Hydrogen concentrations in methane-forming cells probed by the ratios of reduced and oxidized coenzyme F420

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Coenzyme F420 is the central low-redox-potential electron carrier in methanogenic metabolism. The coenzyme is reduced under hydrogen by the action of F420-dependent hydrogenase. The standard free-energy change at pH 7 of F420 reduction was determined to be $-15 \text{kJ mol}^{-1}$, 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 F420 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 F420 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 F420 in thermodynamic equilibrium with external hydrogen. The results of the study demonstrate that F420 is a useful probe to assess in situ hydrogen concentrations in H2-metabolizing methanogens.

INTRODUCTION

Most methanogenic archaea derive their energy for growth from the hydrogen-dependent reduction of CO2 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.

$$4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$$  \hspace{1cm} (1)

A central electron carrier in methane metabolism is the 8-OH-5-deazaflavin derivative coenzyme F420. The compound is present in high concentrations. Oxidized F420 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 F420 a useful probe to measure the pH inside the cell (intracellular pH or pH$_i$) (de Poorter & Keltjens, 2001; von Felten & Bachofen, 2000). F420 is reduced to the non-fluorescent species (F420H2) by the action of F420-reducing hydrogenase (reaction 2) (Fox et al., 1987; Thauer, 1998). F420H2 is the substrate in two consecutive reactions in the methanogenic pathway, viz. the reduction of N$^5$N$^{10}$-methenyl-tetrahydromethanopterin (H$_4$MPT) and N$^5$N$^{10}$-methylene-H$_4$MPT (reactions 3 and 4). The reactions are catalysed by F420-dependent methylene-H$_4$MPT dehydrogenase and methylene-H$_4$MPT 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 F420 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 (pH).

Taking advantage of the fluorescent properties of F420, ratios of reduced and oxidized species were measured in H2–CO2-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 F420 is not only a useful probe to measure pH, but also to determine the in situ hydrogen concentration in H2-metabolizing methanogens.

METHODS

Materials. Coenzyme F420 was purified from whole cells of Methanothermobacter thermautotrophicus and cell extracts of the organism were prepared by using established procedures (te Brömmedstroet 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 catalyzer was a gift from BASF Aktiengesellschaft. All other chemicals used were of the highest grade available.

Culturing methods. Methanothermobacter thermautotrophicus (formerly Methanobacterium thermautotrophicus) strain DH7 = DSM 1053T 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 H2/CO2 (80 : 20%, v/v) at 1500 r.p.m. Mineral medium contained the following constituents (g l−1): KH2PO4 (6.8), Na2CO3 (3.3), NH4Cl (2.1), trace elements as described by Schönheit et al. (1979) and sodium resazurin (0.1 g l−1), and cysteine hydrochloride (0.6–6 g l−1) and Na2S2O3 (0.5–5 g l−1) as reducing agents. At regular time intervals, samples were collected anoxically for the determination of OD600, F420 measurement, pH determination and for cell-suspension incubations. The dissolved pHi and medium pH were monitored online with an amperometric (Ag/AgCl) H2 probe (de Poorter et al., 2003; Schill et al., 1996) and a pH electrode (Ingold, Eilsolab Nederland BV), respectively.

Alternatively, Methanothermobacter thermautotrophicus was cultured in 115 ml serum bottles containing 50 ml mineral medium supplemented with 0.6 g Na2S·2H2O l−1. Growth was performed at various temperatures (50–65 °C) and pH values (6.0–7.5) to an OD600 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 H2/CO2 (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 N2/CO2 (80 : 20%, v/v; 120 kPa) atmosphere to an OD600 of 0.1–0.2.

Reduction of coenzyme F420. Purified coenzyme F420 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% H2, 20% CO2 complemented with N2 (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 OD600 of >1 were diluted with anaerobic mineral medium. After filling, bottles were closed with butyl rubber stoppers and aluminium-crimped seals, evacuated and pressured with mixtures of H2/CO2 (80 : 20%, v/v) and N2/CO2 (80 : 20%, v/v) to obtain the pHi 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 F420 fluorescence analysis.

