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Hydrogen concentrations in methane-forming cells probed by the ratios of reduced and oxidized coenzyme F_{420}

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Coenzyme F_{420} is the central low-redox-potential electron carrier in methanogenic metabolism. The coenzyme is reduced under hydrogen by the action of F_{420}-dependent hydrogenase. The standard free-energy change at pH 7 of F_{420} reduction was determined to be −15 kJ mol^{-1}, irrespective of the temperature (25–65 °C). Experiments performed with methane-forming cell suspensions of *Methanothermobacter thermautotrophicus* incubated under various conditions demonstrated that the ratios of reduced and oxidized F_{420} were in thermodynamic equilibrium with the gas-phase hydrogen partial pressures. During growth in a fed-batch fermenter, ratios changed in connection with the decrease in dissolved hydrogen. For most of the time, the changes were as expected for thermodynamic equilibrium between the oxidation state of F_{420} inside the cells and extracellular hydrogen. Also, methanol-metabolizing, but not acetate-converting, cells of *Methanosarcina barkeri* maintained the ratios of reduced and oxidized coenzyme F_{420} in thermodynamic equilibrium with external hydrogen. The results of the study demonstrate that F_{420} is a useful probe to assess *in situ* hydrogen concentrations in H_2-metabolizing methanogens.

INTRODUCTION

Most methanogenic archaea derive their energy for growth from the hydrogen-dependent reduction of CO_2 into methane (reaction 1). The amount of energy that can be gained in the process depends on the *in situ* hydrogen concentration, which may vary by orders of magnitude in natural habitats and during growth under laboratory conditions.

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \quad (1)$$

$$F_{420} + H_2 \rightleftharpoons F_{420}H_2 \quad (2)$$

$$F_{420}H_2 + N^5,N^{10}\text{-methenyl-}H_4\text{MPT} \rightleftharpoons F_{420} + N^5,N^{10}\text{-methylene-}H_4\text{MPT} \quad (3)$$

$$F_{420}H_2 + N^5,N^{10}\text{-methylene-}H_4\text{MPT} \rightleftharpoons F_{420} + N^5\text{-methyl-}H_4\text{MPT} \quad (4)$$

A central electron carrier in methane metabolism is the 8-OH-5-deazaflavin derivative coenzyme F_{420}. The compound is present in high concentrations. Oxidized F_{420} shows an intense blue fluorescence when excited at 420 nm (DiMarco *et al.*, 1990; Eirich *et al.*, 1978, 1979). UV–visible light and fluorescence spectral properties are pH-dependent, making F_{420} a useful probe to measure the pH inside the cell (intracellular pH or pH_i) (de Poorter & Keltjens, 2001; von Felten & Bachofen, 2000). F_{420} is reduced to the non-fluorescent species ($F_{420}H_2$) by the action of F_{420}-reducing hydrogenase (reaction 2) (Fox *et al.*, 1987; Thauer, 1998). $F_{420}H_2$ is the substrate in two consecutive reactions in the methanogenic pathway, viz. the reduction of N^5,N^{10}-methenyl-tetrahydromethanopterin (H_4MPT) and N^5,N^{10}-methylene-H_4MPT (reactions 3 and 4). The reactions are catalysed by F_{420}-dependent methylene-H_4MPT dehydrogenase and methylene-H_4MPT reductase, respectively. Reactions (2–4) are reversible (Thauer, 1998). The enzymes involved display high turnover numbers (k_{cat}) and each represents as much as 0·5–1 % of the total cellular protein (Enßle *et al.*, 1991; Ma & Thauer, 1990; Schwörer & Thauer, 1991; te Brömmelstroet *et al.*, 1990, 1991a, b). Thus, the catalytic capacities of the hydrogenase, dehydrogenase and reductase substantially exceed the specific rate of methane formation. Under these conditions, the concentration ratios of reduced and oxidized coenzyme F_{420} are

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Abbreviations: p_{H_2}, hydrogen partial pressure; pH_i, intracellular pH.

predicted to be in thermodynamic equilibrium with the hydrogen partial pressure (p_{H_2}).

Taking advantage of the fluorescent properties of F_{420}, ratios of reduced and oxidized species were measured in H_2–CO_2-metabolizing cells of *Methanothermobacter thermautotrophicus* and in methanol- and acetate-utilizing *Methanosarcina barkeri*. It was found that the ratios were, indeed, in close thermodynamic equilibrium with the hydrogen concentrations applied (0–2 %). For reasons discussed, this did not hold for acetate-converting *Methanosarcina barkeri*. The results of the study indicate that coenzyme F_{420} is not only a useful probe to measure pH_i, but also to determine the *in situ* hydrogen concentration in H_2-metabolizing methanogens.

METHODS

Materials. Coenzyme F_{420} was purified from whole cells of *Methanothermobacter thermautotrophicus* and cell extracts of the organism were prepared by using established procedures (te Brömmelstroet *et al.*, 1991b). Gases were supplied by Hoek-Loos. To remove traces of oxygen, hydrogen-containing gases were passed over a BASF RO-20 catalyst at room temperature and nitrogen-containing gases over a pre-reduced BASF R3-11 catalyst at 150 °C. The catalysts were a gift from BASF Aktiengesellschaft. All other chemicals used were of the highest grade available.

