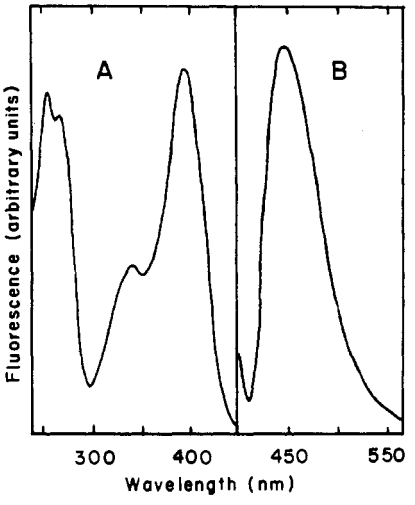


FIGURE 4: Fluorescence spectrum of F_{390}. The uncorrected F_{390} fluorescence spectrum was obtained at pH 3.5. (A) Excitation spectrum of F_{390} while monitoring emission at 460 nm. (B) Emission spectrum of F_{390} using excitation at 390 nm.

fluorescent spectrum for F_{390}-A at pH 3.5 is shown in Figure 4. The excitation spectrum closely matches the absorbance spectrum for the chromophore. The major emission peak at 450 nm is similar to the emission spectrum of coenzyme F_{420} at this pH (Eirich et al., 1979).

Amino Acid Analysis. Coenzyme F_{420} is reported to have two glutamic acids as isolated from *Methanobacterium bryantii* (Eirich et al., 1978) and four to five glutamic acids as obtained from *Methanosarcina barkeri* (van Beelen et al., 1983). The F_{390}-A, F_{390}-G, and F_{420} 5-deazaflavins from *M. thermoautotrophicum* were found to have 2.1, 2.1, and 2.2 glutamic acids by using an extinction coefficient of 14 mM^{-1} at 390 nm for the F_{390}'s (see below) and 25 mM^{-1} at 400 nm for F_{420}. The only other amino acid observed was substoichiometric glycine.

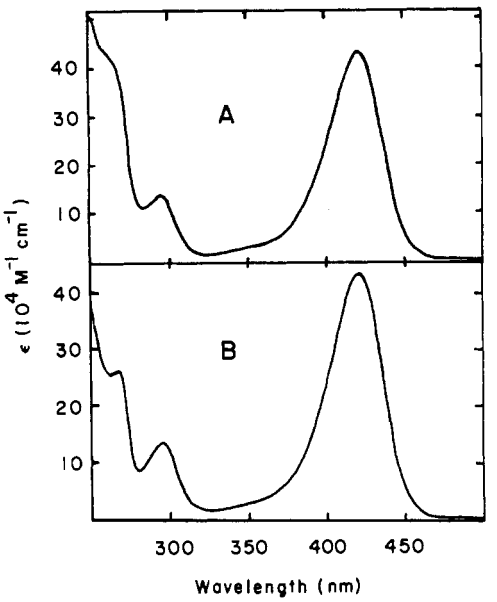


FIGURE 5: UV–visible spectra of phosphodiesterase-digested F_{390}-A and of F_{420}. Spectra were obtained at pH 8.0 for (A) F_{390}-A following phosphodiesterase digestion and for (B) coenzyme F_{420}.

Phosphate Assays. One phosphate (as a phosphodiester) is present in the deazaflavin side chain of the F_{420} molecule (Eirich et al., 1978). In contrast, 1.88 and 1.98 phosphates were present in F_{390}-A and F_{390}-G by phosphate analysis after conversion of organophosphorus species to inorganic phosphate. These data suggest the presence of an additional phosphorylated substituent on the 5-deazaflavin moiety of the F_{390}'s.

^1H NMR. All F_{420} proton resonances were observed in the NMR spectra for F_{390}-A and F_{390}-G. The F_{390} spectra were taken at neutral pH, yet the aromatic protons were shifted as for the spectrum of F_{420} taken below the pK_a (5.8) of the 8-hydroxy group (Eirich et al., 1978). These results are consistent with substitution of the 8-hydroxyl group.

In addition to the F_{420} protons, other resonances were observed in both F_{390} species. The F_{390}-G spectrum included a singlet at 7.12 ppm and a doublet at 5.67 ppm. The F_{390}-A spectrum included aromtic singlets at 7.88 and 7.47 ppm, as well as a doublet at 5.89 ppm. Other resonances, if present, were obscured by the F_{420} ribityl and methylene proton resonances.

The downfield resonances are very similar in position to the proton spectra for GMP and AMP (Pouchert & Campbell, 1974). A singlet at 8.1 ppm and a doublet at 5.85 ppm are observed for the GMP spectrum. The AMP protons resonate as two singlets at 8.5 and 8.0 ppm, as well as a doublet at 6.1 ppm. Upfield shifts due to interaction with the F_{420} ring system could yield spectra similar to those observed for F_{390}-G and F_{390}-A.

^{31}P NMR. Resonances at −0.3 and −5.8 ppm were present in the ^{31}P NMR spectra of F_{390}-A and F_{390}-G. The broad resonances were of equal intensity and do not demonstrate ^{31}P–^{31}P splitting. These results are consistent with two phosphates in each F_{390} as found by assay. In comparison, the single F_{420} phosphodiester phosphate resonated at +0.13 ppm vs. H_3PO_4.