Coenzyme F420 fluorescence measurements. A known volume of cells from the fermenter (~1–5 ml) or from cell-suspension incubations (~1 ml) was injected under anaerobic conditions into a serum bottle closed with a bromobutyl rubber stopper and containing ice-cold anaerobic acetone kept under N2/CO2 (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 F420 present in the samples (F420). To determine the fluorescence of total coenzyme F420 (Ftot), cell samples were mixed, after brief exposure to air, with oxic acetone and spectra were measured under aerobic conditions. To correct for background fluorescence (FB), cell samples were incubated under (H2/CO2) 80 : 20% at 65 °C, added to cold anaerobic 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 F420H2 and F420 were calculated as (F420h–F420)/F420. The experimental values (F420h–F420) were corrected for background fluorescence (FB) measured for the fully (80% H2) reduced cell samples. Acetone extracts were alkaline (pH 9–10). Under these conditions, oxidized F420 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 OD600 value of the culture. Previous research established the linear relationship between both parameters, at which 1 l culture showing an OD600 of 1 equaled 425 mg dry cells (unpublished results). pH values were measured by a previously described method, using the pH-dependent fluorescence properties of oxidized coenzyme F420 (de Poorter & Keltjens, 2001).
Coenzyme F₄₂₀ reduction in methane-forming cell suspensions of Methanothermobacter thermotrophicus

To investigate the effect of the applied pₜₜ on coenzyme F₄₂₀ reduction in methane-producing cells, cell suspensions of Methanothermobacter thermotrophicus were incubated under a variety of conditions and at 0–2 % (v/v) hydrogen in the gas phase (pₜₜ, 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 pₜₜ, the specific rates of methanogenesis in the suspension incubations were linearly dependent on the pₜₜ applied. Specific activities at a pₜₜ of 0–0.02 bar were 5–50 % of the maximal values measured at 80 % H₂ [1–3 μmol CH₄ min⁻¹ (mg DW)⁻¹]. The former percentages depended on the hydrogen concentration at which growth had occurred and reflect changes in the affinities (Kₐ) of the cells for hydrogen. It is known that Methanothermobacter thermotrophicus cells derived from cultures grown under low-hydrogen conditions display a higher hydrogen affinity (Kₐ, approx. 2 % H₂) than cells grown at a high hydrogen concentration (Kₐ, approx. 20 % H₂) (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 % H₂ 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 [F₄₂₀H₂]/[F₄₂₀] ratios and the pₜₜ 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 ϕₑ at +15 kJ mol⁻¹. Above data established a ΔGᵦ of −15 kJ mol⁻¹ at 60 °C and pH 7. From the resulting ΔGᵦ of 0 kJ mol⁻¹ (equation A.1), it is inferred that the concentrations of reduced and oxidized coenzyme F₄₂₀ within the cells are in thermodynamic equilibrium with the pₜₜ in the gas phase.

To investigate the effect of temperature and pH on the hydrogen-dependent reduction of coenzyme F₄₂₀, Methanothermobacter thermotrophicus 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 pₜₜ using medium pH values and temperatures at which culturing had occurred. Separate incubations were performed to measure the pH after incubation. At the experimental conditions, pHₜₜ was found to be equal to the medium pH. As before, [F₄₂₀H₂]/[F₄₂₀] ratios were related linearly to the pₜₜ 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 F₄₂₀ reduction is described by equation (5), in which F₄₂₀ refers to (deprotonated) phenolate anion (Fig. 7):

\[
\frac{[\text{F}_4\text{H}_2]}{[\text{F}_4]} = \frac{K_a}{1 + \frac{p_{\text{H}_2}}{15}}
\]

RESULTS

Hydrogen-dependent reduction of coenzyme F₄₂₀

F₄₂₀ was incubated in the presence of cell extract in a series of serum bottles under different pₜₜ values (°C, v/v), and fluorescence-excitation spectra were recorded after reactions had come to equilibrium (Fig. 1). F₄₂₀ incubated under an N₂/CO₂ atmosphere (80:20 %, v/v) showed maximal fluorescence emission at 427 nm excitation. The same fluorescence intensities of H₂-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 F₄₂₀ within the cells are in thermodynamic equilibrium with the pₜₜ in the gas phase.