Culturing methods. *Methanothermobacter thermautotrophicus* (formerly *Methanobacterium thermautotrophicum*) strain ΔH^T = DSM 1053^T was grown at 65 °C and pH 7·0 in a 3·5 l fermenter (MBR) containing 2·5 l mineral medium and gassed with H_2/CO_2 (80 : 20 %, v/v) at 1500 r.p.m. Mineral medium contained the following constituents (g l^{-1}): KH_2PO_4 (6·8), Na_2CO_3 (3·3), NH_4Cl (2·1), trace elements as described by Schönheit *et al.* (1979) and sodium resazurin (0·1 mg l^{-1}), and cysteine hydrochloride (0·6 g l^{-1}) and $Na_2S_2O_3$ (0·5 g l^{-1}) as reducing agents. At regular time intervals, samples were collected anoxically for the determination of OD_{600}, F_{420} measurement, pH_i determination and for cell-suspension incubations. The dissolved p_{H_2} and medium pH were monitored online with an amperometric (Ag/Ag_2O) H_2 probe (de Poorter *et al.*, 2003; Schill *et al.*, 1996) and a pH electrode (Ingold, Elscolab Nederlands BV), respectively.

Alternatively, *Methanothermobacter thermautotrophicus* was cultured in 115 ml serum bottles containing 50 ml mineral medium supplemented with 0·6 g $Na_2S.2H_2O$ l^{-1}. Growth was performed at various temperatures (50–65 °C) and pH values (6·0–7·5) to an OD_{600} of 0·2–0·3. Incubation took place in a rotary-shaking water bath operating at 150 r.p.m. After inoculation, cultures were pressured daily with H_2/CO_2 (80 : 20 %, v/v; 200 kPa).

Methanosarcina barkeri strain Fusaro (= DSM 804) was cultured in 50 ml amounts in 115 ml serum bottles. Media were prepared as described previously (Hutten *et al.*, 1981) and contained 10 g sodium acetate l^{-1} (122 mM) or 10 ml methanol l^{-1} (200 mM) as a carbon and energy source. Cells were grown without shaking at 35 °C under an N_2/CO_2 (80 : 20 %, v/v; 120 kPa) atmosphere to an OD_{600} of 0·1–0·2.

Reduction of coenzyme F_{420}. Purified coenzyme F_{420} was reduced enzymically by using cell extract of *Methanothermobacter thermautotrophicus* as described previously (Vermeij *et al.*, 1997). Reaction mixtures (3 ml) were incubated in 25 ml serum bottles under 0–80 % H_2, 20 % CO_2, complemented with N_2 (80–0 %). After reactions had come to equilibrium, anoxic acetone was added and fluorescence spectra were recorded immediately as described below.

Cell-suspension incubations. Cells were collected from 3·5 l fed-batch cultures or were obtained from serum-bottle cultures. Inside an anaerobic glove box, 2 ml portions of cells were divided over a series of 115 ml serum bottles. Cell suspensions with an OD_{600} of >1 were diluted with anoxic mineral medium. After filling, bottles were closed with butyl rubber stoppers and aluminium-crimped seals, evacuated and pressured with mixtures of H_2/CO_2 (80 : 20 %, v/v) and N_2/CO_2 (80 : 20 %, v/v) to obtain the p_{H_2} values specified in the text. Hereafter, titanium citrate (1 mM) was added to remove traces of oxygen (Zehnder & Wuhrmann, 1976). Ethane (1 ml) was added as an internal standard for methane measurements (Gijzen *et al.*, 1991). Serum bottles were subsequently placed in a water bath without shaking at the specified temperatures. At regular times, headspace samples were withdrawn to follow methane formation. As soon as methanogenesis had started, incubations were continued for 30 min at 150 r.p.m. (*Methanothermobacter thermautotrophicus*) or 100 r.p.m. (*Methanosarcina barkeri*) rotation. Reactions were then stopped by cooling the serum bottles rapidly in ice-cold water and samples were immediately withdrawn with a gas-tight syringe for F_{420} fluorescence analysis.

Coenzyme F_{420} fluorescence measurements. A known volume of cells from the fermenter (1–5 ml) or from cell-suspension incubations (1 ml) was injected under anoxic conditions into a serum bottle closed with a bromobutyl rubber stopper and containing ice-cold anoxic acetone kept under N_2/CO_2 (80 : 20 %, v/v). Before use, acetone was stored overnight in an anaerobic glove box to remove traces of oxygen. Immediately afterwards, cell–acetone mixtures were pipetted into cuvettes placed inside the glove box. Cuvettes were closed with bromobutyl stoppers and the contents were analysed by anaerobic fluorescence spectroscopy. This gave the fluorescence intensities of oxidized F_{420} present in the samples (F_{ox}). To determine the fluorescence of total coenzyme F_{420} (F_{tot}), cell samples were mixed, after brief exposure to air, with oxic acetone and spectra were measured under aerobic conditions. To correct for background fluorescence (F_b), cell samples were incubated under (H_2/CO_2) 80 : 20 % at 65 °C, added to cold anoxic acetone and measured anaerobically.

Fluorescence emission was recorded at room temperature on an Aminco SPF-500 fluorimeter with excitation wavelength at 427 nm (band pass, 4 nm) and emission wavelength at 471 nm (band pass, 2 nm). Alternatively, excitation spectra (340–470 nm) were recorded at an emission wavelength of 471 nm. The concentration ratios of $F_{420}H_2$ and F_{420} were calculated as $(F_{tot}-F_{ox})/F_{ox}$. The experimental values (F_{tot}, F_{ox}) were corrected for background fluorescence (F_b) measured for the fully (80 % H_2) reduced cell samples. Acetone extracts were alkaline (pH 9–10). Under these conditions, oxidized F_{420} is measured exclusively as the phenolate–quinoid anionic species (see Appendix).

