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VALINOMYCIN INHIBITED METHANE SYNTHESIS IN METHANOBACTERIUM THERMOAUTOTROPHICUM

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SUMMARY: Methanobacterium thermoautotrophicum cells, incubated anaerobically under H_2 in 0.1 M KCl or 0.1 M NaCl, above pH 7.5, are interior acid with respect to the incubation medium. The pH gradient thus established can be discharged by either carbonyl cyanide m-chlorophenylhydrazone or valinomycin at high concentration (17μM). In these cells, which actively synthesize CH_4 from CO_2 and H_2, methanogenesis is strongly inhibited when the pH gradient is discharged.

INTRODUCTION: Under standard conditions, the overall reduction of CO_2 with H_2 to CH_4 is an energy yielding reaction. The first step, i.e. the reduction of CO_2 to the level of formaldehyde is endergonic (+ 25 kJ/mol)(1). The reduction of formaldehyde to CH_4 is exergonic and Thauer et al (1) calculate that the $\Delta G^{0'}$=-157 kJ/mol for this reaction. Although in intact cells, CH_4 synthesis results in an increased energy charge ([ATP] + [0.5 ADP])/([ATP] + [ADP] + [AMP]), vesicles, carefully isolated from these cells, synthesize CH_4 without the apparent involvement of ATP (2,3). No CH_4 was synthesized if the vesicles were disrupted with deoxycholate (2) or by high pressure in a Ribi cell fractionator (3). Chemical reagents which decrease the transmembrane electrochemical proton potential, $\Delta\bar{\mu}H^+$, also inhibit CH_4 synthesis. Of those tested, the proton conducting reagents, 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone (CCCP), which abolish both electrical potential ($\Delta\psi$) and pH gradient (ΔpH) were most inhibitory (2). Lipophylic cations, triphenylmethyl phosphonium bromide and tetrabutyl ammonium bromide, which are thought to abolish $\Delta\psi$ (4) also inhibited CH_4 synthesis.

ABBREVIATIONS:
CCCP carbonyl cyanide m-chlorophenylhydrazone; $TPMP^+$ triphenylmethylphosphonium (bromide salt); $\Delta\psi$, membrane potential; ΔpH, transmembrane proton gradient; $\Delta\bar{\mu}H^+$, proton motive force.

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The present results show that above pH 7.5, M. thermoautotrophicum cells, incubated in 0.1 M K^+ or Na^+, are interior acid (with respect to the incubation medium) and when the pH gradient is abolished, CH_4 synthesis is inhibited.

MATERIALS AND METHODS: Cells of M. thermoautotrophicum (A.T.C.C. 29183) were cultured, harvested and stored as described (3). Frozen cells were thawed under H_2, sedimented at 9000g for 15 min and resuspended in the appropriate buffer. All steps were done at 0-5° in an atmosphere of O_2 free H_2. H_2 was purified by passage through a heated copper column.

For pH measurements, a Corning combination electrode (no.476050) was inserted through a black rubber stopper (Bellco-Glass Co., Vineland, N.J.) which was fitted on a Wheaton 10 ml serum bottle. The sealed bottle had an inlet (23 ga. needle attached to polyethylene tubing, 0.58 mm ID) and an outlet (17 ga. needle attached to 1.27 mm ID tubing). The bottle was repeatedly evacuated and filled with O_2 free H_2, the inlet tubing was clamped and the outlet tubing submerged under 8 cm water to prevent pressure changes during incubation. The appropriate buffers which contained 2 mM Na_2S (4.0 ml), and cells (1.0 ml, 4-10 mg protein), were injected through the stopper with a 23 ga. needle. After temperature and pressure equilbration, pH measurements were made with a Corning Digital 112 pH meter and an Instrument Specialties recorder, calibrated to give full scale deflection for 0.045 pH units. Incubations were done at 60° with constant shaking. After each incubation, the system was calibrated by recording the deflection for known quantities of acid injected through the inlet tube. Uncoupling reagents were injected through the rubber stopper at specified times, with a Hamilton microsyringe. CH_4 determination and radioactivity analyses were done as described previously (2,5).