Phosphodiesterase Treatment of F_{390}'s. Digestion of F_{390}-A and F_{390}-G with phosphodiesterase resulted in development of yellow color and F_{420}-like fluorescence in both samples. As shown in Figure 5, the UV–visible spectrum of phosphodi-

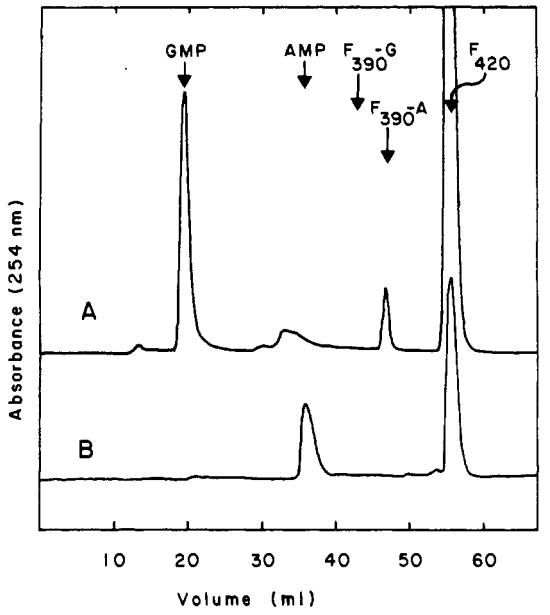


FIGURE 6: Reverse-phase HPLC of phosphodiesterase-treated F_{390}'s. Samples were digested with phosphodiesterase and chromatographed by HPLC using conditions described in the text: (A) digestion products of F_{390}-G and (B) phosphodiesterase-digested F_{390}-A. Also shown for comparison are the elution positions for nondigested F_{390}-A and F_{390}-G and authentic standards of GMP, AMP, and F_{420}.

esterase-treated F_{390}-A closely resembled the F_{420} absorbance spectrum (Eirich et al., 1978) except in the 250–280-nm region. A similar absorbance peak shift from 390 to 420 nm was obtained for F_{390}-G. For both samples the final spectra differ from the F_{420} spectrum due to the presence of an additional aromatic chromophore in each sample (see below).

By use of the known extinction coefficient for F_{420} (420 nm, 43 nM^{-1}; 400 nm, 25 mM^{-1}) (Ashton et al., 1979), the 390-nm extinction coefficients were then calculated for F_{390}-A (14.3 mM^{-1}) and F_{390}-G (13.6 mM^{-1}). These values compare well with the value for 5-deazariboflavin (7,8-dimethyl) (12.5 mM^{-1}) at its absorbance maximum of 397 nm (Walsh et al., 1978).

The phosphodiesterase-digested F_{390} samples were chromatographed by using preparative, reverse-phase HPLC as shown in Figure 6. Two chromophores absorbing at 254 nm were present in each sample. The second chromophore to elute from the column was, in both cases, identical with F_{420} on the basis of UV–visible absorbance spectra, fluorescence spectra, and HPLC elution position. The first chromophore to elute from the column differed for the two samples. The F_{390}-G digestion product absorbed maximally at 253 nm with a shoulder at 275 nm. This spectrum is that of GMP. In contrast, the digestion product for F_{390}-A possessed a single absorbance peak at 259 nm, identical with the spectrum of AMP. The reverse-phase elution positions for the GMP- and AMP-like species matched the elution positions for authentic AMP and GMP standards.

No changes in HPLC elution position were observed when the intact F_{390} samples were treated with alkaline phosphatase.

Nucleotide Identification. The proton NMR evidence from intact F_{390}'s and the UV–visible spectra of the phosphodiesterase cleavage products were consistent with the presence of adenosine 5′-phosphate and guanosine 5′-phosphate components. These assignments were confirmed by anion-exchange HPLC of the phosphodiesterase products using conditions that separate all nucleotide 5′-monophosphates. The digestion products from F_{390}-A and F_{390}-G coeluted with authentic AMP and GMP, respectively.

Further confirmation for the presence of AMP and GMP used enzymatic methods. The putative AMP species was a substrate for adenylate-5′-phosphate deaminase as shown by the characteristic spectral shift from 258 to 250 nm as IMP was produced. The presence of stoichiometric GMP was established by using the very specific enzyme (Grassl, 1974) guanylate-5′-phosphate kinase in a coupled assay with pyruvate kinase and lactate dehydrogenase.

Mass Spectrometry. Fast atom bombardment mass spectrometry in the negative ion mode was used to analyze the 5-deazaflavin species. Coenzyme F_{420} gave rise to molecular anions consistent with the known structure (Eirich et al., 1978); i.e., species observed include the molecular monoanion with three associated protons (m/z 772), with one sodium cation and two protons (m/z 794), and with the potassium cation and two protons (m/z 810).

The sample of F_{390}-A yielded a molecular anion at m/z 1101 and the sodium- and potassium-containing anions at m/z 1123 and 1139, respectively. In addition, fragment ions were observed consistent with loss of a lactyldiglutamyl group (m/z 771) and loss of a ribityl phosphate and lactyldiglutamate (m/z 557).