Whole cells incubated under hydrogen revealed excitation and emission spectra that were indistinguishable from those obtained for purified F₄₂₀ (data not shown). This demonstrated that other cellular components did not interfere with F₄₂₀ fluorescence measurements. The fluorescence characteristics were subsequently used to determine the concentration ratios of reduced and oxidized F₄₂₀ in metabolizing cells.

Hydrogen metabolism and F₄₂₀ reduction
H₂ + H⁺ + F₄₂₀ − ⇌ F₄₂₀H₂

(5)

The plot of $RT \ln q_i$ versus pH gave a straight line (Fig. 3b). The slope (−6·4 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 $\Delta G^0$ 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 ($\Delta G_{60.0}$) sums as $\Delta G_{60.0} = RT \ln q_i$ (equation A.1), a $\Delta G_{60.0}$ 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 $RT \ln q_i$ term was constant (+ 15 kJ mol⁻¹) and exactly opposite to the (temperature-independent) $\Delta G^0$ of −15 kJ mol⁻¹, again demonstrating thermodynamic equilibrium ($\Delta G^0 = 0$) (Figs 4a and b).

By routine, cell-suspension incubations were performed at relatively low $p_{H₂}$ values (0–0·02 bar). When incubated at higher headspace-hydrogen concentrations, large variations in [F₄₂₀H₂]/[F₄₂₀] ratios were found among repeated experiments and the ratios were generally lower than expected. At the higher $p_{H₂}$ 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 [F₄₂₀H₂]/[F₄₂₀] ratios.
Changes in the ratios of reduced and oxidized coenzyme F<sub>420</sub> 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<sub>2</sub>/CO<sub>2</sub> (Fig. 6). Growth was characterized by an exponential increase of cell density up to an OD<sub>600</sub> of 1:7 [specific growth rate, 0.024 h<sup>-1</sup>; doubling time (t<sub>d</sub>), 1.9 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<sup>-1</sup> (mg DW)<sup>-1</sup>. Considering that 4 mol hydrogen is used (mol methane formed)<sup>-1</sup> (equation 1), the specific hydrogen-consumption rate amounted to 6–10 mol min<sup>-1</sup> mg<sup>-1</sup>. Together with the increase in biomass, the overall hydrogen-consumption rate increased tenfold (0.6–6 mmol min<sup>-1</sup>). The increase in hydrogen consumption was accompanied by the decline in the dissolved p<sub>H<sub>2</sub></sub> from 70 to 3 % (0.07 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<sub>H<sub>2</sub></sub> became constant at 6 mmol min<sup>-1</sup> and 0.03 bar, respectively. Now, pH was about equal to the medium pH of 7.0.

At regular time intervals, cells were collected anoxically from the fermenter and analysed for the [F<sub>420H</sub>]<sub>2</sub>/[F<sub>420</sub>] ratios (Fig. 6). The apparent ratios tended to decrease, but became somewhat higher during the linear-growth phase. From the recorded p<sub>H<sub>2</sub></sub> and pH values, [F<sub>420H</sub>]<sub>2</sub>/[F<sub>420</sub>] ratios were calculated theoretically, assuming thermodynamic equilibrium. It can be seen that experimental and theoretical ratios were about equal during the early-exponential (0–3 h) and linear (10–12 h) phases, where p<sub>H<sub>2</sub></sub> was as high as 0.70 bar and as low as 0.03 bar, respectively. During the intermediary-exponential phase, however, experimental [F<sub>420H</sub>]<sub>2</sub>/[F<sub>420</sub>] 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
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 ($\Delta G^\circ$, $-15$ kJ mol$^{-1}$). As the midpoint potential of the $H^+/H_2$ couple varies with temperature, $E_mT$ for the $F_{420}/F_{420}H_2$ couple has to show the same temperature dependency. On the basis of the experimental $\Delta G^\circ$ 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).

$$4 \text{CH}_3\text{OH} \rightarrow 3 \text{CH}_4 + \text{CO}_2 + 2 \text{H}_2\text{O} \quad (6)$$