Other analytical methods. Methane-production rates during the fermenter culturing were calculated from the flow rate and methane content of the outflow gas, which were measured by use of a soap-film meter and by GC, respectively. GC was performed on an HP 5890 gas chromatograph equipped with a Poropak Q column and a flame-ionization detector. Cellular dry weights (DW) to determine specific methane-forming activities were derived from the OD_{600} value of the culture. Previous research established the linear relation between both parameters, at which 1 l culture showing an OD_{600} of 1 equalled 425 mg dry cells (unpublished results). pH_i values were measured by a previously described method, using the pH-dependent fluorescence properties of oxidized coenzyme F_{420} (de Poorter & Keltjens, 2001).

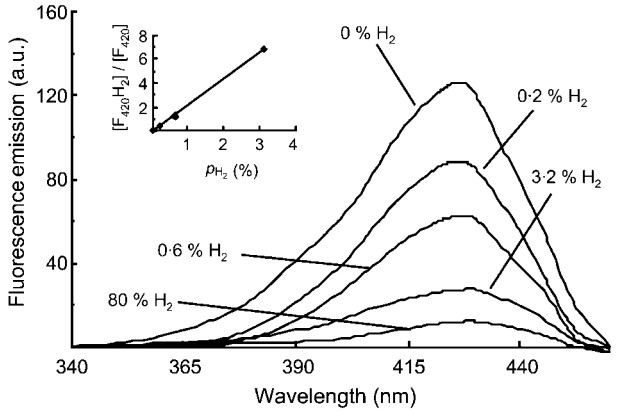

Fig. 1. Hydrogen-dependent reduction of coenzyme F_{420}. F_{420} (5 μM) was reduced at the indicated p_{H_2} values (%, v/v), using cell-free extract (15 μg protein) as described in Methods. Reactions were performed at 60 °C and pH 7·0. Excitation spectra were recorded at 471 nm emission. In the inset, concentration ratios of reduced ($F_{420}H_2$) and oxidized F_{420} are plotted against the applied hydrogen partial pressures (p_{H_2}). a.u., Arbitrary units.

RESULTS

Hydrogen-dependent reduction of coenzyme F_{420}

F_{420} was incubated in the presence of cell extract in a series of serum bottles under different p_{H_2} (0–80 %; 0–0·8 bar), and fluorescence-excitation spectra were recorded after reactions had come to equilibrium (Fig. 1). F_{420} incubated under an N_2/CO_2 atmosphere (80 : 20 %, v/v) showed maximal fluorescence emission at 427 nm excitation. The same fluorescence intensities of H_2-incubated reaction mixtures were found after exposure to air or after mixing with aerobic acetone. Incubations at increased hydrogen concentrations resulted in the concomitant decrease of the excitation spectra, characteristic of F_{420} reduction. Under 80 % H_2, the spectrum was bleached almost completely. Concentration ratios of $F_{420}H_2$ and F_{420}, determined as described in Methods, were related linearly to the p_{H_2} applied (Fig. 1, inset). From the slope of the plot and by using equation (A.8) from the Appendix, a $\Delta G^{0\prime}$ of -15 kJ mol^{-1} was calculated at the experimental conditions (pH 7·0, 60 °C). Remarkably, the same value of $\Delta G^{0\prime}$ was found under standard conditions (pH 7·0, 25 °C).

Whole cells incubated under hydrogen revealed excitation and emission spectra that were indistinguishable from those obtained for purified F_{420} (data not shown). This demonstrated that other cellular components did not interfere with F_{420} fluorescence measurements. The fluorescence characteristics were subsequently used to determine the concentration ratios of reduced and oxidized F_{420} in metabolizing cells.

Coenzyme F_{420} reduction in methane-forming cell suspensions of *Methanothermobacter thermautotrophicus*

To investigate the effect of the applied p_{H_2} on coenzyme F_{420} reduction in methane-producing cells, cell suspensions of *Methanothermobacter thermautotrophicus* were incubated under a variety of conditions and at 0–2 % (v/v) hydrogen in the gas phase (p_{H_2}, 0–0·02 bar). Cell suspensions were obtained from different growth stages in the fed-batch fermenter (see below) or from serum-bottle cultures. At low p_{H_2}, the specific rates of methanogenesis in the suspension incubations were linearly dependent on the p_{H_2} applied. Specific activities at a p_{H_2} of 0·02 bar were 5–50 % of the maximal values measured at 80 % H_2 [1–3 μmol CH_4 min^{-1} (mg DW)$^{-1}$]. The former percentages depended on the hydrogen concentration at which growth had occurred and reflect changes in the affinities (K_m) of the cells for hydrogen. It is known that *Methanothermobacter thermautotrophicus* cells derived from cultures grown under low-hydrogen conditions display a higher hydrogen affinity (K_m approx. 2 % H_2) than cells grown at a high hydrogen concentration (K_m approx. 20 % H_2) (Pennings *et al.*, 2000). In addition, maximal specific activities of the cultured cells varied in a growth phase- and growth condition-related way (Pennings *et al.*, 2000; L. M. I. de Poorter & J. T. Keltjens, unpublished observations). This explains the differences in values measured at 80 % H_2 during the suspension incubations.