The F_{390}-G sample yielded a molecular anion at m/z 1117 with the sodium- and potassium-containing anions at m/z 1139 and 1155. The same fragmentation as above was observed, which resulted in species with m/e 787 and 573.

Discussion

F_{390} was initially isolated as a chromophoric species that we observed copurifying with methyl coenzyme M reductase. Enzyme purification to homogeneity (Hausinger et al., 1984) removed all F_{390}, and F_{390} plays no obvious role in methyl coenzyme M reductase function. Nonetheless, we pursued the structural identification of this chromophore from methanogen cells.

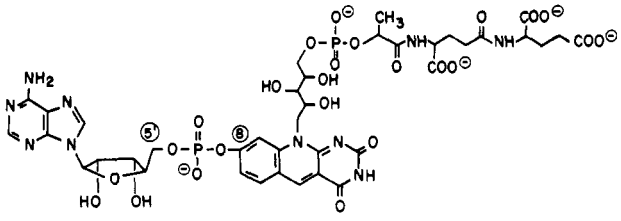
UV–visible and fluorescence spectra suggested that F_{390} was a novel 5-deazaflavin species. This compound was only present in extracts of *Methanobacterium thermoautotrophicum* cells that had not been carefully protected from oxygen. Under these conditions, methanogen cells are known to lose their F_{420} by apparent oxidative degradation of unknown mechanism (Schonheit et al., 1981). Conversion of F_{420} to an F_{390}-like species has been suggested (Keweloh, 1980), but the identity of the F_{420} "degradation" product was not elucidated. A second form of F_{390} has now been detected. Because of our interest in the biological chemistry of 5-deazaflavins (Jacobson & Walsh, 1984; Fisher et al., 1976), the identities and properties of these novel compounds were determined.

We propose that F_{390}-A and F_{390}-G are the adenosine 5′-phosphate and guanosine 5′-phosphate derivatives of F_{420} in which the nucleotides are in phosphodiester linkage to the 5-deazaflavin 8-hydroxyl functionality.

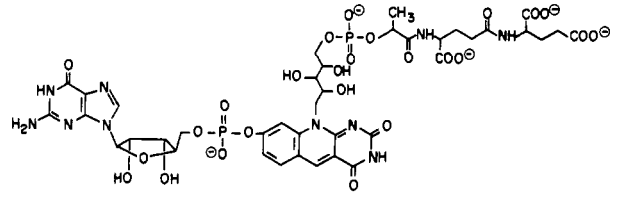
The presence of intact F_{420} within the F_{390} structures is demonstrated by (i) the presence of two glutamic acids by amino acid analysis, (ii) the presence of all F_{420} proton resonances in the ^1H NMR spectra, and (iii) the release of authentic F_{420} by phosphodiesterase treatment as shown by UV–visible spectroscopy, fluorescence spectroscopy, and cochromatography on HPLC.

Evidence for adenosine 5′-phosphate and guanosine 5′-phosphate as substructures of F_{390}-A and F_{390}-G released by phosphodiesterase action includes the (i) ^1H NMR spectra, (ii) UV–visible spectra, (iii) reverse-phase and anion-exchange HPLC elution position, (iv) conversion of AMP to IMP by adenylate 5′-deaminase, and (v) stoichiometric GMP by assay.

The F_{420} and nucleotide monophosphates are linked by a



F_{390}-A



F_{390}-G

phosphodiester bond with the deazaflavin 8-hydroxyl group. Substitution of the 8-hydroxy position prevents the paraquinoid structure observed in the F_{420} anion above its pK_a (5.8). Thus, the long wavelength absorbance maximum of F_{420} is replaced by a spectrum similar to that of 5-deazariboflavin (397 nm maximum) and 8-methoxy-5-deazariboflavin (393 nm maximum) (Yamazaki et al., 1982). Fluorescence quenching at pH 7 may be due to stacking between the purine and deazaflavin or to solvation of the 8-hydroxy phosphodiester anion.

Further support for the proposed structures includes the results from phosphate analyses and ^{31}P NMR spectroscopy which demonstrate the presence of two phosphodiesters in the F_{390} molecules. Finally, the mass spectrometry results are consistent with the structures shown (possessing proton counterions).

M. thermoautotrophicum is a strict anaerobe tolerating only brief exposure to air. Yet, the two novel 5-deazaflavins characterized above were only obtained in quantity from cells exposed to oxygen—conditions which result in a complete loss of F_{420} in cells [observed here and by Schonheit et al. (1981)]. The significance of F_{390} formation under these conditions is not known. If the F_{390} species are present at low levels in anaerobic cells, a role in metabolic control can be envisaged. Alternatively, the F_{390} species may be formed only during oxidative stress and may act as alarmones (Lee et al., 1983). Other oxygen protective mechanisms are known to exist in methanogens such as superoxide dismutase purified from *Methanobacterium bryantii* (Kirby et al., 1981). It remains to be determined whether F_{390} is ever present under normal anaerobic growth conditions and what role F_{390} may play.

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