Changes in Concentrations of Coenzyme F$_{420}$ Analogs during Batch Growth of *Methanosarcina barkeri* and *Methanosarcina mazei*

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Coenzyme F$_{420}$ has been assayed by high-performance liquid chromatography with fluorimetric detection; this permits quantification of individual coenzyme F$_{420}$ analogs whilst avoiding the inclusion of interfering material. The total intracellular coenzyme F$_{420}$ content of *Methanosarcina barkeri* MS cultivated on methanol and on H$_2$-CO$_2$, and of *Methanosarcina mazei* S-6 cultured on methanol remained relatively constant during batch growth. The most abundant analogs in *M. barkeri* were coenzymes F$_{420-2}$ and F$_{420-4}$, whilst in *M. mazei* coenzymes F$_{420-2}$ and F$_{420-3}$ predominated. Significant changes in the relative proportions of the coenzyme F$_{420}$ analogs were noted during batch growth, with coenzymes F$_{420-2}$ and F$_{420-4}$ showing opposite responses to each other and the same being also true for coenzymes F$_{420-3}$ and F$_{420-5}$. This suggests that an enzyme responsible for transferring pairs of glutamic acid residues may be active. The degradation fragment FO was also detected in cells in late exponential and stationary phase. Coenzyme F$_{420}$ analogs were present in the culture supernatant of both methanogens, in similar proportions to that in the cells, except for FO which was principally located in the supernatant.

The final step in the multistage anaerobic digestion process involves methane formation from suitable substrates by methanogenic bacteria. The stability of the entire digestion process is highly dependent upon the activity of the methanogens; therefore, an effective control procedure requires a reliable quantification of the methanogenic biomass. Due to problems encountered with direct counts, indirect assays involving serological techniques (13, 17), the analysis of polar lipids (23), and assay of coenzyme F$_{420}$ (13, 28, 34) have been developed.

The molecular structure of coenzyme F$_{420}$ (a 7.8-didemethyl-8-hydroxy-5-deazaflavin) from * Methanobacterium bryantii* was established by Eirich et al. (9) and had two glutamic acid residues in the side chain (Fig. 1). Three coenzyme F$_{420}$ analogs, identical except for apparently containing three, four, and five glutamic acid residues, respectively, in the side chain (14), and a degradation fragment FO (9) have also been described (Fig. 1). Coenzyme F$_{420}$ has been detected in all methanogenic bacteria examined (10, 14) and is virtually unique to the methanogens, having been detected at trace amounts in only a few other organisms (e.g., *Streptomyces griseus* [12], actinomycyes and related bacteria [6], and other archaebacteria [11, 21, 30]). In its oxidized state, coenzyme F$_{420}$ exhibits a specific blue-green fluorescence which has been used for the identification of methanogenic bacteria by fluorescence microscopy (8, 24) and permits the selective determination of coenzyme F$_{420}$ in cell extracts. Coenzyme F$_{420}$ principally acts as an electron carrier in metabolism (2, 16), although secondary roles have also been proposed (19, 20). Initial methods of coenzyme F$_{420}$ quantification involved a simple extraction with direct fluorescence measurement. However, an alternative procedure involves coenzyme F$_{420}$ extraction and analysis by high-performance liquid chromatography (HPLC) with fluorimetric detection (for examples, see references 13, 25, 26, and 33). Advantages of this procedure are that interfering fluorescent material is not included (25) and that the four coenzyme F$_{420}$ analogs and FO (Fig. 1) may be quantified individually. The independent quantification of the coenzyme F$_{420}$ analogs may permit some identification of the methanogens present (14, 26). One factor influencing the applicability of the coenzyme F$_{420}$ assay is variation in the total and individual coenzyme F$_{420}$ content of methanogenic species. This study has examined the relationship between the individual and total coenzyme F$_{420}$ content of methanogenic cells and the culture supernatant, and cell protein and the methane production rate during growth of *Methanosarcina barkeri* MS and *Methanosarcina mazei* S-6 in batch culture. These two methanogens were studied as they contain all four coenzyme F$_{420}$ analogs described (14, 26).