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 $N^9$-methyl-$H_4MPT$ and $N^9,N^{10}$-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 $N^9,N^{10}$-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 rate during acetate metabolism. Apparently, hydrogen does not equilibrate with the intermediary $F_{420}$ metabolism, serving now only some specific anabolic steps.
In nature, methanogenic archaea form part of densely packed, complex microbial consortia that degrade organic matter into methane and CO₂ (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 F₄₂₀ 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 F₄₂₀ into 1,5-dihydro-F₄₂₀ (F₄₂₀H₂) with hydrogen. The (Gibbs) free-energy change, ΔG (kJ mol⁻¹), at specified reaction conditions (suffixed r: temperature, pH) of the reaction is:

\[
\Delta G_r = \Delta G_0^r + RT \ln q_r
\]  

(A.1)

in which R is the gas constant (8·314·10⁻³ kJ mol⁻¹ K⁻¹), T is the absolute temperature (K) and q_r is the mass-action ratio:

\[
q_r = [\text{F}_420\text{H}_2]/[\text{F}_420] \quad \text{PH}_2
\]  

(A.2)

q_r equals the slope in the experimental [F₄₂₀H₂]/[F₄₂₀] versus pH plots. It should be noted that [F₄₂₀] and [F₄₂₀H₂] represent total concentrations of the oxidized and reduced species, respectively. In the physiological pH range, the 5-deazaflavin chromophore of oxidized coenzyme F₄₂₀ contains one ionizable group, viz. 8-OH (pK_a1 6·3–6·47, 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 F₄₂₀, NH(1) (pK_a2 6·9) and the 8-hydroxyl group (pK_a1 9·7) are of relevance. Thus, oxidized and reduced F₄₂₀ are composed of a mixture of species that will affect the redox potential of the F₄₂₀/F₄₂₀H₂ couple in a pH-dependent fashion.

\[
\text{[F}_{420}\text{]}_{\text{tot}} = [\text{F}_{420}](1 + K_{a1}/[\text{H}^+])
\]  

(A.3)

\[
\text{[F}_{420}\text{H}_2]\text{tot} = [\text{F}_{420}\text{H}_2](1 + K_{a2}/[\text{H}^+] + K_{a2}.K_{a1}/[\text{H}^+]^2)
\]  

(A.4)

In addition, the free-energy changes of coenzyme F₄₂₀ reduction with hydrogen will vary with the pH:

\[
\text{H}_2 \pm n\text{H}^+ + \text{F}_{420}\text{H}_2 \rightarrow \text{F}_{420}\text{H}_2
\]  

(A.5)

Defining ΔGₐₙₖᵣ (kJ mol⁻¹) as the free-energy change at pH 7 and at the

**Fig. 7. Structure of coenzyme F₄₂₀ and its (de)protonation and redox reactions.**
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:

\[
\Delta G^0_r = \Delta G^0_i \pm 2.303 \, m \, RT \, (pH) \tag{A.6}
\]

\[
\Delta G^0_r = nF \Delta E_m,7 \tag{A.7}
\]

In equation (A.6), the sign of the term is minus in a proton-consuming reaction. In equation (A.7), \( n \) is the number of electrons involved, \( F \) is the Faraday constant (96,484 J V\(^{-1}\) mol\(^{-1}\)) and \( \Delta E_{m,7} \) is the difference between the midpoint potentials (V) of the \( H^{+}/H_2 \) (\( E_{m,7} \)) and \( F_420/F_421H_2 \) (\( E_{m,7} \)) redox couples, respectively, at pH 7 and the specified temperature. \( E_{m,7} \) is derived for each given temperature from the Nernst equation: \( E_{m,7} = -2.303(7RT/F) \). \( E_{m,F} \) should be measured, or it can be calculated if \( \Delta G^0_r \) (at pH 7) is known. The latter can be determined from the reaction at equilibrium. Considering that, under these conditions, \( \Delta G^0_r = 0 \) and that the mass-action ratio \( (q_r) \) equals the equilibrium constant \( K_r \), it follows from equation (A.1):

\[
\Delta G^0_r = -RT \ln K_r \tag{A.8}
\]

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