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Proton translocation coupled to methanogenesis from methanol + hydrogen in *Methanosarcina barkeri*

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Addition of methanol to resting cells of *Methanosarcina barkeri* incubated under an atmosphere of molecular hydrogen resulted in an acidification of the medium. This acidification was not observed when H_2 was replaced by N_2 or air, or when the uncoupler tetrachlorosalicylanilide was present. 2-Bromoethanesulfonate completely inhibited both methanogenesis and proton extrusion. *N,N'*-Dicyclohexylcarbodiimide, an inhibitor of the proton-translocating ATPase in *M. barkeri*, did not affect proton extrusion. Therefore, it could be concluded that proton translocation was coupled to the terminal methylcoenzyme M methylreductase reaction and that it was not due to an H^+-translocating ATPase. A maximum value of 4 H^+ translocated per CH_4 formed was calculated.

Methanogenesis; Proton translocation; (*M. barkeri*)

1. INTRODUCTION

Compared to other methanogenic organisms *Methanosarcina barkeri* has a broad substrate spectrum: besides $H_2 + CO_2$ also methanol, acetate [1], CO [2], methanol + H_2 [3] as well as methylamines [4] may be utilized as substrates. The organism's ability to directly reduce methanol to methane in the presence of molecular hydrogen allowed investigations of the energetics of the terminal methylcoenzyme M methylreductase reaction in intact cells [5]. These investigations provided evidence that *M. barkeri* employs a chemiosmotic mechanism for the synthesis of ATP [6,7]. However, the transmembrane electrochemical gradient of protons was measured only indirectly using the equilibrium distribution method of the lipophilic tetraphenylphosphonium cation (for $\Delta\psi$) or of benzoic acid (for ΔpH). Here we report on experiments directly demonstrating proton ejection coupled to methane formation.

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2. MATERIALS AND METHODS

2.1. *Organism and cell preparation*

M. barkeri, strain Fusaro (DSM 804) was grown on methanol as described before [4]. Cells were harvested by centrifugation, washed once with 1 mM imidazole-HCl buffer, pH 6.9, containing 1 mg resazurin and 2 ml titanium (Ti)(III) citrate solution [8]. The resulting cell suspension contained 10–20 mg of protein per ml and was stored on ice until used in the experiments. All manipulations were done in an anaerobic hood. Protein was determined according to [9] using bovine albumin as a standard.

2.2. *Measurement of proton translocation*

For proton translocation experiments a double-sided glass vessel (190 ml) thermostatted at 37°C was used. A pH electrode (Orion, Cambridge,

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USA) was inserted into the vessel from the side through a rubber stopper. The electrode was connected with an Orion model 720 pH meter and a chart recorder (Kipp & Zonen, Kronenberg, FRG). The vessel was filled aerobically with 24–27 ml of 1 mM imidazole-HCl buffer, pH 6.7, containing 200 mM choline chloride, 50 mM KSCN, and 1 mg resazurin/l. This buffer was subsequently gassed for 20 min with H_2 by means of two needles inserted from the top through a rubber stopper. Following the reduction of the medium with 50 μl of titanium (Ti)(III) citrate solution [8], 3–6 ml of the cell suspension of *M. barkeri* described above were added to give a final volume of 30 ml. The medium was continuously stirred and if necessary the pH was adjusted to 6.6–6.8 with either HCl or carbonate-free KOH. After incubation for at least 20 min, pulses of methanol were added to the cells with a microliter syringe. The pH changes were calibrated with either 10 mM HCl or 10 mM KOH prepared from standard solutions (Fluka and Merck, respectively). *N,N'*-Dicyclohexylcarbodiimide (DCCD) and tetrachlorosalicylanilide (TCS) were added as ethanolic solutions.

2.3. *Measurement of methane formation*

For measurement of methane formation, 1–2 ml of the cells prepared as described above were added to 9 or 8 ml of the same anaerobic buffer used for proton translocation experiments. The experiments were done in 58-ml bottles closed with rubber stoppers and previously gassed with H_2 or N_2. Methanol was added as indicated for each experiment. Methane was determined by gas chromatography as described [6].