When cell suspensions collected from different growth stages in the fed-batch fermenter were incubated at 60 °C and pH 7, a linear relationship was found between the $[F_{420}H_2]/[F_{420}]$ ratios and the p_{H_2} values applied (Fig. 2). Slopes of the graphs measured with cells from different growth stages were identical. The mass–action ratio was associated with $RT \ln q_r$ at $+15$ kJ mol^{-1}. Above data established a $\Delta G_r^{0\prime}$ of -15 kJ mol^{-1} at 60 °C and pH 7. From the resulting $\Delta G_r'$ of 0 kJ mol^{-1} (equation A.1), it is inferred that the concentrations of reduced and oxidized coenzyme F_{420} within the cells are in thermodynamic equilibrium with the p_{H_2} in the gas phase.

To investigate the effect of temperature and pH on the hydrogen-dependent reduction of coenzyme F_{420}, *Methanothermobacter thermautotrophicus* was cultured in serum bottles at a range of temperatures (50–65 °C) and pH values (6·0–7·5). Cells were subsequently incubated under various p_{H_2}, using medium pH values and temperatures at which culturing had occurred. Separate incubations were performed to measure the pH_i after incubation. At the experimental conditions, pH_i was found to be equal to the medium pH. As before, $[F_{420}H_2]/[F_{420}]$ ratios were related linearly to the p_{H_2} values applied (Figs 3a and 4a). Slopes were pH-dependent and an approximately tenfold decrease in the mass–action ratio was observed when medium pH increased by 1 unit (Fig. 3a). This indicates that coenzyme F_{420} reduction is described by equation (5), in which F_{420}^- refers to (deprotonated) phenolate anion (Fig. 7):

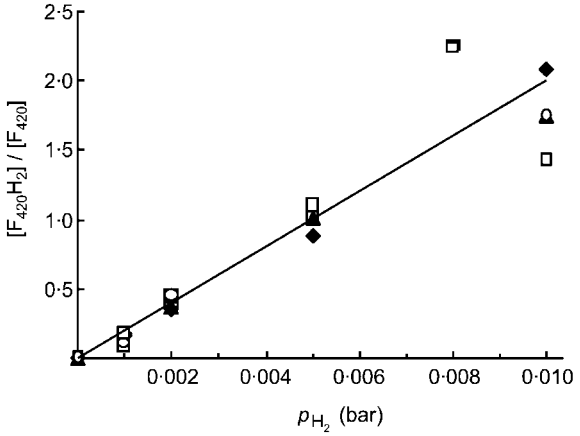

Fig. 2. Effect of p_{H_2} on coenzyme F_{420} reduction in *Methanothermobacter thermautotrophicus*. Cell suspensions were incubated under 20 % CO_2 and the indicated gas-phase p_{H_2} values. Incubations took place at 60 °C and pH 7·0 as described in Methods. Suspensions were collected from fed-batch fermenter cultures at the different growth phases. Symbols: ◆, early-exponential phase ($OD_{600}=0{\cdot}2$); □, exponential phase ($OD_{600}=0{\cdot}6$); ▲, linear phase ($OD_{600}=2$); ○, stationary phase ($OD_{600}=6$).

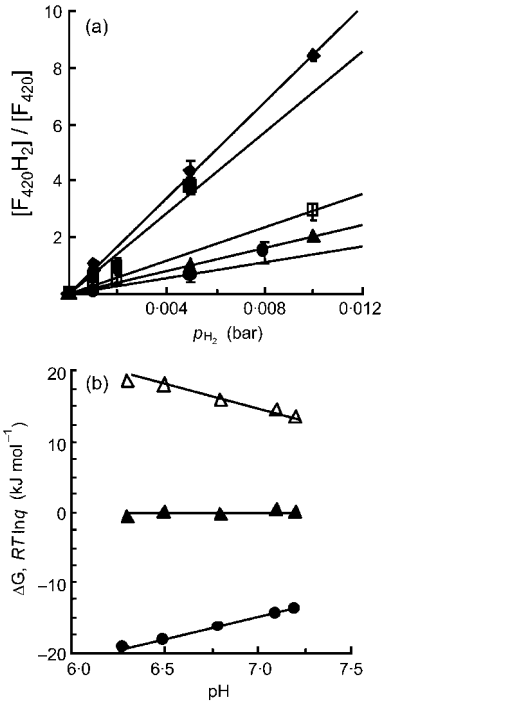

Fig. 3. Effect of pH on the thermodynamics of F_{420} reduction in *Methanothermobacter thermautotrophicus*. (a) Cell suspensions were incubated at 60 °C under 20 % CO_2 and the indicated gas-phase p_{H_2} values as described in Methods. Reactions took place at the following pH values of the medium: 6·3 (◆), 6·5 (■), 6·8 (□), 7·1 (▲) or 7·2 (●). Data represent the means and errors of triplicate experiments. (b) Plots of mass–action ratio terms (RT ln q) (△) derived from the slopes presented in (a), calculated pH-dependent ΔG^0 values (●) and net Gibbs free-energy changes (ΔG) (▲).

