Continuous monitoring of the cytoplasmic pH in *Methanobacterium thermoautotrophicum* using the intracellular factor F₄₂₀ as indicator

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The absorption spectrum of factor F₄₂₀ changes depending on the pH and the redox state of the cytoplasm. Specific wavelengths were used to calibrate absorption changes to allow the measurement of changes in the cytoplasmic pH in *Methanobacterium thermoautotrophicum*. Upon a hydrogen pulse, a rapid efflux of protons was observed. Under these energized conditions, the ΔpH amounts to 0–2–0 pH units at pH 6–6, and 0–6–0 pH units at pH 6–0. It decays within 10–20 s. In parallel, a sodium gradient is formed which has a slightly longer lifetime. Both ΔpH and ΔΨ contribute to the proton-motive force present during methanogenesis. The energy-conversion rate, as indicated by the decay of the energized state of the cell, is fastest under growth conditions, i.e. at pH 6–9 and at a temperature of 58 °C.

**Keywords:** cytoplasm, pH gradient, intracellular pH, proton-motive force, methanogenic bacteria

**INTRODUCTION**

The pH of the cell cytoplasm is a critical parameter controlling a variety of cellular processes. In most organisms, the pH in the cytoplasm is maintained over a range of approximately two pH units around neutrality (Padan *et al.*, 1981). However, this pH homeostasis is dependent on energy. The pH surrounding cells or cell aggregates is the main environmental factor that strongly determines growth and metabolism. It is crucial to the size of the pH gradient across the cell membrane, which forms an essential part of the proton-motive force driving biological energy conversion.

In suspension cultures of micro-organisms, the external pH is easily followed by using electrodes. In contrast, the determination of cytoplasmic pH in cells as small as bacteria is more difficult. Certain methods are based on the distribution of radiolabelled weak acids or bases whereby the cells (after incubation) are separated from the medium by rapid filtration or centrifugation. As sampling is periodic, the data from these methods are not continuous (Padan *et al.*, 1981). Spectrometric methods, however, either absorption or fluorescence spectrometry, use specific pH indicators to monitor the cytoplasmic pH continuously.

The first descriptions of pH estimations using dyes (fluorogenic esters) were given by Thomas *et al.* (1979, 1982) for tumour cells and bacteria. The prerequisites of the method are as follows: (1) the cells must be permeable to these colourless and non-fluorescent esters; (2) the indicators must be concentrated in the cells; (3) an intracellular esterase must cleave off an absorbing or fluorescing species; (4) the membrane must be impermeable to the negatively charged species formed by the hydrolysis (so that the indicator remains in the cells, at least for the duration of the experiment); (5) a calibration curve must be obtained; and (6) no other cellular compounds should interfere (by absorbance or fluorescence) with the marker compound. Generally, not all of these prerequisites are fulfilled. To prevent leakage of the pH-indicator, a dye forming covalent bonds with cytoplasmic compounds has been developed (Breeuwer *et al.*, 1996). However, the question as to whether or not indicator dyes interact with cytoplasmic proteins and cause erroneous results remains open to debate (Yassine *et al.*, 1997). To compensate for unequal uptake of the dye and varying esterase activity, fluorescence ratios from two wavelengths were analysed to follow the pH (Aono *et al.*, 1997). Recently, fluorescence ratio microscopy imaging even made it possible to follow the pH of single bacterial cells in a mixed culture.

**Abbreviations:** BESA, bromoethanesulfonic acid; CF, carboxyfluorescein; TCS, tetrachlorosalicylanilide.
(Siegumfeldt et al., 1999). These studies demonstrate the versatility of spectroscopic techniques in the investigation of pH homeostasis and the dynamics of pH changes during energy transduction.

The metabolism (including energy transduction) of methanogenic bacteria has been studied intensively in recent decades, as reviewed, for example, by Deppenmeier et al. (1996) and Schäfer et al. (1999). In the process of the stepwise reduction of CO₂ to CH₄ by H₂, protons are extruded, giving rise to a pH gradient, which, along with a Na⁺ gradient and the membrane potential, is an important component of the driving force for ATP synthesis in a chemiosmotic mechanism. Changes in membrane potential upon the energization of whole cells of *Methanobacterium thermoautotrophicum* have been measured by Butsch & Bachofen (1984). Using the dye carboxyfluorescein (CF), Bachofen & Butsch (1986) demonstrated, qualitatively, the formation of a pH gradient upon cell energization. The length of the signal correlated with the partial pressure of hydrogen gas introduced, whereas its magnitude was independent of the partial pressure over the range 20–80 % (v/v) hydrogen in the gas mixture.