Carbonyl cyanide m-chlorophenyl-hydrazone was purchased from Sigma Chemical Co. Valinomycin and gramicidin were purchased from Calbiochem-Behring Corp.

RESULTS: M. thermoautotrophicum cells incubated under H_2 at pH 7.5 or higher, show a transient uptake of H^+ which makes the cell interior acid with respect to the incubation medium (Fig.1; A,B,C). With cells incubated in 0.1 M KCl, valinomycin completely discharged a pH gradient (interior acid) and the addition of CCCP caused no further pH change (Fig. 1A). Valinomycin did not completely discharge the pH gradient (interior acid) when cells were suspended in 0.1 M NaCl (Fig. 1B). In this case H^+ release followed the addition of CCCP. The addition of gramicidin resulted in the release of intracellular H^+ when cells were incubated in 0.1 M NaCl (Fig. 1C). The further addition of CCCP resulted in only a small increase in extracellular $[H^+]$. When CCCP was added to M. thermautotrophicum cells before

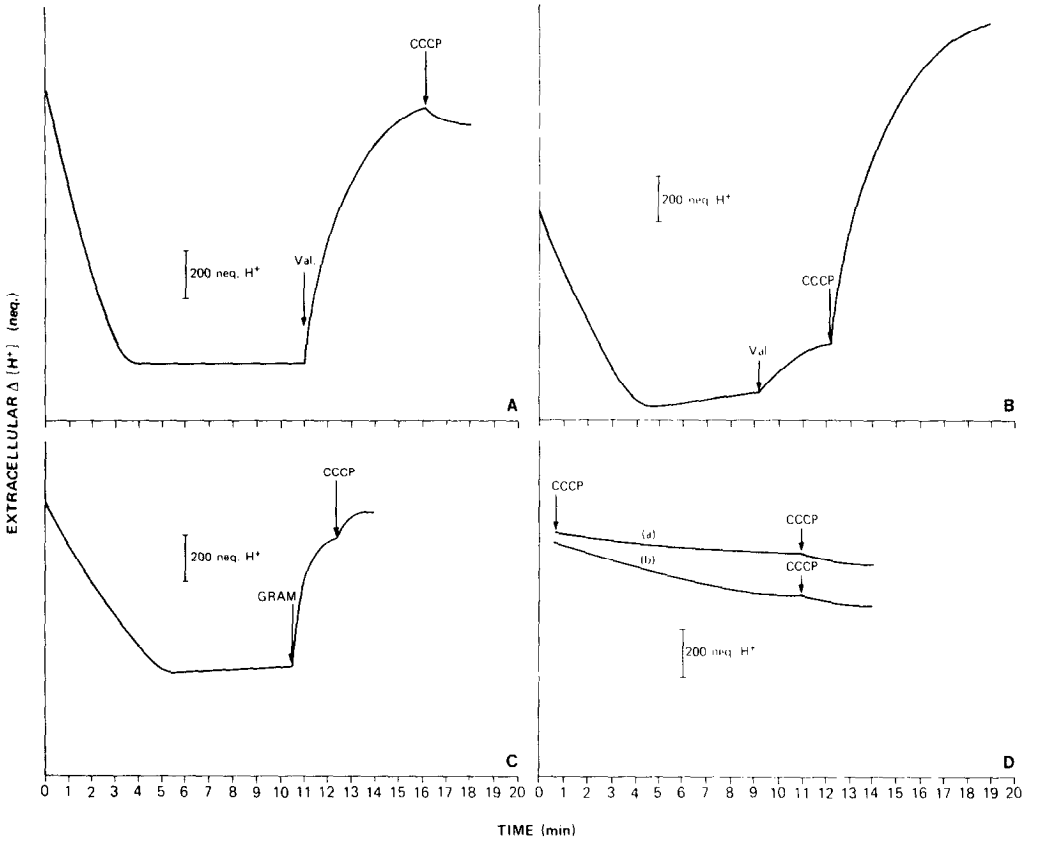

Fig. 1. Extracellular pH changes in M. thermoautotrophicum cells which synthesize CH_4 (A) Washed cells were incubated in a 10 ml Wheaton flask (actual internal vol. 13.5 ml) in KCl, 0.1 M; Na_2S, 2 mM; $NaHCO_2$, 0.4 mM; KPi, 0.25 mM, cell protein 7.0 mg in a final vol. of 5.0 ml, with the initial pH 7.60. Incubations were done under an atmosphere of O_2 free H_2, purified over a hot copper column, at 60°. Valinomycin (17μM) and CCCP (100μM) were added at indicated time. Acid deflection (↑) was calibrated with the addition of dilute HCl. (B) The incubation was exactly as in (A) except 0.1M NaCl was substituted for 0.1M KCl. Cell protein, 8.4 mg, starting pH 7.82. (C) The incubation was exactly as described in (A) Cell protein, 8.6 mg, starting pH 7.74. Gramicidin, (17μM) and CCCP (100μM) were added at indicated time. Gramicidin, unlike valinomycin, gave a stepwise acid deflection (not shown) (D) In (a) the cells (4.4 mg protein) were uncoupled with CCCP (100μM) prior to the incubation which was exactly as described for (A). For (b) the cells (6.0 mg protein) were incubated as described for (A) except that an argon gas phase was substituted for O_2 free H_2.

the incubation, both the initial H^+ uptake and the H^+ release after CCCP addition, were absent (Fig. 1D). There was no measurable pH deflection with cells incubated under argon.