$$H_2 + H^+ + F_{420}{}^- \rightleftharpoons F_{420}H_2 \quad (5)$$

The plot of $RT \ln q_r$ versus pH gave a straight line (Fig. 3b). The slope ($-6{\cdot}4$ kJ mol^{-1} pH^{-1}) at the incubation temperature (60 °C) was in full agreement with the net uptake of one proton. By use of equation (A.6) and the experimental $\Delta G^{0\prime}$ of -15 kJ mol^{-1}at pH 7, $\Delta G_{60}{}^0$ could be calculated for the different pH values (Fig. 3b). Again considering that the Gibbs free-energy change at 60 °C (ΔG_{60}) sums as $\Delta G_{60}{}^0 + RT \ln q_r$ (equation A.1), a ΔG_{60} of 0 kJ mol^{-1} was derived for all pH values tested, indicative of thermodynamic equilibrium (Fig. 3b). When incubated at pH 7, mass–action ratios varied with the incubation temperatures (50–65 °C), but the $RT \ln q_r$ term was constant ($+15$ kJ mol^{-1}) and exactly opposite to the (temperature-independent) $\Delta G^{0\prime}$ of -15 kJ mol^{-1}, again demonstrating thermodynamic equilibrium ($\Delta G' = 0$) (Figs 4a and b).

By routine, cell-suspension incubations were performed at relatively low p_{H_2} values (0–0·02 bar). When incubated at higher headspace-hydrogen concentrations, large variations in $[F_{420}H_2]/[F_{420}]$ ratios were found among repeated experiments and the ratios were generally lower than expected. At the higher p_{H_2} values, methane production and, in direct connection, hydrogen uptake took place at correspondingly enhanced rates. The consumption of dissolved hydrogen during the brief but variable period between rotary incubation and cooling of the samples (5–15 s) probably caused the variation in and underestimation of the $[F_{420}H_2]/[F_{420}]$ ratios.

Coenzyme F_{420} reduction in methanol- and acetate-metabolizing cell suspensions of *Methanosarcina barkeri*

Methanosarcina barkeri was grown in serum bottles on methanol (200 mM) or acetate (122 mM) as substrates to an OD_{600} of 0·1–0·2. At this time, cultures still contained approximately 150 mM methanol and 80 mM acetate, whilst methane was formed with specific activities of 0·4 and 0·1 μmol min^{-1} (mg DW)$^{-1}$, respectively. Portions (2 ml) of the cultures were subsequently incubated under 0–80 % hydrogen at 35 °C. Determination of the $[F_{420}H_2]/[F_{420}]$ ratios revealed a linear relationship between the ratios and the p_{H_2} values applied in the case of methanol-grown cells (Fig. 5). From the slope of the curve, an $RT \ln q_r$ of $+15$ kJ mol^{-1} could be calculated, which equals the above-determined values. From this, we conclude that methanol-metabolizing *Methanosarcina barkeri* cells maintain their $[F_{420}H_2]/[F_{420}]$ ratios in thermodynamic equilibrium with the p_{H_2} in the environment. In acetate-grown cells, the situation was different. Although clearly detectable by the sensitive fluorescence method used, the F_{420} content was lower by

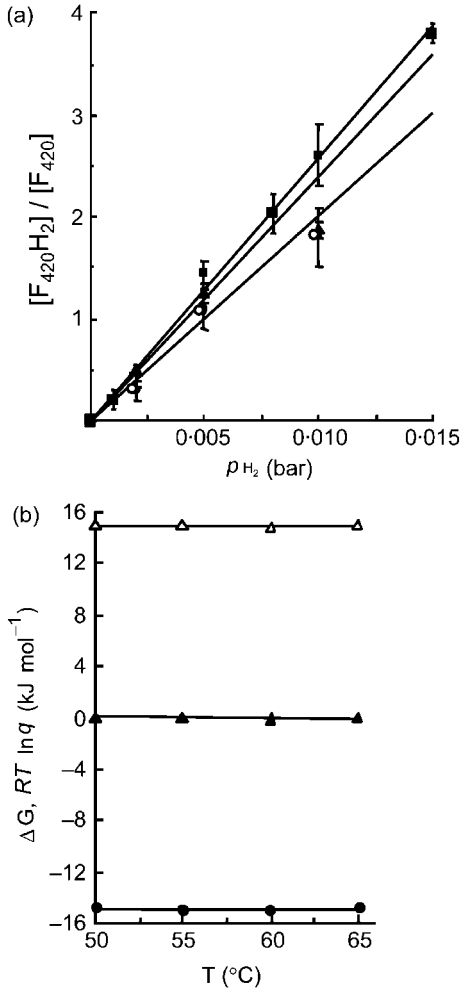

Fig. 4. Effect of temperature on the thermodynamics of F_{420} reduction in *Methanothermobacter thermautotrophicus*. (a) Cell suspensions were incubated at pH 7·0 under 20 % CO_2 and the indicated headspace p_{H_2} values as described in Methods. Reactions took place at the following temperatures: 50 °C (■), 55 °C (▲) and 65 °C (○). Data represent the means and errors of triplicate experiments. (b) Plots of mass–action ratio terms (RT ln q) (△) derived from the slopes presented in (a), calculated ΔG^0 values (●) and net Gibbs free-energy changes (ΔG) (▲). The mass–action ratio at 60 °C was obtained from data presented in Fig. 2.

more than a factor of ten than that in methanol-grown cells. Moreover, coenzyme F_{420} was only present in the oxidized state $[(F_{420}H_2)/(F_{420}) = 0]$ (Fig. 5), even if incubations were performed under high hydrogen concentrations (up to 80 %).