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MATERIALS AND METHODS

Culture procedure. *M. barkeri* MS (DSM 800) and *M. mazei* S-6 (DSM 2053) were maintained on the energy source used for experimentation, and the contents of a single Hungate tube (4.5 ml) were used to inoculate 400-ml cultures of modified MET3 prepared in 1-liter Duran bottles. Modified MET3 contained CaCl$_2$·6H$_2$O at 0.01 g liter$^{-1}$ instead of 2 g liter$^{-1}$ previously (7). Cultures were grown at 37°C and were shaken when H$_2$-CO$_2$ (4:1 [vol/vol]) at 172.4 kPa served as the energy source but not when methanol (68 mM) was provided. Methane production was monitored daily (3), and samples (40 ml on days 1 and 2 and 20 ml thereafter) were removed (in an anaerobic chamber) for coenzyme F$_{420}$ and protein determinations. Cells were immediately harvested by centrifugation (25,000 × g, 10 min, 15°C), and the pellets were washed with phosphate-buffered saline (1 PBS) pH 7.4 prior to suspension in a final volume of 2 ml of ice-cold glass-distilled water. A 0.5-ml sample for protein determination was washed twice further with PBS and suspended in...
0.5 ml of PBS. Complete cell breakage was achieved by sonication (MSE Soniprep 150) and was checked under a phase-contrast microscope (3). The protein content was measured according to standard procedures (22).

Coenzyme \( F_{420} \) was extracted from 1.5-ml samples by boiling for 10 min and then cooling rapidly on ice. Cooled samples were vigorously shaken with 3.5 ml of ice-cold methanol, and the pellet was extracted twice further with 5 ml of 70% (vol/vol) ice-cold methanol. The volume of pooled extracts was reduced by rotary evaporation and freeze-drying, and the extracts were suspended in 0.5 ml of solution 1 (4% [vol/vol] methanol, 24 mM sodium acetate [pH 6]) with particulate material removed by centrifugation. The extracts were stored at \(-20^\circ\text{C}\), thawed, and held at 4°C immediately prior to measurement. Injecting samples of concentrated culture supernatant directly onto the HPLC column reduced column efficiency, and a desalting procedure was devised. Samples of culture supernatant (8 to 20 ml) were adjusted to pH 4 with hydrochloric acid and applied to a prewetted (with methanol, glass-distilled water, and 100 mM sodium acetate [pH 4]) C\(_{18}\) Sep-pak cartridge (Waters Associates, Inc., Northwich, Cheshire, United Kingdom). Contaminating polar material was eluted with a 3-ml portion of 100 mM sodium acetate (pH 4) and 3 ml of glass-distilled water, and coenzyme \( F_{420} \) was eluted with 5 ml of 50% (vol/vol) methanol and 5 ml of 100% methanol. This procedure eluted at least 99.9% of the coenzyme \( F_{420} \) and FO and led to the removal of over half of the contaminating material (as judged by fluorescence at 400 to 468 nm and \( A_{254} \)). Methanolic eluants were pooled, rotary evaporated and freeze-dried, and suspended in 0.5 ml of solution 1, as described previously.