Apparently, the oxidation of H_2 is required for the establishment of a transmembrane H^+ gradient in these cells when incubated at pH 7.5 in the presence of high K^+ or Na^+ concentrations.

Table 1. Effect of valinomycin and carbonyl cyanide m-chlorophenylhydrazone on methane synthesis in M. thermoautotrophicum cells

	Methane Synthesis	
	32 min	42 min
	dpm x 10^{-3}(nmol)	dpm x 10^{-3}(nmol)
'KCl' cells	1087 (1680)	2247 (2110)
'KCl' cells + valinomycin	145 (980)	242 (1060)
'KCl' cells + CCCP	210 (1080)	362 (1290)
'Phosphate' cells + valinomycin	1288 (3050)	2738 (3920)

'KCl' cells were incubated in a 10 ml flask in 0.1M KCl;2 mM Na_2S; 0.4 mM $NaHCO_3$; 0.25 mM KPi; 11.0 mg cell protein in a final volume of 5.0 ml. Starting pH was adjusted to 7.5 with 0.1 N HCl. 'Phosphate' cells were incubated in 0.1 M potassium phosphate (pH 7.0); 2 mM Na_2S and 6.0 mM $NaHCO_3$. Some endogenous CO_2 was always present in cells and buffers. Valinomycin (17μM) or CCCP (100μM) was added after 15 min incubation where indicated.

The flasks were incubated for 15 min. At this time 840 ± 43 n mol CH_4 had been synthesized and the inhibitor additions (CCCP or valinomycin) were made as indicated. The incubations were continued for an additional 7 min (total time elapsed, 22 min) and $NaH^{14}CO_2$ (4.27×10^6 dpm) was added to each flask. After an additional 10 and 20 min (total incubation times, 32 and 42 min) gas samples, were removed, for mass and radioactivity measurements. Numbers in brackets indicate total CH_4 produced (in nmol).

Cells suspended in 0.1M KCl actively synthesized CH_4 (Table 1). Production of $^{14}CH_4$ was linear for the 10 and 20 min intervals (32 and 42 min total incubation time). When valinomycin was added to the cells suspended in 0.1 M KCl after 15 min, the 10 and 20 min $^{14}CH_4$ production rates were inhibited 87% and 89% below control rates, respectively. The addition of CCCP inhibited $^{14}CH_4$ production 81% and 84%, respectively. Some CH_4 synthesis persisted even after the M. thermoautotrophicum cells were uncoupled with CCCP. Valinomycin did not inhibit CH_4 synthesis when the cells were incubated in 0.1 M phosphate buffer at pH 7.0.