Changes in the ratios of reduced and oxidized coenzyme F_{420} during growth of *Methanothermobacter thermautotrophicus* in a fed-batch fermenter

Methanothermobacter thermautotrophicus was cultured in a fed-batch fermenter at constant gassing with 80 : 20 % H_2/CO_2 (Fig. 6). Growth was characterized by an exponential increase of cell density up to an OD_{600} of 1·7 [specific growth rate, 0·24 h^{-1}; doubling time (t_d), 2·9 h]. Hereafter, cell density increased linearly with time. During exponential growth, methane was formed with a specific activity of 1·5–2·5 mol min^{-1} (mg DW)$^{-1}$. Considering that 4 mol hydrogen is used (mol methane formed)$^{-1}$ (equation 1), the specific hydrogen-consumption rate amounted to 6–10 mol min^{-1} mg^{-1}. Together with the increase in biomass, the overall hydrogen-consumption rate increased tenfold (0·6–6 mmol min^{-1}). The increase in hydrogen consumption was accompanied by the decline in the dissolved p_{H_2} from 70 to 3 % (0·7 to 0·03 bar). Remarkably, the intracellular pH of the cells decreased as well, in particular during the mid-exponential phase (Fig. 6). During the linear-growth phase (10–12 h), the hydrogen-consumption rate and p_{H_2} became constant at 6 mmol min^{-1} and 0·03 bar, respectively. Now, pH_i was about equal to the medium pH of 7·0.

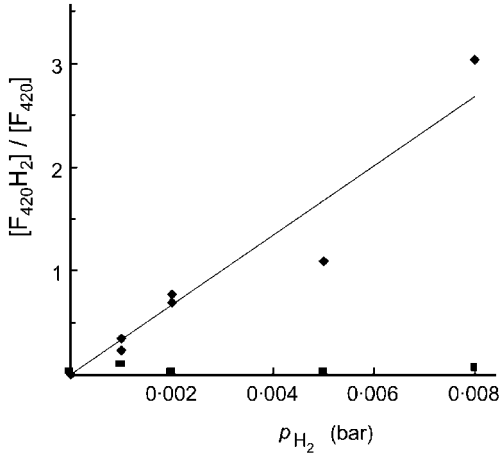

Fig. 5. Effect of p_{H_2} on coenzyme F_{420} reduction in *Methanosarcina barkeri*. Methanol (◆) and acetate (■)-metabolizing cell suspensions were incubated under 20 % CO_2 and the indicated headspace p_{H_2} values. Reactions took place at 37 °C and pH 7·0 as described in Methods.

At regular time intervals, cells were collected anoxically from the fermenter and analysed for the $[F_{420}H_2]/[F_{420}]$ ratios (Fig. 6). The apparent ratios tended to decrease, but became somewhat higher during the linear-growth phase. From the recorded p_{H_2} and pH_i values, $[F_{420}H_2]/[F_{420}]$ ratios were calculated theoretically, assuming thermodynamic equilibrium. It can be seen that experimental and theoretic ratios were about equal during the early-exponential (0–3 h) and linear (10–12 h) phases, where p_{H_2} was as high as 0·70 bar and as low as 0·03 bar, respectively. During the intermediary-exponential phase, however, experimental $[F_{420}H_2]/[F_{420}]$ ratios were five- to 15-fold lower than the theoretical values. It is conceivable that, during this stage, the hydrogen concentration inside the cells was lower than that in the medium. However, at least part of the difference

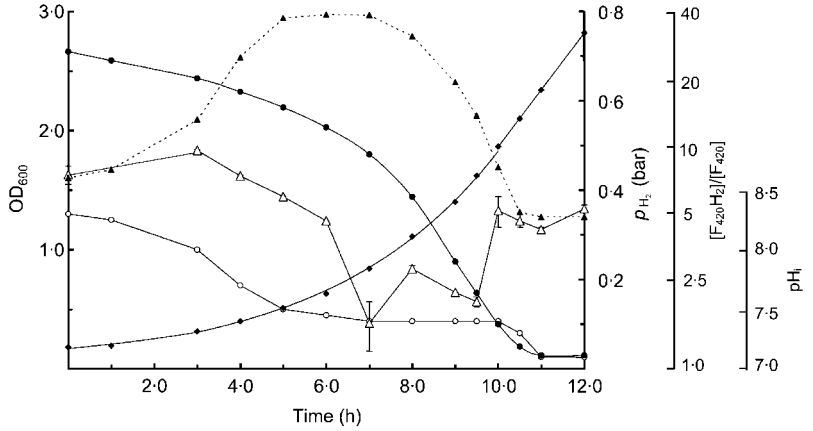

Fig. 6. Changes in the concentration ratios of reduced and oxidized coenzyme F_{420} during growth of *Methanothermobacter thermautotrophicus* in a fed-batch fermenter. The organism was grown under 80 : 20 % H_2/CO_2 at a constant gassing rate of 218 ml min^{-1}. Culturing took place at 65 °C and pH 7·0 as described in Methods. Measurements started ($t=0$) 12 h after inoculation. Symbols: ◆, OD_{600}; ●, p_{H_2} in growth medium; ○, intracellular pH (pH_i); △, experimental concentration ratios of reduced and oxidized F_{420} (means and errors of triplicate fluorescence measurements); ▲, $[F_{420}H_2]/[F_{420}]$ ratios (assuming thermodynamic equilibrium).

could be due to an underestimation of the $[F_{420}H_2]/[F_{420}]$ ratios as a result of the sampling procedure. Sampling included the passage of the culture liquid through the device interconnecting the fermenter and the acetone-containing sample bottle, which took about 5 s. During the passage, a substantial part of the dissolved hydrogen could have been utilized, especially at high cellular hydrogen-uptake rates and at high medium p_{H_2}, conditions that typically apply to the exponential phase. Indeed, when acetone mixtures were analysed by GC for dissolved hydrogen, levels in samples collected during the intermediary-exponential phase were lower by a factor of 5–15 than measured with the hydrogen probe. In contrast, GC determinations on liquids from early-exponential and linear-phase cells agreed well with those recorded in the fermenter (data not shown).