2.4. *Determination of $\Delta\psi$*

$\Delta\psi$ was estimated from the equilibrium distribution of [^{14}C]tetraphenylphosphonium (Ph_4P^+) as described [6]. 1 μCi [^{14}C]Ph_4PBr was added to 10 ml of the resting cell suspension mentioned above to give a final Ph_4PBr concentration of 10 μM. The internal and total water spaces of *M. barkeri* were determined from the distribution of ^3H_2O (10 μCi) and [^{14}C]sucrose (1 μCi, 27 μM). The internal water space was 3.2 ± 0.1 μl/mg protein; the total water space was 7.8 ± 0.3 μl/mg protein. At the times indicated in fig.2, 0.5-ml samples of the cell suspensions were transferred into 1.5-ml microfuge tubes containing 0.2 ml silicone oil (d = 1.023) which had been preincubated for at least 12 h in an anaerobic chamber. The cells were separated from the medium by centrifugation through silicone oil. The supernatant and the pellet were assayed for ^{14}C and ^{3}H using a liquid scintillation counter model LS 7500 (Beckman, Fullerton, USA). Correction for nonspecific binding was made as described [6].

2.5. *Chemicals*

All radiochemicals were obtained from NEN (Dreieich, FRG). DCCD was purchased from Sigma (Taufkirchen, FRG) and 3,5,4',5'-TCS from Kodak (Rochester, USA). 2-Bromoethanesulfonic acid (sodium salt) was obtained from Fluka (Buchs, Switzerland) and the silicone oil from Roth (Karlsruhe, FRG).

3. RESULTS AND DISCUSSION

Cell suspensions of *M. barkeri* were prepared in a weakly buffered medium containing 50 mM KSCN (to ensure electroneutral movement of protons) and incubated under H_2. When methanol was added to these suspensions, typical acidification traces were recorded (fig.1a). The slopes of the baselines in fig.1a–d were due to electrode drift. Contact of the syringe with the reaction vessel caused a sudden deflection of the recorder pen as seen on the traces immediately before the additions to the reaction mixture. The maximal rate of proton ejection observed was 35 nmol $H^+ \cdot min^{-1} \cdot (mg\ protein)^{-1}$. When the cells were preincubated for 5 min with TCS, which conducts protons across the cytoplasmic membrane, such an acidification was not observed (fig.1b), although methane formation was not inhibited under these conditions (not shown). This indicated that a transmembrane pH gradient could not be established in the presence of TCS. Incubation under air resulted in a complete loss of the acidification (not shown) usually occurring upon methanol addition. This was probably due to the inhibition of methanogenesis by O_2.

In order to show that the observed acidification was specifically coupled with methanogenesis, cells were preincubated with 2-bromoethanesulfonate (BrES). BrES is known as an effective and specific inhibitor of the methylcoenzyme M methylreduc-

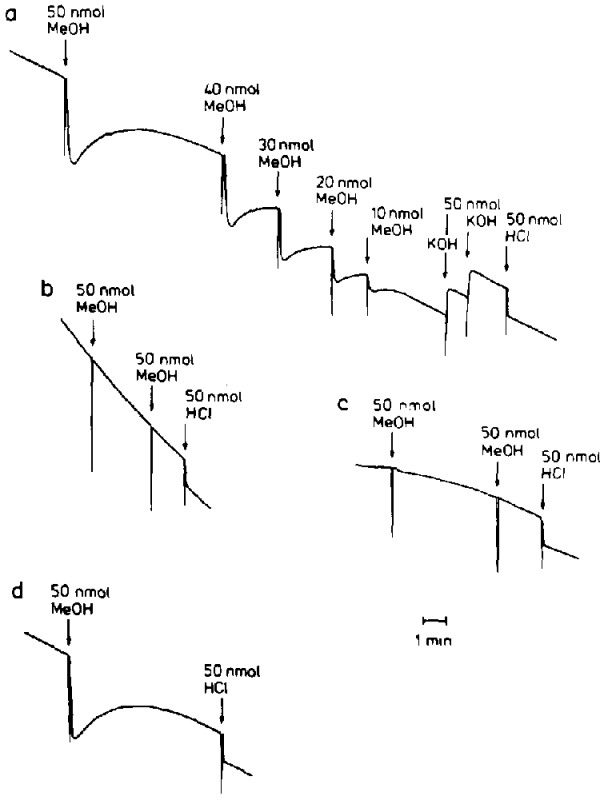

Fig.1. Electron transport-driven proton translocation in resting cells of *M. barkeri*. Anaerobic cell suspensions (protein content: 2 mg/ml) incubated under H_2 were pulsed with methanol; the acidification traces were calibrated by HCl and KOH addition. The cells were preincubated (a) without additions, (b) for 5 min with 10 μM TCS, (c) for 15 min with 10 mM BrES, or (d) for 20 min with 30 nmol DCCD/mg protein before methanol addition.