DISCUSSION: The electrochemical proton gradient, $\Delta\bar{\mu}H^+$, consists of a membrane potential, $\Delta\psi$, a proton gradient ΔpH, and is described by:

$$\Delta\bar{\mu}H^+ = \Delta\psi - (\frac{2.3RT}{F})\Delta pH$$

Bacterial cells and right side out vesicles are interior negative and alkaline with respect to the suspending medium and $\Delta\bar{\mu}H^+$ is expressed in mV. In Escherichia coli cells, ΔpH changes with external pH, from a calculated 118 mV at pH 6, to 0 at pH 7.65 (6). At pH 8 a reversal of ΔpH occurs (-12 mV) which is subtracted from Δψ and decreases the $\Delta\bar{\mu}H^+$ value (7). A decrease in ΔpH as a result of increasing external pH, is generally compensated for by an increased Δψ so that $\Delta\bar{\mu}H^+$ remains relatively constant for E. coli cells maintained between pH 6-8 (7). Similar results are observed with vesicles prepared from E. coli cells (8,9) although there is no inversion of the pH gradient above pH 7.5.

M. thermoautotrophicum cells suspended in 0.1 M KCl at pH 7.5 or higher became interior acid (with respect to the incubation medium) when incubated under H_2. An inverse proton gradient is thus established which can be discharged by a proton conductor such as CCCP. When uncoupled, these cells released up to 158 neq. H^+ per mg protein. This suggests that an inverse proton gradient of considerable magnitude can be established. Abolishing this proton gradient resulted in an inhibition of CH_4 synthesis by more than 80%.

With E. coli cells incubated above pH 8, the small, inverse ΔpH value (in mV), subtracted from a large Δψ value does not greatly decrease $\Delta\bar{\mu}H^+$ (7). It remains to be established if Δψ in M. thermoautotrophicum cells incubated at high pH and salt concentration is sufficiently large to cancel out the inverse ΔpH and maintain a sufficiently large $\Delta\bar{\mu}H^+$ to meet the energy requirements of the cell. If not, then the possibility must be considered that in these cells energy can be derived with a proton gradient that is inversed, i.e. interior acid.

Initial attempts to measure Δψ in these cells were unsuccessful. There was no measurable [^{3}H] triphenylmethylphosphonium ($TPMP^+$) uptake even after pretreatment of the cells (10). In the absence of KCl, and at pH 7.2 there was no detectable H^+ release from the cells after the addition of

either CCCP or valinomycin (data not shown). This suggests that without KCl, and at an external pH below 7.5, ΔpH is decreased.

Valinomycin, which is a mobile K^+ carrier, and gramicidin, which forms channels in membranes (11) are not H^+/cation exchangers (such as monensin or nigericin) when used at low concentration. Valinomycin, at low concentrations, when added to respiring E. coli cells (2μg/mg cells protein) (6) or respiring E. coli vesicles (1-5 μM), (9) decreases transmembrane $\Delta\psi$ and increase transmembrane ΔpH in a reciprocal manner so that $\Delta\bar{\mu}H^+$ remains relatively constant. Valinomycin used at high concentrations (17μM) with actively metabolizing M. thermoautotrophicum apparently acts as an H^+/K^+ exchanger since the addition of this ionophore triggers the release of intracellular H^+ when these cells are suspended in 0.1 M K^+ at pH>7.5. This has an "uncoupling" effect and results in inhibited CH_4 production like that obtained with the addition of CCCP. Valinomycin is known to have an uncoupling effect in the presence of high K^+ (6,12). In valinomycin treated E. coli cells incubated in 0.15 M KCl, the $\Delta\bar{\mu}H^+$ decreased from the control value of 122 mV to 70 mV, however, it was $\Delta\psi$ and not ΔpH which decreased (6).

With M. thermoautotrophicum the valinomycin effect was cation specific. When cells were suspended in 0.1 M NaCl, gramicidin, but not valinomycin, triggered the H^+ release. The proton conductor CCCP discharged the pH gradient with either K^+ or Na^+. These results show that M. thermoautotrophicum cells can establish an inverse pH gradient when incubated in high salt concentration above pH 7.5 and that when the pH gradient is discharged. CH_4 synthesis is effectively inhibited.

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