DISCUSSION

Hydrogen-metabolizing cells of *Methanothermobacter thermautotrophicus* consistently maintained the concentration ratios of reduced and oxidized coenzyme F_{420} in thermodynamic equilibrium with the p_{H_2}, if below approximately 0·02 bar. However, equilibrium was also observed at p_{H_2} values as high as 0·7 bar and at high specific hydrogen-consumption rates (see Fig. 6, early-exponential phase). Therefore, the relationship could be valid for all conditions, but this could not be substantiated by the method applied, due to the time delay in our sampling procedure. Online *in situ* fluorescence measurements might clarify this issue.

In the temperature range tested (25–65 °C), the standard free-energy change at pH 7 related to the hydrogen-dependent reduction of coenzyme F_{420} was constant ($\Delta G^{0\prime}$, −15 kJ mol^{-1}). As the midpoint potential of the H^+/H_2 couple varies with temperature, $E_{m,F}$ for the $F_{420}/F_{420}H_2$ couple has to show the same temperature dependency. On the basis of the experimental $\Delta G^{0\prime}$ values, the H^+/H_2 midpoint potentials and by using equation (A.7), E_m values of −340 and −385 mV are then calculated for the $F_{420}/F_{420}H_2$ couple at 25 and 60 °C, respectively, by the biochemical assay described here. The former value equals reported data (−340 to −350 mV) determined at ambient temperature by electrochemical methods (Jacobson & Walsh, 1984; Pol *et al.*, 1980).

Thermodynamic equilibrium was also found in methanol-utilizing *Methanosarcina barkeri* cells. This is remarkable, as the conversion of methanol into methane and CO_2 does not involve hydrogen (equation 6).

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O \qquad (6)$$

However, methanol-grown cells contain high levels of F_{420}-reducing hydrogenase (Michel *et al.*, 1995), whilst F_{420} serves as the electron carrier in two reactions of the methyl group-oxidation pathway, notably N^5-methyl-H_4MPT and N^5,N^{10}-methylene-H_4MPT oxidation (reversed reactions 3 and 4) (Enßle *et al.*, 1991; Schwörer & Thauer, 1991; te Brömmelstroet *et al.*, 1991a; Thauer, 1998). During growth on methanol, the compound serves as both the energy and carbon source. As cell carbon is formally more oxidized than that in methanol, anabolism is associated with a net electron production. It is conceivable that the generation (or consumption) of hydrogen gas is required to balance electron flows in catabolic and anabolic reactions at which F_{420}-hydrogenase could act as a redox valve. Indeed, it is known that *Methanosarcina* growing on methanol accumulates small concentrations of hydrogen gas in the gas atmosphere (Lovley & Ferry, 1985). In contrast, acetate catabolism does not involve F_{420}-dependent reactions. Under these conditions, F_{420}-reducing hydrogenase, as well as F_{420}-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase and reductase, are repressed (Schwörer & Thauer, 1991; Vaupel & Thauer, 1998). As expected for a limited role in cellular metabolism, F_{420} is present at only low levels (Heine-Dobbernack *et al.*, 1988; this study). Furthermore, it was found here that hydrogen had no effect on the F_{420} reduction state during acetate metabolism. Apparently, hydrogen does not equilibrate with the intermediary F_{420} metabolism, serving now only some specific anabolic steps.