tase, which catalyzes the terminal and energy-conserving reaction of methanogenesis. Preincubation with BrES resulted in inhibition of both methanogenesis (not shown) and acidification following methanol addition (fig.1c). This inhibition turned out to be a time-dependent process (fig.2). 6 min after BrES addition the amount of protons produced was only 25% of that observed before or shortly after BrES addition. 12 min after BrES addition proton production was completely inhibited although always the same amount of methanol was administered to the cells. This shows that methanogenesis was a prerequisite for H^+ liberation by the cells.

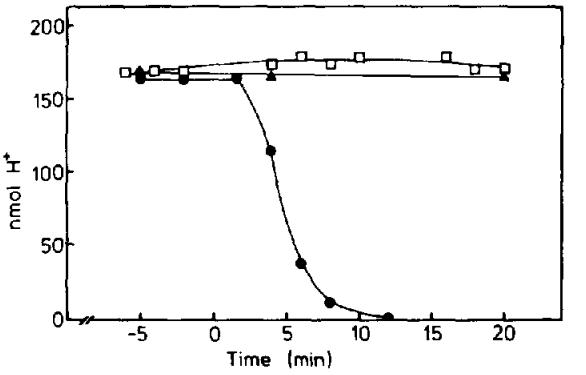

Fig.2. Effect of BrES or DCCD on H^+ ejection by resting cells of *M. barkeri* pulsed with methanol. Each value in the graph represents one determination of the amount of protons liberated after the addition of 50 nmol of methanol. At zero time either BrES (●) or DCCD (□) was added to final concentrations of 10 mM and 30 nmol/mg protein, respectively; no additions were made to the control (▲). The protein content of the cell suspension was 2 mg/ml.

Under H_2, *M. barkeri* cells convert methanol completely to methane according to: $CH_3OH + H_2 \longrightarrow CH_4 + H_2O$ (eqn 1). In the absence of H_2, however, methanol is disproportionated according to: $4\ CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$ (eqn 2). To exclude that the observed acidification was due to CO_2 production according to eqn 2 (in spite of the presence of H_2) the methanol conversion was reexamined under the conditions employed for the proton extrusion experiments by measuring the methane formation under both H_2 or N_2. It is evident from fig.3 that in the buffer system used methanol was not converted to methane unless H_2 was present. This was most probably due to the absence of Na^+ and the presence of KSCN, the latter of which abolished the transmembrane electrical potential $\Delta\psi$ (fig.4). This observation is in agreement with the previous finding that the first step of methanol oxidation depends on both the protonmotive force and sodium ions [10]. Hence, under the condition employed here the observed H^+/CH_4 stoichiometry must be equal to the H^+/CH_3OH stoichiometry.

In order to determine the apparent H^+/CH_4 stoichiometry methanol pulses of 10–50 nmol were administered to *M. barkeri* cells and the corresponding liberation of protons was registered. A typical series of measurements is given in table 1.

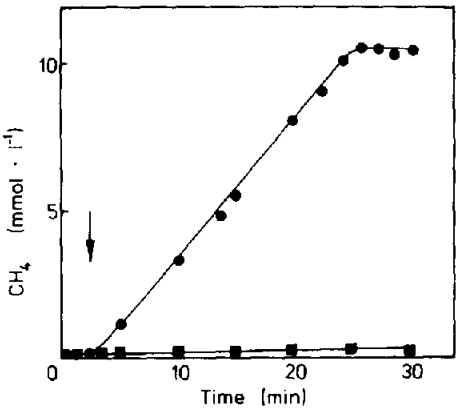

Fig.3. Methane formation from methanol by resting cells of *M. barkeri* under H_2 or N_2 in the presence of 50 mM KSCN. Cell suspensions (protein content: 2 mg/ml) were incubated 30 min prior to zero time under H_2 (●) or N_2 (■). At the time indicated by the arrow methanol was added to each suspension to a final concentration of 10 mM.