In the present work, the endogenous factor F₄₂₀ was used as an intrinsic pH indicator. It fulfils most of the requirements cited above for a cytoplasmic pH indicator.

**METHODS**

All chemicals used were of highest analytical grade and were obtained from Fluka, Sigma or Merck. Gases were obtained from Butsch & Bachofen (1984). Stock cultures were kept at −80 °C. The medium was based on that of Schönheit et al. (1979), as modified by Butsch & Bachofen (1984), and contained the following: NH₄Cl (40 mM), MgCl₂·6H₂O (1-5 mM), nitritotriacetate (0-15 mM), NaCl (10 mM), KH₂PO₄ (10 mM), K₂CO₃ (0-15 mM), Na₂MoO₄·2H₂O (1 mM), NiCl₂·6H₂O (1 mM) and FeCl₃·4H₂O (25 mM). Cells were grown in chemostat mode in a 2 l bioreactor equipped with controls for temperature, pH and redox potential, as described by Jud et al. (1997). The temperature was held at 58 °C and the pH at 6-9. The culture was supplied with Na₂S (310 mM) at intervals, producing a final concentration of 0.5 mM sulfide in the reactor. The gas supplied was H₂/CO₂ 80%:20% (v/v); the rate was controlled electronically and kept at 220 ml min⁻¹ (equal to 0.12 vol. per vol. per min). Traces of oxygen were removed by a BASF catalyst, R0–20, sealed in an iron tube in the gas supply line. The sterile media were kept under nitrogen.

**Spectroscopic investigations.** The absorption spectra of solutions were obtained with a Unicam 810 spectrophotometer. Optical measurements of cell suspensions were obtained with an Aminco DW-2 dual-wavelength spectrophotometer using either specially made anaerobic cuvettes with rubber septa or a 30 ml minibioreactor built in our workshop and coupled to the DW-2 optics by light pipes. The spectrophotometer was connected to a computer with an ADALAB A/D converter (Interactive Microwave).

**Gas analysis.** Hydrogen, methane, oxygen, carbon monoxide and carbon dioxide were quantified by gas chromatography [using a Shimadzu GC-R1A with integrator RPR-G1 and a CSS column Carbosieve S 120/140 (Supelco)] with a TCD detector. The gas was supplied reproducibly to the cuvettes and the minibioreactor through a stainless steel needle as pulses of 10 s at a flow rate of 240 ml min⁻¹ by a computer-controlled valve.

**Cell preparations.** Cells were harvested by centrifugation (15 min at 1800 g, 4 °C) and washed twice with growth medium. All steps were performed under anoxic conditions using bottles flushed nine times with nitrogen (cycling between 0.5 and 2 bar). For the experiments, the cell concentration, measured as OD₄₅₀ was set between 1.5 and 2, which is equivalent to 0.8–1.1 g cells (dry weight) l⁻¹. The suspension was transferred either into an anaerobic cuvette equipped with a valve for gas pulses or into the minibioreactor in an anaerobic box (Forma Scientific 1024). To ensure the complete absence of oxygen, all glassware was kept in the anaerobic box for 24 h prior to the experiments. All manipulations were done under strictly anaerobic conditions, in closed vessels under purified nitrogen or in the anaerobic glove box. If these precautions for anoxic conditions were observed, the cells could be used reproducibly in experiments over a period of at least 4 h.

**Measurement of the pHₘ.** The cuvette or the minibioreactor was stirred and kept at constant temperature under strictly anaerobic conditions during the measurements. The Aminco DW-2 spectrophotometer was used in the dual-wavelength mode, which allows the quantification of small absorption changes in the presence of a large optical background signal (cell scatter). The determination of the relevant wavelengths and the calibration of the intracellular pH (pHₘ) are described in Results.

**Isolation of F₄₂₀.** F₄₂₀ was isolated, according to Cheeseman et al. (1972) and Schönheit et al. (1981), by extracting the compound using 50% (v/v) acetone followed by chromatography twice on QAE-Sephadex A-25.