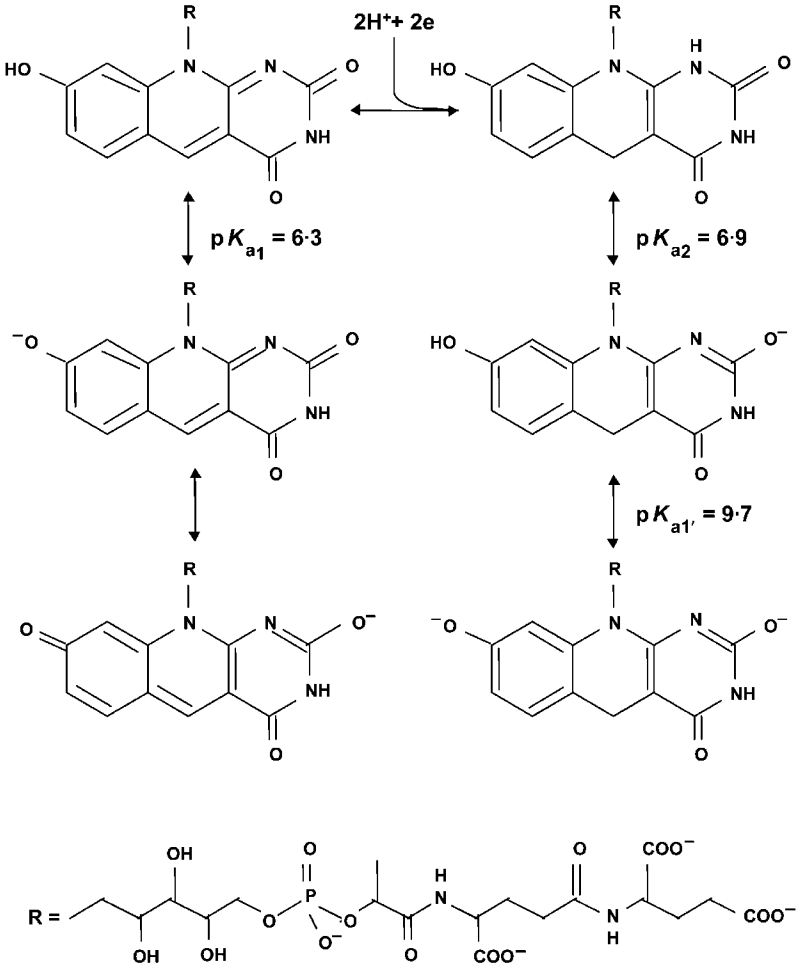

Fig. 7. Structure of coenzyme F_{420} and its (de)protonation and redox reactions.

In nature, methanogenic archaea form part of densely packed, complex microbial consortia that degrade organic matter into methane and CO_2 (Zinder, 1993). Hydrogen is a central intermediate in the degradation and the gas is presumably present as steep spatial-concentration gradients. Detailed understanding of the processes will require methods to measure *in situ* hydrogen concentrations within the microsystems. By taking advantage of its fluorescent properties, coenzyme F_{420} could serve as a probe to assess hydrogen concentrations by using, for example, non-invasive laser techniques.

APPENDIX

Theory

Equation (2) in the Introduction formally describes the reduction of coenzyme F_{420} into 1,5-dihydro-F_{420} ($F_{420}H_2$) with hydrogen. The (Gibbs) free-energy change, ΔG_r (kJ mol^{-1}), at specified reaction conditions (suffix *r*; temperature, pH) of the reaction is:

$$\Delta G_r = \Delta G_r^0 + RT \ln q_r \quad (A.1)$$

in which R is the gas constant ($8{\cdot}314{\cdot}10^{-3}$ kJ mol^{-1} K^{-1}), T is the absolute temperature (K) and q_r is the mass–action ratio:

$$q_r = [F_{420}H_2]/[F_{420}]\ p_{H_2} \quad (A.2)$$

q_r equals the slope in the experimental $[F_{420}H_2]/[F_{420}]$ versus p_{H_2} plots. It should be noted that $[F_{420}]$ and $[F_{420}H_2]$ represent total concentrations of the oxidized and reduced species, respectively. In the physiological pH range, the 5-deazaflavin chromophore of oxidized coenzyme F_{420} contains one ionizable group, viz. 8-OH (pK_{a1} 6·18–6·47, depending on the temperature) (Jacobson & Walsh, 1984; Purwantini *et al.*, 1992). Deprotonation of 8-OH results in the phenolate anion, which tautomerizes into the conjugated paraquinoid anion (Fig. 7). In (non-fluorescent) reduced F_{420}, NH(1) (pK_{a2} 6·9) and the 8-hydroxyl group (p$K_{a1'}$ 9·7) are of relevance. Thus, oxidized and reduced F_{420} are composed of a mixture of species that will affect the redox potential of the $F_{420}/F_{420}H_2$ couple in a pH-dependent fashion.

$$[F_{420}]_{tot} = [F_{420}]\ (1 + K_{a1}/[H^+]) \quad (A.3)$$

$$[F_{420}H_2]_{tot} = [F_{420}H_2]\ (1 + K_{a2}/[H^+] + K_{a2}.K_{a1'}/[H^+]^2) \quad (A.4)$$

In addition, the free-energy changes of coenzyme F_{420} reduction with hydrogen will vary with the pH:

$$H_2 \pm m H^+ + {F_{420}}^{m-} \rightleftharpoons F_{420}H_2 \quad (A.5)$$

Defining $\Delta G_r^{0'}$ (kJ mol^{-1}) as the free-energy change at pH 7 and at the

temperature at which the reaction is followed and m as the net number of protons that are consumed or produced per reaction, the following relations hold:

$$\Delta G^0 = \Delta G^0_r \pm 2.303\ mRT\ (7\text{-pH}) \quad (A.6)$$

$$\Delta G^0_r = nF\Delta E_{m,7} \quad (A.7)$$

In equation (A.6), the sign of the term is minus in a proton-consuming reaction. In equation (A.7), n is the number (2) of electrons involved, F is the Faraday constant (96·49 kJ V^{-1} mol^{-1}) and $\Delta E_{m,7}$ is the difference between the midpoint potentials (V) of the H^+/H_2 ($E_{m,H}$) and $F_{420}/F_{420}H_2$ ($E_{m,F}$) redox couples, respectively, at pH 7 and the specified temperature. $E_{m,H}$ is derived for each given temperature from the Nernst equation: $E_{m,H} = -2{\cdot}303(7RT/F)$. $E_{m,F}$ should be measured, or it can be calculated if $\Delta G_r^{0\prime}$ (at pH 7) is known. The latter can be determined from the reaction at equilibrium. Considering that, under these conditions, $\Delta G_r = 0$ and that the mass–action ratio (q_r) equals the equilibrium constant K_r, it follows from equation (A.1):

$$\Delta G^{0\prime}_r = -RT \ln K'_r \quad (A.8)$$

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