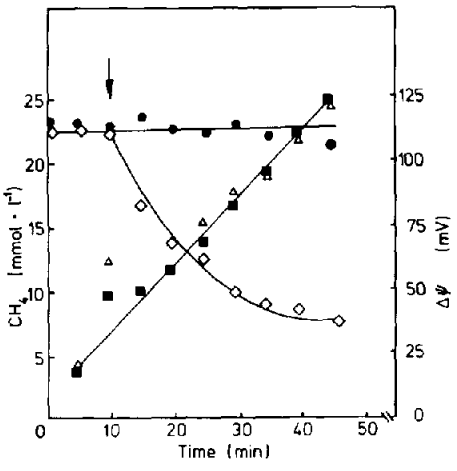

Fig.4. Effect of KSCN on $\Delta\psi$ during methane formation from methanol + H_2. Resting cells of *M. barkeri* (protein content: 1.2 mg/ml) were preincubated for 15 min under H_2. 15 min before zero time methanol was added to a final concentration of 35 mM. At the time indicated by the arrow KSCN (final concentration: 50 mM) was added. CH_4 + KSCN, △; CH_4, control, ■; $\Delta\psi$ + KSCN, ◇; $\Delta\psi$, control, ●.

For the sake of clarity it must be emphasized that the observed stoichiometry was realtively constant within a series of experiments from the same batch of cells but varied between different preparations

Table 1

H^+/CH_4 ratios determined with whole cells of *Methanosarcina barkeri*

MeOH added (nmol)	No. of experiments	H^+/CH_4	SDEV (+/−)
50	14	3.31	0.24
40	5	3.32	0.19
30	5	3.35	0.29
20	5	3.47	0.24
10	6	3.70	0.45

Resting cells incubated under H_2 were pulsed with a given amount of methanol as described in section 2. H^+/CH_4 values were calculated based on methanol conversion according to eqn 1

and depended on the age, the physiological state, and the density of the cell suspensions as well as on the amount of methanol added to the cells. Usually, higher amounts of methanol added resulted in smaller apparent H^+/CH_4 ratios. From more than 20 series of such experiments each comprising at least 30 determinations most H^+/CH_4 values obtained ranged between 3 and 4, although there were also measurements under comparable conditions showing values smaller than 3. On the basis of these measurements an H^+/CH_4 stoichiometry of 4 can be envisaged. It was not possible to rule out that 2 H^+ were directly released at the outer side of the cytoplasmic membrane by a membrane-bound hydrogenase. Previous studies in *Methanobacterium thermoautotrophicum* suggest that the protons derived from H_2 via the hydrogenase are liberated in the cytoplasm [11]. If this finding is also valid for *M. barkeri*, 4 vectorial protons may be translocated per pair of electrons transferred from H_2 to methylcoenzyme M via yet unknown carriers.

It was crucial to exclude the DCCD-sensitive proton-translocating ATPase from playing a role in proton extrusion. The presence of this enzyme has been indirectly demonstrated in whole cells of *M. barkeri* [6,12]. Very recently, Inatomi [13] purified the soluble part of an ATPase from *M. barkeri* which displays similarities with the F_1 part from eubacterial sources: when this F_1-like protein is stripped off the membrane it becomes DCCD-insensitive. However, as long as it is attached to the membrane (probably to the integral F_0 part) the

ATPase activity is inhibited by DCCD [13]. If this enzyme were involved in proton translocation one would expect DCCD to inhibit the acidification usually observed upon methanol addition. From figs 1d and 2 it is evident that the proton extrusion was not affected by DCCD at a concentration of 30 nmol/mg protein which is sufficient in whole cells to completely inhibit ATP synthesis driven by an artificially imposed pH gradient [6]. Hence, the DCCD-sensitive proton-translocating ATPase cannot be responsible for this process.

Our experiments are in agreement with earlier results which have provided evidence that the electron transfer from H_2 along unknown carriers in the membrane to the terminal acceptor methylcoenzyme M drives the synthesis of ATP via a chemiosmotic mechanism [6]. The direct demonstration of H^+ ejection independent from an H^+-translocating ATPase confirms these findings and implicates that the methyl reductase plays a role in the energy-conserving process. In accordance with these results the methylcoenzyme reductase which has been isolated as a soluble protein was recently shown in *Methanococcus voltae* to be attached to the inner aspect of the cytoplasmic membrane [14].

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