**HPLC measurement of coenzyme \( F_{420} \).** The coenzyme \( F_{420} \) content was determined by reversed-phase HPLC with fluorimetric detection. The gradient HPLC system used was based on the isocratic system described previously (26), which required modification in order to elute FO. A 10-\( \mu \)l injection loop was used to apply extracts onto a column (15 cm \( \times \) 0.46 cm) of 3-\( \mu \)m Apex Octadecyl packing material (Jones Chromatography, Llanbradach, Mid Glamorgan, United Kingdom). A saturator and guard column of the same material were also employed, and all three columns were maintained at 25°C. The solvent was delivered at a flow rate of 0.8 ml min\(^{-1} \) and was filtered (0.45-\( \mu \)m-pore membrane) and degassed prior to use. The solvent was initially 4.8% (vol/vol) methanol in 23.8 mM sodium acetate (pH 6). At 10 min after injection, the concentration of methanol was increased and the sodium acetate concentration was decreased according to linear gradients. The concentration of methanol reached 19.2% (vol/vol) (20.2 mM sodium acetate) after 20 min and a final concentration of 79.2% (vol/vol) (5.2 mM sodium acetate) at 25 min, which was maintained until 35 min after injection. Fluorescence detection was achieved at excitation-emission wavelengths of 400 to 468 nm (the optimal wavelengths of this system). The quantification and identification of the fluorescence peaks was achieved with purified coenzyme \( F_{420} \) and FO standards kindly supplied by G. D. Vogels, University of Nijmegen, The Netherlands. FO was not included as an internal standard, as previously (13, 14), and was quantified in cells and culture supernatants.

**RESULTS**

**Relationship of intracellular** coenzyme \( F_{420} \) **to growth and metabolic activity.** An example of the HPLC separation is given in Fig. 2; each coenzyme \( F_{420} \) analog is separated from each other and from interfering non-coenzyme \( F_{420} \) fluorescent material. The respective growth curves (of cell protein and methane production) were virtually parallel to each other and to that of total intracellular coenzyme \( F_{420} \) in each case (Fig. 3). For *M. barkeri* cultured on \( \text{H}_2-\text{CO}_2 \), the doubling times were 15.0 h for the methane production rate, 13.7 h for cell protein, and 11.6 h for total cell coenzyme \( F_{420} \).
or methanol, production rate (Fig. 3A). The provided substrate, doubling growth, the underestimate of again generally predominated on phase, profile F420-3 coenzyme pressure and 7.4 h times of coenzyme obtained with coenzyme whilst the proportions F420-5 whereas the proportions process a enzyme F420 during a process cultivated barkeri grown on constant methanol, and maintained F420-F420 during stationary phase (Fig. 3). In the exponential phase of growth, the total intracellular coenzyme F420 concentration (nmol g protein) remained constant for each culture. A mean value of 530 was obtained with M. barkeri cultivated on H2-CO2, 474 with M. barkeri grown on methanol, and 230 for M. mazei cultivated on methanol. A loss of intracellular coenzyme F420 during stationary phase (Fig. 3) was greatest with cells cultured on methanol and was accompanied by a reduction in cellular protein (Fig. 3B and C).

Variations in relative abundance of coenzyme F420 analogs during growth. The relative abundance of the coenzyme F420 analogs varied with species, substrate, and during batch growth (Fig. 4). For M. barkeri cultured on H2-CO2, coenzymes F420-4 and F420-2 initially predominated (Fig. 4A). However, during exponential growth the proportion of coenzyme F420-4 increased whilst that of coenzyme F420-2 fell, a process which continued at an accelerated rate in stationary phase, when an increase in coenzyme F420-5 and a fall in coenzyme F420-3 were also evident (Fig. 4A). A different coenzyme profile was observed when M. barkeri was cultured on methanol, although coenzymes F420-2 and F420-4 again generally predominated (Fig. 4B). During exponential growth, the proportions of coenzymes F420-2 and F420-3 increased whilst the proportions of coenzymes F420-4 and F420-5 fell (Fig. 4B). Small amounts of the degradation fragment FO were detected in late exponential and stationary phases when M. barkeri was cultured on H2-CO2 (Fig. 4A) and on methanol (Fig. 4B). In the exponential phase of growth, coenzymes F420-2 and F420-3 were the most abundant analogs in cell extracts of M. mazei (Fig. 4C), with coenzymes F420-4 and F420-5 each contributing less than 10% to total coenzyme F420. Changes in the proportions of the coenzyme F420 analogs during exponential and stationary phases included an increase in FO, which represented a quarter of the intracellular coenzyme F420 complement by day 10 (Fig. 4C).