**Other determinations.** The minibioreactor was equipped with electrodes for the continuous measurement of pH, redox potential and Na⁺ ions (Ingold). The OD₄₅₀ of the cell suspension was calibrated by using dry-weight determination.

**RESULTS AND DISCUSSION**

Previous experiments using CF demonstrated a hydrogen-induced change in absorption which was interpreted as a rise in intracellular pH (Bachofen & Butsch, 1986). However, the spectrum of CF overlaps with that of the intrinsic electron carrier, F₄₃₀ and no useful calibration was obtained with CF. Similarly, carboxynaphthofluorescein could not be used satisfactorily as an indicator for the intracellular pH, although it has an absorption maximum shifted 100 nm to longer wavelengths. Factor F₄₃₀ has a defined absorption spectrum (Fig. 1) that changes upon reduction and also shows a typical fluorescence. This allows methanogens to be easily distinguished from other bacteria in environmental samples (Cheeseman et al., 1972). Furthermore, the signal has been found to be proportional to the biomass (Reuter et al., 1986). As the absorption spectrum of F₄₃₀ is also dependent on pH (Cheeseman et
calibration curve was obtained for whole cells of *Methanobacterium thermoautotrophicum* pH 5 energized conditions in the absence of an electron donor, and Bachofen & Butsch (1986) demonstrated that no measurable proton potential is present under de-...%#!To compensate for variations in F$_{420}$ concentration in different preparations, both wavelengths, 400 and 420 nm, are related to the absorption at 450 nm as the reference wavelength [ratio ($A_{420} - A_{450}$)/($A_{400} - A_{450}$)]. For isolated F$_{420}$, the calibration curve is linear between pH 5–8 and pH 7–5. Using the same wavelengths, a calibration curve was obtained for whole cells of *M. thermoautotrophicum*. Schönheit & Beimborn (1985) and Bachofen & Butsch (1986) demonstrated that no measurable proton potential is present under de-energized conditions in the absence of an electron donor, and that the pH$_{in}$ is then in equilibrium with, and thus equal to, the external pH (pH$_{out}$). Measurements were taken with cell suspensions equilibrated under nitrogen at medium pH (pH$_{out}$) values between 5–8 and 7–2. This ratio method makes the pH determinations independent of the intracellular concentration of the marker, F$_{420}$, and compensates for parallel absorption changes of unknown compounds or variable scattering effects of cell suspensions (Kotyk & Slavík, 1989).

The experiment presented in Fig. 2 is an example showing the kinetics of the changes in pH$_{in}$ as calculated from the calibration curve. The rapid alkalization of the cytoplasm upon the substrate pulse cannot be visualized because bubbles produced during gas injection make optical measurements impossible. Under defined conditions, the duration of the alkalization until the pH$_{in}$ returned to the initial pH of the medium was highly reproducible within the same cell preparation but it could vary between cell batches. The redox potential in the medium did not change after a hydrogen pulse (not shown). The size of the pH increase in the cytoplasm was dependent on the pH$_{out}$: it is larger at acid values of the medium pH and becomes smaller towards neutrality. Around neutrality, Schönheit & Beimborn (1985) found, using *M. thermoautotrophicum*, that they could not measure a ΔpH under metabolically active conditions; in a more acidic environment (pH 5), however, the cytoplasm was found to be more alkaline, resulting in a pH gradient of 1–1.3 pH units under energized conditions. This is in close agreement with our observations. In contrast, the ΔpH reported by Dybas & Konisky (1992) for *Methanococcus voltae* under growing conditions was only a few millivolts.

The duration of the signal change upon hydrogen addition characterizes the length of time for which the cells stay in an energized state, conditions when a pH gradient and a membrane potential are present across the membrane. This is governed by the amount of H$_2$ taken up by the cells, the concentration of H$_2$ reached in...
the medium after flushing, and (mainly) by the speed at which the electrons are used up in cellular metabolism. The length of the signal is determined from the time immediately after hydrogen injection (pulses of H₂/CO₂, 80:20, 10 s duration) to the time at which 50% decay is reached; it was strongly dependent on environmental factors such as temperature (Fig. 3). The decay of the hydrogen-induced proton gradient is rapid between 45°C and the growth temperature of 58°C, but slows down rapidly at lower temperatures – a temperature-dependence similar to that of the growth rate of the organism.