Relationship of intracellular and extracellular coenzyme F420. The extracellular coenzyme F420 concentration increased slowly in early and mid-exponential phase but rapidly during late exponential and stationary phase (Fig. 3), and it was accompanied by a decrease in intracellular coenzyme F420 (Fig. 3). The total concentration in the culture supernatant reached 52 nM for M. barkeri cultured on H2-CO2, 270 nM for M. barkeri grown on methanol, and 228 nM for M. mazei cultivated on methanol. The relative abundance of the coenzyme F420 analogs in the culture supernatants generally reflected those in the cells. Consequently, the proportion of each analog located intracellularly and extracellularly was similar to that reported for total coenzyme F420. An exception to this was the degradation fragment FO, of which at least 97% of the total complement was present in the culture supernatant for each methanogen, where it represented up to 60% of the total coenzyme F420.

DISCUSSION

The total intracellular coenzyme F420 concentration per methanogenic biomass remained relatively constant for each methanogen during exponential batch growth, this relationship having been demonstrated previously for other metha-
nogens where total fluorescence was quantified rather than coenzyme F₄₂₀ alone. Methanogens examined have included Methanobacterium thermoautotrophicum, Methanobrevibacter arboriphilus (31), M. bryantii, M. barkeri FUSARO (15), and Methanobacterium formicicum cultivated in continuous culture (5). The intracellular coenzyme F₄₂₀ content of M. maezi (230 nmol.g of protein⁻¹), M. barkeri MS cultured on methanol (474 nmol.g of protein⁻¹), and M. barkeri MS cultured on H₂-CO₂ (530 nmol.g of protein⁻¹) agree well with previous values (Table 1). Growth substrate had little effect on the coenzyme F₄₂₀ content of M. barkeri MS (this study) and M. barkeri 227 (Table 1); however, some differences have been noted previously (Table 1), and a clear pattern has not yet emerged. A consistent pattern has also not been identified when growth on formate and growth on H₂-CO₂ have been compared; whilst M. formicicum contained more coenzyme F₄₂₀ when cultured on formate (14, 29), the reverse was true for Methanococcus voltae and Methanogenium thermophilicum (14). A significant influence of medium composition on the coenzyme F₄₂₀ content of methanol-grown M. barkeri FUSARO (15) and M. maezi (13) has also been established.

In this study, coenzyme F₄₂₀-4 followed by coenzyme F₄₂₀-2 were the most abundant analogs in M. barkeri MS, whilst previously coenzymes F₂₋₅ and F₄₋₇ were thought to be most common (14). Furthermore, in extracts from M. maezi, coenzymes F₄₋₇ and F₄₋₃ were the predominant analogs rather than coenzyme F₂₋₅ (14). These differences are unlikely to be caused by small variations in technique but probably by differences in the growth media. An effect of substrate on the relative abundance of the analogs has also been noted for M. barkeri MS (Fig. 4A and B) and other

**TABLE 1.** Summary of total intracellular coenzyme F₄₂₀ content of M. barkeri and M. maezi

<table>
<thead>
<tr>
<th>Methanogen</th>
<th>Substrate*</th>
<th>Total coenzyme F₄₂₀ nmol.g of protein⁻¹</th>
<th>Reference</th>
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<tr>
<td>M. barkeri MS</td>
<td>H₂-CO₂/acetate</td>
<td>190</td>
<td>10</td>
</tr>
<tr>
<td>M. barkeri 227</td>
<td>H₂-CO₂</td>
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<td>4</td>
</tr>
<tr>
<td>M. barkeri 227</td>
<td>Acetate</td>
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<td>4</td>
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<tr>
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<td>4</td>
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<tr>
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<td>33</td>
</tr>
<tr>
<td>M. barkeri isolate</td>
<td>H₂-CO₂/methanol</td>
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<td>32</td>
</tr>
<tr>
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<td>15</td>
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<td>32</td>
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<tr>
<td>M. maezi S-6</td>
<td>Methanol</td>
<td>130</td>
<td>13</td>
</tr>
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</table>

* ND. Not disclosed.