Control experiments with a nitrogen/CO₂ mixture (80:20) and with inactivated denatured cells (stirred for 48 h under air, conditions in which F_{420} is rapidly inactivated and converted to factor_{190} (Hausinger et al., 1985; Schönheit et al., 1981) prove that the hydrogen-induced pH changes are driven by the bacterial metabolism.

It has been suggested that F_{420} is not homogeneously distributed within the cell but is, rather (for functional reasons) concentrated near the membrane at the site of the hydrogenase (Muth, 1988). Thus, an averaging method such as the distribution of weak acids and bases (Schönheit & Beimborn, 1985) or measurement with homogeneously distributed indicator dyes may not give the same values as a more localized pH indicator (Kotyk & Slavik, 1989). The pH_{in} measured by F_{420} probably does not indicate the mean pH within the cell but, rather, represents the pH found close to the membrane. This would support the suggestion that the pumped protons are held along the membrane by anionic lipids and are not in equilibrium with the cytoplasm (Haines, 1983).

The concentration of Na⁺ ions in the medium, important for the formation of the ΔΨ in methanogens, was measured simultaneously. It increases when hydrogen is supplied, and the return to the original value is delayed relative to the decay of the ΔpH. The decay clearly accelerates after the ΔpH has dropped to below approximately 50% of the original size (Fig. 4). At the cell concentration chosen for the experiments, the ratio between the volume of medium and the volume of cells has been estimated to be approximately 600. Thus, an Na⁺ increase of a few millimoles per litre in the medium upon an H₂ pulse would represent a drastic decrease in the ion concentration in the cell. Although most of the Na⁺ ions may have been bound to cell components, it indicates the formation of a noticeable membrane potential. Indeed, it has been suggested that Na⁺ and K⁺ ions are strongly complexed with specific lipids of the cell membrane (Kramer et al., 1988). The Na⁺ efflux is probably the result of a directly coupled Na⁺ pump, whereas the Na⁺/H⁺ antiporter driven by H⁺ extrusion during methanogenesis acts as a mechanism for regulating the pH_{in} (Deppenmeier et al., 1996; Schäfer et al., 1999). Because of the presence of CO₂ in the gas pulse, the pH of the medium drops slightly and returns slowly (within 30–40 s) to the original value before the pulse (Fig. 4).

Inhibitors of methanogenesis, such as the uncoupler tetrachlorosalicylanilide (TCS), the Na⁺ ionophore monensin, the inhibitor of the sodium–proton antiporter amiloride (for a review, see Kleyman & Craigoe, 1988), and the analogue of Coenzyme M and inhibitor of the methylreductase, bromoethanesulfonic acid (BESA), were tested for effects on the hydrogen-induced pH_{in} changes. With TCS at 10 µM, the formation of a ΔH was completely abolished, which is typical of an uncoupling action. The other reagents altered the reaction kinetics less drastically (Table 1). Monensin at 10 µM reduced the initial ΔpH upon the H₂ pulse and slowed down the signal recovery after the pulse. BESA showed no effects on the size of the ΔpH up to 50 mM, but, again, the decay time also had increased. Amiloride at 10 µM had some effect on the initial size of the ΔpH and also retarded the decay kinetics. A prolonged signal duration is indicative of a longer lifetime of the pH.
gradient. In the presence of amiloride, the Na\textsuperscript{+}/H\textsuperscript{+} antiporter, and thus the conversion of a \(\Delta p\text{H} \) into a \(\Delta N\text{a}\textsuperscript{+} \) is blocked. Similar effects of amiloride were noted by Müller et al. (1987) for Methanosaeta barkeri. Monensin abolishes part of the \(p\text{H} \), probably as a consequence of the higher Na\textsuperscript{+} permeability.

Conclusions

Following absorption changes of the endogenous marker F\textsubscript{120} at three wavelengths allows the determination of p\text{H} changes in the cytoplasm of whole cells of methanogenic bacteria. The \(\Delta p\text{H} \) is dependent on the external p\text{H}; its decay is determined by the metabolism and affected by external factors. Furthermore, it is influenced by inhibitors of proton and sodium transport.

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REFERENCES


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