* The total cellular coenzyme F₄₂₀ has been converted from the original units (where necessary) assuming a molecular weight of 860.7 g·mol⁻¹ (9), and that protein represents 16% wet weight and 50% dry weight.
methanogens (14). However, unlike the total coenzyme F₄₂₀ content of methanogenic bacteria, the concentration of each coenzyme F₄₂₀ analog (Fig. 1) did not remain constant during batch growth. This has not been reported previously, as studies in which individual coenzyme F₄₂₀ analogs have been quantified have sampled at only one point in the growth curve. The changes in the proportions of analogs followed no discernable pattern except that an increase in coenzyme F₄₂₀-2 was generally accompanied by a fall in coenzyme F₄₂₀-4 and vice versa, whilst a change in the concentration of coenzyme F₄₂₀-3 was accompanied by an opposite response in the concentration of coenzyme F₄₂₀-5 (Fig. 1). This suggests that an enzyme responsible for transferring pairs of glutamic acid residues may be active. The advantage to the cell of adjusting the relative abundance of its coenzyme F₄₂₀ analogs is at present unclear. It is tempting to speculate that the number of glutamic acid residues in the side chain may influence the interaction of this coenzyme with the appropriate enzyme, and that each coenzyme analog may be active with only a limited number of enzymes. A further difference between this and previous studies was that the degradation fragment FO (Fig. 1) was not included as an internal standard and was quantified. Towards the end of stationary phase, FO represented 1 to 3% total coenzyme F₄₂₀ in M. Barkeri and 25% in M. mazei (Fig. 4).

The final extracellular concentration of coenzyme F₄₂₀ and FO reached 270 nM with M. Barkeri grown on methanol and is considerably less than the value of approximately 5.8 μM previously recorded for M. thermoaerotrophicum (18). The higher concentration noted with M. thermoaerotrophicum may reflect a greater amount of coenzyme F₄₂₀ present in this methanogen (10, 14), a larger proportion (70 to 90%) of total fluorescence in the supernatant (27), and a higher cell concentration. The relative proportions of the coenzyme F₄₂₀ analogs were similar in the cells and supernatant with the exception of FO, which was principally located in the culture supernatant. This excretion of FO has been observed previously with M. thermoaerotrophicum and was attributed to its being a precursor of coenzyme F₄₂₀ (18). The nonpolar nature of FO may also facilitate its permeation of the cell membrane, which will act as a barrier to the negatively charged coenzyme F₄₂₀ analogs. Extracellular fluorescence has also been detected in anaerobic sludge samples (28; W. J. de Zeeuw, Ph.D. thesis, University of Wageningen, Wageningen, The Netherlands, 1984), although the possibility of the microbial degradation of coenzyme F₄₂₀ in some systems cannot be eliminated. When assaying methanogenic bacteria in pure cultures, anaerobic digesters, or with landfill extracts, it is therefore prudent to ensure full removal of extracellular coenzyme F₄₂₀ before quantification.

Measurement of the methanogenic biomass by coenzyme F₄₂₀ assay represents a significant improvement on quantification by traditional microbiological techniques. The assay is more rapid, avoids imposing a selective pressure on the organisms, and may be conducted without recourse to specialized microbiological techniques. The applicability of the coenzyme F₄₂₀ assay is dependent upon consistent values for the coenzyme F₄₂₀ content of methanogenic bacteria. This relationship has been demonstrated for the total intracellular coenzyme F₄₂₀ content of methanogens during batch growth, although some variation in the relative abundance of the coenzyme F₄₂₀ analogs has been identified. Substrate, growth medium, and strain may also influence the total and individual coenzyme F₄₂₀ content of methanogenic species, a subject which merits further investigation.